Involvement of the Carboxyl Groups of Glutathione in the Catalytic Mechanism of Human Glutathione Transferase A1-1†

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ABSTRACT: The present study proposes the participation of both carboxylate groups of the glutathione molecule as functional entities in the catalytic apparatus of human glutathione transferase (GST) A1-1. Functional studies in combination with structural data provide evidence for the α-carboxylate of the Glu residue of glutathione acting as a proton acceptor in the catalytic mechanism. The Glu carboxylate is hydrogen-bonded to a protein hydroxyl group and a main-chain NH, as well as to a water molecule of low mobility in the active site region. The Glu α-carboxylate of glutathione is bound in a similar manner to the active sites of mammalian glutathione transferases of classes Alpha, Mu, and Pi, for which three-dimensional structures are known. Mutation of the hydroxyl group that is hydrogen-bonded to the α-carboxylate of the Glu residue of glutathione (Thr68→Val) caused a shift of the pH dependence of the enzyme-catalyzed reaction, suggesting that the acidic limb of the pH−activity profile reflects the ionization of the carboxylate of the Glu residue of glutathione. The second carboxylate group of glutathione, which is part of its Gly residue, interacts with two Arg side chains in GST A1-1. One of these residues (Arg45) may influence an ionic interaction (Arg221/Asp42), which appears to contribute to binding of the second substrate by fixing the C-terminal α-helix as a lid over the active site. Removal of the Gly residue from the glutathione molecule caused a 13-fold increase in the K_M value for the electrophilic substrate. Thus, the Gly carboxylate of glutathione, by way of influencing the topology of the active site, contributes to the binding of the second substrate of the enzyme. Consequently, the glutathione molecule has several functions in the glutathione transferase catalyzed reactions, not only as a substrate providing the thiol group for different types of chemical reactions but also as a substrate contributing a carboxylate that acts as a proton acceptor in the catalytic mechanism and a carboxylate that modulates binding of the second substrate to the enzyme.

The glutathione transferases (GSTs)[1] (Mannervik, 1985; Mannervik & Danielson, 1988; Armstrong, 1991; Ketterer, 1988) form a multigene family of enzymes that catalyze the nucleophilic conjugation of glutathione (γ-L-Glu-L-Cys-Gly; GSH) with a variety of electrophilic compounds (Chasseaud, 1979). In mammals GSTs occur both as a membrane-bound form (Morgenstern *et al.*, 1990) and as cytosolic enzymes. On the basis of their primary structure, the mammalian cytosolic forms have been divided into four classes named Alpha, Mu, Pi, and Theta (Mannervik *et al.*, 1985; Meyer *et al.*, 1991).

The three-dimensional structures of members of mammalian GSTs from classes Alpha (Sinning *et al.*, 1993; Cameron *et al.*, 1995), Mu (Ji *et al.*, 1992; Raghunathan *et al.*, 1994) and Pi (Reinemer *et al.*, 1991, 1992; Gárcia-Sáez *et al.*, 1994; Dirr *et al.*, 1994) have provided the structural basis for investigations of the enzyme active site, and amino acid residues putatively involved in the catalytic mechanism have been examined by mutational studies [*cf.* Widersten *et al.* (1994)]. Common structural features have been observed among GSTs from classes Alpha, Mu, and Pi, particularly in the compartment of the active site where the thiol substrate GSH is bound, the "G-site". Binding of the electrophilic substrate occurs at an adjacent site, the "H-site" (*i.e.*, the site for binding of the second, often hydrophobic, substrate). The amino acid residues that are involved in binding of the electrophilic substrate may also, if correctly positioned, contribute to the chemical steps on the reaction pathway. In any case, the structure of the H-site governs the substrate specificity of a GST.

The conjugation of GSH with an aryl chloride, such as the commonly used GST substrate CDNB (Figure 1), involves the nucleophilic attack by the GSH thiolate on the electrophilic carbon on the phenyl ring followed by expulsion of the chloride ion. The transition state of the reaction is postulated to involve a σ-complex in which the glutathione sulfur is linked to the same carbon as the leaving group chloride (Figure 1A). Upon the decomposition of the σ-complex, the reaction products, Cl^- and the *S*-(2,4-dinitrophenyl)glutathione conjugate, are formed.

The catalysis of nucleophilic aromatic substitution reactions can be divided into steps involving binding of substrates to the enzyme active site, activation of GSH by deprotonation of the thiol to form the nucleophilic thiolate (Graminski *et al.*, 1989a), and nucleophilic attack by the thiolate at the

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[1] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; *o*-CF_3CDNB, 2-chloro-3,5-dinitro-1,1,1-(trifluoromethyl)benzene; *p*-CF_3CDNB, 4-chloro-3,5-dinitro-1,1,1-(trifluoromethyl)benzene; GSH, glutathione; GSHO-Et, GSH glycyl ethyl ester; GST, glutathione transferase; NCA, 4-nitrocinnamaldehyde; TNB, 1,3,5-trinitrobenzene. The nomenclature used for human GSTs is that described in Mannervik *et al.* (1992).

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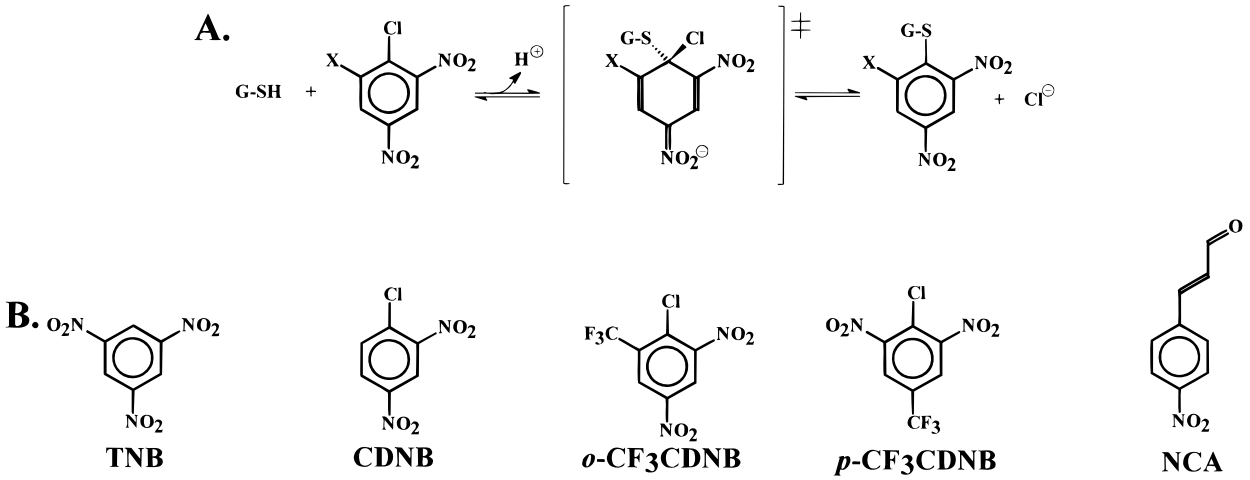


FIGURE 1: (A) Aromatic substitution reaction of a nitro-substituted aryl chloride. Within brackets is the postulated transition state σ-complex in which the sulfur atom and the chlorine are linked to the same carbon. X denotes a substituent such as H or CF_3. (B) Electrophilic compounds used in the study. TNB forms a dead-end σ-complex with a thiol nucleophile which does not decompose into the thioether conjugate due to the lack of a good leaving group. The other compounds shown are enzyme substrates. Abbreviations: TNB, 1,3,5-trinitrobenzene; CDNB, 1-chloro-2,4-dinitrobenzene; *o*-CF_3CDNB, 2-chloro-3,5-dinitro-1,1,1-(trifluoromethyl)benzene; *p*-CF_3CDNB, 4-chloro-3,5-dinitro-1,1,1-(trifluoromethyl)benzene; NCA, 4-nitrocinnamaldehyde.

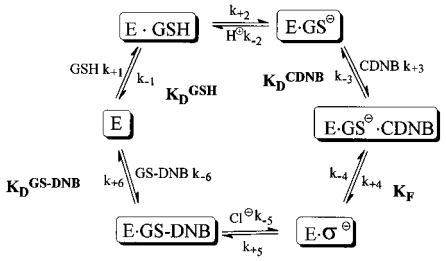


FIGURE 2: Reaction scheme for the reaction between GSH and CDNB catalyzed by GST A1-1.

electrophilic center to form a σ-complex. The reaction proceeds further through decomposition of the σ-complex to form the product and release of reaction products from the active site (Figure 2). Any one of these steps may, to a certain degree, influence the reaction rate.

GST A1-1 is the predominant GST form in human liver. This enzyme is therefore probably of crucial importance for the detoxifying capacity of this tissue, regarding conjugation of noxious electrophiles with glutathione. The present study is focused on the catalytic mechanism of human GST A1-1 in S_NAr-type reactions as described in Figure 1A. The aromatic substitution reactions are by far the most widely used for measuring GST activity and are therefore of fundamental interest in the understanding of the catalytic function of these enzymes. In the present work, three structurally related dinitro-substituted aromatic chlorides were investigated as electrophilic substrates in order to probe the catalysis of S_NAr reactions.

Human GST A1-1 catalyzes the reaction between GSH and CDNB with a rate enhancement of approximately 1×10^7 (Widersten *et al.*, 1994). Several amino acid residues important for catalysis have been identified in the active site of the enzyme, both by structural investigations (Sinning *et al.*, 1993) and by functional studies of the effects of site-specific mutations (Stenberg *et al.*, 1991; Widersten *et al.*, 1994; Björnestedt *et al.*, 1995). A Tyr residue, Tyr9, is required for maintaining high catalytic turnover (k_{cat}). The proposed role of Tyr9 is to provide a hydrogen bond via its phenolic hydroxyl group to the sulfur of enzyme-bound GSH, thereby stabilizing the ionized form of the thiol. Further, an Arg residue, Arg15, fulfills several important roles such as contributing to a positive electrostatic potential of the G-site, as well as positioning the thiolate properly for catalysis. The side-chain methylene groups of Arg15 contribute to the H-site, and the backbone amide of Arg15 is involved in a hydrogen bond to the hydroxyl of Tyr9. However, the basic understanding of the catalysis is still incomplete, *e.g.*, with regard to determinants on the reaction pathway that influence the k_{cat} and K_M values. Further information is required on structural components that are important for ionization of enzyme-bound GSH, features that influence binding of the electrophilic substrate, interactions that are important for efficient formation of the σ-complex, and factors that influence release of the reaction product from the enzyme.

EXPERIMENTAL PROCEDURES

Construction of Mutant GST A1-1 T68V. A PCR with 1.5 μg/mL pGΔETacA1 (Widersten & Mannervik, 1995) as template DNA, 0.35 mM dNTPs, 1.3 μM primer A1T68DV (GAG AGA *GAA TTC* GTT GAG *ATC GAT* GGG ATG AAG CTG GTG CAG GWT AGA GCC ATT CTC, where W is A or T), 1.9 μM primer A1I60ClaI (CAT CCC *ATC GAT* CTC AAC CAT), 1.75 mM $MgCl_2$, and 17.5 units/mL *Taq*-*Pwi*-mix DNA polymerases (Boehringer Mannheim, Mannheim, Germany) was performed using the following temperature cycle: 94 °C for 30 s, 58 °C for 1 min, and 68 °C for 6 min, repeated 35 times. Italicized letters in the primer sequences indicate recognition sites for restriction endonucleases. The PCR product, consisting of the entire plasmid, including the mutated coding region for GST A1-1, was digested with *Cla*I, religated, and transformed into electrocompetent *Escherichia coli* XL1-Blue (Stratagene, La Jolla, CA). A mutant cDNA encoding the Thr68→Val replacement was identified by dideoxynucleotide sequencing (Sanger *et al.*, 1977). To avoid any spurious undesired mutations inserted into vector DNA during PCR, the mutant cDNA was digested with *Eco*RI and *Bcl*I and the fragment containing the Thr68→Val mutation was subcloned into pGΔETacA1 digested with the same enzymes. The proper

sequence of the coding part as well as the *tac* promoter region of the mutated GST A1 cDNA was subsequently confirmed.

Construction of Mutant GST A1-1 Y132F and Y132L. A PCR with 1 μM primer A1Y132FL (TT*G AAT TCT GAT CA*A GGA GAA AAT AAA AAA TCG CTT MTT CCC TGC CTT T, where M is A or C; italicized letters indicate restriction enzyme cleavage sites) and M13pUC-reverse primer (AAC AGC TAT GAC CAT G) and 10 ng/mL pGΔETacA1 as template, in the presence of 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 25 units/mL *Taq* DNA polymerase (Boehringer Mannheim), was performed for 30 cycles under the following conditions: 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. The product formed was digested with *Bcl*I and *Xba*I, and the fragment was purified by agarose gel electrophoresis followed by adsorption to silica beads. The pGΔETacA1 plasmid was purified from *E. coli* JM110 (*dam*$^-$) (Strategene), and the plasmid DNA was digested with *Bcl*I and *Xba*I and gel purified as above. The digested fragment and vector were ligated in a molar ratio of 10:1 and transformed into electrocompetent *E. coli* XL1-Blue. Mutant clones were identified by dideoxy sequencing. Mutant DNA used for subsequent protein expression was sequenced through the PCR-derived region, to exclude production of protein with unwanted mutations.

Protein Expression and Purification. The construction and purification of the H-site mutants M208A and M208W have been reported elsewhere (Widersten *et al.*, 1994). Purified wild-type recombinant GST A1-1 (Stenberg *et al.*, 1992) was generously provided by Ms. Birgit Olin in our laboratory. The enzymes were purified by affinity chromatography on *S*-hexylglutathione Sepharose (Mannervik & Guthenberg, 1981). Wild-type GST A1-1, mutants T68V, Y132F, and mutant Y132L were eluted from the affinity matrix using 50 mM glycine−NaOH, pH 10. The eluate was neutralized by addition of 2 M Tris-HCl, pH 7.2, in a final ratio of 1:10 (v/v). The eluate was subsequently dialyzed against 10 mM Tris-HCl, pH 7.8. Mutants M208A and M208W were eluted with 5 mM *S*-hexylglutathione and dialyzed extensively against 10 mM Tris-HCl, pH 7.8. Removal of *S*-hexylglutathione was completed by two subsequent rounds of gel filtration over Sephadex G-25 equilibrated with the above Tris-buffer. The enzyme active site concentration was determined by the UV absorbance at 280 nm using ϵ_{280} = 24 700 M^{-1} cm^{-1} for wild-type GST A1 subunit and mutant subunits T68V and M208A, ϵ_{280} = 30 000 M^{-1} cm^{-1} for mutant subunit M208W (Widersten *et al.*, 1994), and ϵ_{280} = 23 300 M^{-1} cm^{-1} for mutant subunits Y132F and Y132L. Protein purity was confirmed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Laemmli, 1970).

Kinetic Properties. Steady-state kinetic measurements were performed in 0.1 M sodium phosphate, pH 6.5, in the presence of either 5 mM GSH (Sigma Chemical Co., St. Louis, MO), 5 mM GSH glycyl ethyl ester (GSHOEt) (a gift from Drs. Marika Eller and Ulf Ragnarsson of our department), or 20 mM γ-L-Glu-L-Cys (Kohjin Co. Ltd., Tokyo, Japan). Electrophilic substrates were used in the following concentration ranges: 0.05−1.6 mM CDNB (Figure 1B) (Sigma Chemical Co.), 0.025−0.7 mM *o*-CF_3-CDNB (Figure 1B) (Aldrich Chemical Co., Milwaukee, WI), 0.025−0.7 mM *p*-CF_3CDNB (Figure 1B) (Aldrich Chemical Co.), and 0.05−0.4 mM NCA (Figure 1B) (Aldrich Chemical Co.). Alternatively, *o*-CF_3CDNB was used at a fixed concentration (0.7 mM), and the GSH concentration was varied in the range 0.08−2.1 mM. The reactions were followed at 30 °C in a Varian 2290 spectrophotometer at 340 nm for substrates CDNB, *o*-CF_3CDNB, and *p*-CF_3CDNB and at 360 nm for NCA. The molar extinction coefficients of the thioether adducts of CDNB, $\Delta\epsilon_{340}$ = 9600 M^{-1} cm^{-1} (Habig *et al.*, 1974), *o*-CF_3CDNB, $\Delta\epsilon_{340}$ = 5600 M^{-1} cm^{-1}, *p*-CF_3CDNB, $\Delta\epsilon_{340}$ = 1600 M^{-1} cm^{-1}, and NCA, $\Delta\epsilon_{360}$ = −3200 M^{-1} cm^{-1}, were determined spectrophotometrically in the presence of limiting amounts of either GSH or electrophile. The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M were determined by nonlinear regression analysis of experimental steady-state data using the SIMFIT program package (Bardsley *et al.*, 1989). The rate constant for the uncatalyzed reactions, k_2, was determined from the slope of v versus [varied substrate] graphs divided by the concentration of the substrate used in excess.

pH Dependence of k_{cat} and k_{cat}/K_M. The steady-state kinetic parameters for wild-type GST A1-1 and the T68V mutant were determined by nonlinear regression analysis of data collected in the presence of 5 mM GSH and varying concentrations of CDNB (50−1600 μM) in 0.1 M sodium phosphate ranging in pH from 5.5 to 8.1 at 30 °C. pK_a values were estimated by fitting eq 1 to experimentally determined

$$L_H = \frac{[H^+]^2 L_{H_2A} + [H^+]K_1 L_{HA^-} + K_1K_2L_{A^{2-}}}{K_1K_2 + [H^+]K_1 + [H^+]^2} \quad (1)$$

k_{cat} and k_{cat}/K_M values, where L is the pH-dependent parameter k_{cat} or k_{cat}/K_M (Fersht, 1985).

Titration of the Phenolic Hydroxyl Group of Tyr9 in Mutant T68V. The ionization of the active site Tyr9, which can be monitored separately from other Tyr residues bacause of the unusually low pK_a of its phenolic hydroxyl group (Björnestedt *et al.*, 1995), was determined by difference spectrophotometry on a Varian 2290 instrument as previously described (Björnestedt *et al.*, 1995). The absorbance of the phenolate formed was mesured at 293 nm at 30 °C. The phenolate concentration was calculated using $\Delta\epsilon$ = 2400 M^{-1} cm^{-1} (Glazer, 1976). The sample contained 10 μM GST A1-1 T68V subunit at pH values ranging from 5.5 to 9.5, in 0.1 M sodium phosphate in the pH interval from 5.5 to 8.1 and 0.1 M glycine−NaOH above pH 8.1. A pK_a value for the deprotonation was determined after fitting the experimental data to a modified form of eq 1.

Formation of the σ-Complex between Thiol Substrate and 1,3,5-Trinitrobenzene. The formation of the σ-complex between TNB (Figure 1B) and GSH or GSH derivatives was studied by difference spectrophotometry. The σ-complex formation was followed at pH 6.5 in the presence of either 5 mM GSH, 5 mM GSHOEt, or 20 mM γ-L-Glu-L-Cys and varying concentrations of TNB essentially as described by Graminski *et al.* (1989a). TNB was a generous gift from Mr. Göran Karlsson, Nobel Chemicals AB, Karlskoga, Sweden. The constant for formation of the σ-complex, K_F, was determined by nonlinear regression analysis of the experimental data using eq 2. The concentration of GST

$$A_{450} = \frac{A_{max}/[E]_0[TNB]}{1/K_F + [TNB]} \quad (2)$$

A1-1 subunits in the sample cuvette was 20−50 μM. A_{max} is the absorbance at 450 nm at saturating concentrations of

TNB and thiol substrate used, and $[E]_0$ is the GST A1 subunit concentration. The formation constant for the GSH−TNB complex at pH 6.5, 30 °C, in the absence of enzyme was determined from the slope of [σ-complex] versus [TNB] divided by the GSH concentration used. The concentration of the σ-complex was determined using an extinction coefficient of 25 000 M^{-1} cm^{-1} (Crampton, 1968).

Effect of Medium Viscosity on k_{cat}. The reaction rate was measured in the presence of 5 mM GSH or GSHOEt and varying concentrations of CDNB in 0.1 M sodium phosphate, pH 6.5, 8.0, and 8.5, fortified with variable concentrations of sucrose. The reaction was followed in a ThermoMax microtiter plate reader thermostated at 30 °C. k_{cat} and K_M values were determined by nonlinear regression analysis. The relative viscosity, η_{rel}, of the medium was determined from a standard curve [*cf.* Brouwer and Kirsch (1982) and Cooper (1977)].

Equilibrium Binding of GSH and a GSH Conjugate. *S*-[2,4-Dinitro-6-(trifluoromethyl)phenyl]glutathione was synthesized by mixing GSH and *o*-CF_3CDNB in equimolar amounts in 70% (v/v) ethanol in water containing 3 equiv of NaOH, at room temperature overnight. The GSH conjugate was precipitated by lowering the pH by dropwise addition of concentrated HI during incubation on ice. The product was washed on a sintered-glass funnel with ethanol. The dissociation constant, K_D, for the reaction product was determined by nonlinear regression analysis of data obtained by measuring the quenching of the intrinsic Trp fluoresence upon binding of the ligand tested. The measurements were performed after addition of increasing concentrations of *S*-[2,4-dinitro-6-(trifluoromethyl)phenyl]glutathione to 0.6 μM active site at pH 6.5, 30 °C, in an Aminco spectrofluorometer using $\lambda_{excitation} = 278$ nm and $\lambda_{emission} = 340$ nm.

The apparent K_D for enzyme-bound GSH was determined by measuring the suppression of the ionization of the phenol of Tyr9 upon binding of GSH (Björnestedt *et al.*, 1995). Data were collected from difference spectra of 10 μM GST A1 subunit at pH 5.5 (reference cuvette) and at pH 7.3 or 8.1. GSH was present in the concentration range of 100−1300 μM. K_D values were obtained by nonlinear regression analysis.

Molecular Modeling. Structural models of GST A1-1 (and mutants) were constructed with programs O (Jones *et al.*, 1991) and InsightII (Biosym Technologies, San Diego, CA) using the atomic coordinates 1GUH (Sinning *et al.*, 1993) and 1GSD and 1GSE (Cameron *et al.*, 1995) in the Brookhaven Protein Data Bank. Coordinates for water oxygens were kindly provided by Drs. T. Alwyn Jones and Alexander D. Cameron, Department of Molecular Biology, Uppsala University, Sweden. Energy calculations (AMBER force field) on the molecular models, involving 100 iterations of conjugate gradient minimization, followed by 0.1 ps molecular dynamics (303 K), were performed using the Discover program in InsightII. All molecular modeling was performed on an Indigo2 graphical workstation.

RESULTS

Construction and Purification of Active Site Mutants of GST A1-1. In mutant T68V the side-chain hydroxyl of Thr68, interacting with the α-carboxylate of the Glu residue of GSH, was mutated into Val by an ACC→GTT codon replacement. Tyr132 is interacting with crystal water molecules in the active site and is strictly conserved within the Alpha class of GSTs. This residue was mutated into Phe (Y132F; TAC→TTC) and Leu (Y132L; TAC→TTA) in order to remove any hydrogen bond interactions with the active site water molecules. The purified proteins were homogeneous as judged from sodium dodecyl sulfate/polyacrylamide gel electrophoresis with Coomassie Brilliant Blue R-250 staining (data not shown).

Kinetic Properties. Six forms of human GST A1-1 were characterized: wild-type enzyme as well as five mutants involving amino acid substitutions in the G-site (mutants T68V, Y132F, and Y132L) and in the H-site (M208A and M208W). The kinetic parameters k_{cat}, K_M, and k_{cat}/K_M determined by steady-state kinetic analysis are presented in Table 1. The effects of the mutations of GST A1-1 on the reaction involving GSH and the *p*-trifluoromethyl derivative *p*-CF_3CDNB were similar to those observed with the *p*-nitro-substituted compounds CDNB and *o*-CF_3CDNB.

Replacing GSH as thiol substrate with γ-L-Glu-L-Cys in the reaction with *o*-CF_3CDNB caused an >10-fold increase in $K_M^{o-CF_3CDNB}$ and an 8-fold decrease in k_{cat} for the wild-type enzyme (Table 1). Values for k_{cat} and $K_M^{o-CF_3CDNB}$ could not be determined for mutant M208A when this GSH fragment was used as thiol substrate, due to the lack of appreciable rate saturation of this mutant within the concentration range used.

Use of GSHOEt as an alternative thiol substrate caused a decrease in $k_{cat}^{o-CF_3CDNB}$ by 7-fold, a result similar to that obtained with γ-L-Glu-L-Cys. However, in the presence of GSHOEt, $K_M^{o-CF_3CDNB}$ was not increased and was comparable to the $K_M^{o-CF_3CDNB}$ value determined in the presence of GSH proper (Table 1).

The replacement of a hydroxyl group by a methyl group in the T68V mutant caused a decrease in the k_{cat} value of 3−6-fold, depending on the aryl chloride used. However, k_{cat}^{NCA} was not affected as compared to the wild-type value. The K_M values for all electrophiles used were increased 3−5-fold in the T68V mutant (Table 1), but the K_M^{GSH} value was only increased by 1.6-fold as compared to that of the wild-type GST A1-1.

The mutations of Tyr132 (Y132F and Y132L) increased the K_M values for the electrophile by approximately 2-fold.

The kinetic effects caused by mutations of the enzyme were essentially additive with effects of using alternative GSH derivatives, as expressed in the differences in Gibbs free energy calculated from k_{cat}/K_M values (Figure 3).

pH Dependence of the Reaction with GSH and CDNB. The dependence of k_{cat}^{CDNB} and k_{cat}/K_M^{CDNB} on pH is shown in Figure 4. k_{cat}^{CDNB} versus pH shows an optimum at approximately pH 7.1 and describes the ionization of the (E·GSH·CDNB → E·GS−DNB → E + GS−DNB) complexes. The curve is dependent on at least two ionizations with pK_a values determined to be p$K_{a1} = 5.6 \pm 0.3$ and p$K_{a2} = 8.1 \pm 0.3$ (Figure 4A). The pH dependence of k_{cat} in mutant T68V was distinct from that of the wild type (Figure 4A). The larger experimental error in the values for the T68V mutant did not allow accurate estimation of the pK_a values. However, the acidic limb of the pH profile had been shifted approximately 1.5 pH units to a more basic value as compared to that of the the wild-type enzyme (Figure 4A).

The pH dependence of k_{cat}/K_M^{CDNB} is expected to reflect the titration of groups involved in the process (E·GSH + CDNB → E + GS−DNB). The pH dependence of k_{cat}/

Table 1: Kinetic Properties of GST A1-1 and Active Site Mutants[a]

	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} mM^{-1})	$\Delta\Delta G^b$ (thiol) (kJ/mol)	$\Delta\Delta G^c$ (mutant) (kJ/mol)
			Varied [CDNB] + 5 mM GSH		
wild type[d]	88 ± 3	0.56 ± 0.04	160 ± 8.0		(0)
T68V	32 ± 5	1.5 ± 0.34	22 ± 2.1		+5.0
Y132F	80 ± 3	1.0 ± 0.09	79 ± 3.6		+1.8
Y132L	69 ± 4	0.93 ± 0.11	74 ± 4.7		+1.9
M208A[d]	110 ± 11	3.4 ± 0.5	34 ± 1.1		+3.9
M208W[d]	99 ± 2	0.18 ± 0.02	540 ± 40		−3.1
			Varied [*o*-CF_3CDNB] + 5 mM GSH		
wild type	69 ± 2	0.084 ± 0.008	820 ± 55	(0)	(0)
T68V	11 ± 0.65	0.23 ± 0.034	47 ± 4.3	(0)	+7.2
Y132F	59 ± 4	0.15 ± 0.03	390 ± 59	(0)	+1.9
M208A	150 ± 15	0.65 ± 0.11	220 ± 15	(0)	+3.3
M208W	65 ± 4	0.020 ± 0.004	3300 ± 480		−3.5
			Varied [GSH] + 0.7 mM *o*-CF_3CDNB		
wild type	51 ± 1.9	0.41 ± 0.046	120 ± 10		(0)
T68V	8.0 ± 0.50	0.67 ± 0.10	12 ± 1.2		+5.8
			Varied [*o*-CF_3CDNB] + 5 mM GSHOEt		
wild type	9.4 ± 0.28	0.042 ± 0.006	224 ± 28	+3.3	(0)
T68V	2.1 ± 0.16	0.29 ± 0.047	7.4 ± 0.70	+4.6	+8.6
Y132F	4.3 ± 0.26	0.091 ± 0.015	47 ± 5.6	+5.3	+3.9
			Varied [*o*-CF_3CDNB] + 20 mM γ-L-Glu-L-Cys		
wild type	8.1 ± 2.6	1.1 ± 0.4	7.6 ± 0.76	+11.8	(0)
M208A	>1.2	>0.7	1.9 ± 0.33	+12.0	+3.5
			Varied [*p*-CF_3CDNB] + 5 mM GSH		
wild type	240 ± 20	0.080 ± 0.026	2800 ± 690		(0)
M208A	180 ± 13	0.31 ± 0.04	600 ± 45		+3.9
M208W	140 ± 3.5	0.033 ± 0.005	4300 ± 500		−1.1
			Varied [NCA] + 5 mM GSH		
wild type	0.41 ± 0.038	0.26 ± 0.053	1.6 ± 0.76	(0)	(0)
T68V	0.54 ± 0.20	1.3 ± 0.62	0.41 ± 0.036	(0)	+3.4
Y132F	0.55 ± 0.068	0.35 ± 0.084	1.6 ± 0.19	(0)	± 0
M208A	0.76 ± 0.19	0.47 ± 0.20	1.6 ± 0.31	(0)	± 0
			Varied [NCA] + 5 mM GSHOEt		
wild type	0.14 ± 0.015	0.11 ± 0.038	1.3 ± 0.31	+0.6	(0)
T68V	0.066 ± 0.026	1.4 ± 0.68	0.046 ± 0.0040	+5.5	+8.4
Y132F	0.071 ± 0.0056	0.26 ± 0.044	0.28 ± 0.027	+4.4	+3.9
M208A	0.13 ± 0.037	0.49 ± 0.24	0.27 ± 0.059	+4.6	+4.0

[a] The second-order rate constants, k_2, for the uncatalyzed reactions at pH 6.5 were determined to be the following: GSH + CDNB, 0.0145 ± 0.002 s^{-1} M^{-1} (see footnote *d*); GSH + *o*-CF_3CDNB, 0.118 ± 0.004 s^{-1} M^{-1}; GSHOEt + *o*-CF_3CDNB, 0.128 ± 0.031 s^{-1} M^{-1}; γ-Glu-Cys + *o*-CF_3CDNB, 0.0274 ± 0.007 s^{-1} M^{-1}; GSH + *p*-CF_3CDNB, 0.282 ± 0.007 s^{-1} M^{-1}; GSH + NCA, 0.215 ± 0.013 s^{-1} M^{-1}; GSHOEt + NCA, 0.250 ± 0.010 s^{-1} M^{-1}. [b] As calculated from the formula $\Delta\Delta G = -RT \ln [(k_{cat}/K_M^{GSH\ derivative})/(k_{cat}/K_M^{GSH})]$. [c] As calculated from the formula $\Delta\Delta G = -RT \ln [(k_{cat}/K_M^{mutant})/(k_{cat}/K_M^{wild\ type})]$. [d] From Widersten *et al.* (1994).

K_M^{CDNB} could not be fitted well to a simple rational function in the regression analysis. However, it appears to involve at least two ionization states in the wild-type GST A1-1 (Figure 4B) with the acidic $pK_{a1} = 5.3 \pm 0.7$. The more basic pK_{a2} could not be estimated in the analysis. The pH profile of the T68V mutant showed only one clear ionization with a pK_a of 6.6 ± 0.5 (Figure 4B).

Titration of the Phenolic Hydroxyl Group of Tyr9 in Mutant T68V. The pK_a for the Tyr9 ionization in mutant T68V was determined to be 8.4 ± 0.1 (Figure 4C), a value 0.3 pH unit higher than that previously determined for the wild-type enzyme ($pK_a = 8.1 \pm 0.1$; Björnestedt *et al.*, 1995).

Formation of the σ-Complex Involving Thiol Substrate and 1,3,5-Trinitrobenzene. The enzyme-bound 1:1 σ-complex involving TNB and either GSH, GSHOEt, or γ-L-Glu-L-Cys could be determined from the characteristic spectrum (Crampton, 1968) involving a peak at 450 nm and a shoulder around 550 nm (Figure 5). The formation constants determined, K_F, and the corresponding A_{max} values for enzyme-bound thionyltrinitrocyclohexyldienate are shown in Table 2. No accurate value for K_F or A_{max} could be obtained for M208A due to lack of saturation with this mutant with any of the thiol substrates used.

Effect of Medium Viscosity on k_{cat} and K_M for the Reaction with CDNB. The values for k_{cat}^{CDNB} determined in the presence of 5 mM GSH at pH 6.5, 8.0, and 8.5 or 5 mM GSHOEt at pH 6.5 at different relative viscosities, η_{rel}, are given in Table 3.

Equilibrium Binding of GSH and Reaction Products. The dissociation constant, K_D, for *S*-[2,4-dinitro-6-(trifluoromethyl)phenyl]glutathione, determined by measuring the Trp fluorescence quenching, is given in Table 4. Values for *S*-(2,4-dinitrophenyl)glutathione (Widersten *et al.*, 1994) have been included for comparison.

Measuring the quenching of the UV absorbance of Tyr ionization upon addition of GSH resulted in hyperbolic curves which allowed determination of K_D for GSH to be 180 ± 48 μM at pH 7.3 and 230 ± 5 μM at pH 8.1.

DISCUSSION

The Nature of the Proton Acceptor for the Ionization of GSH at the Active Site. Available evidence, from previously

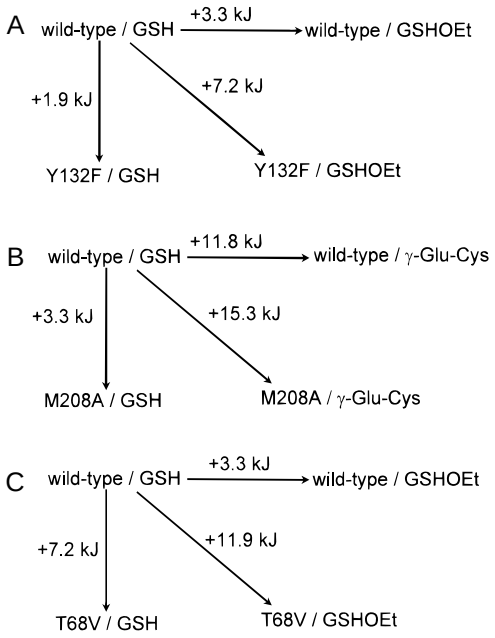


FIGURE 3: Additive effects of mutations in the enzyme structure combined with modifications of the thiol substrate: (A) Tyr132→Phe, GSH→GSHOEt; (B) Met208→Ala, GSH→γ-L-Glu-L-Cys; (C) Thr68→Val, GSH→GSHOEt. The incremental Gibbs free energy for the stabilization of the transition state involving thiol substrate and *o*-CF_3CDNB was calculated from the equation $\Delta\Delta G = -RT$ ln {[k_{cat}/K_M(mutant, GSH derivative)]/[k_{cat}/K_M(wild type, GSH)]}.

published work as well as from the present investigation, identifies the α-carboxylate of the γ-glutamyl portion of GSH as a possible acceptor of the proton released from the sulfhydryl group upon binding of GSH to the active site. The data suggest that GSH itself promotes the deprotonation of its cysteinyl thiol and becomes protonated at a distinct chemical group, *i.e.*, the Glu carboxylate.

The functional requirement of the Glu carboxylate of GSH in catalysis is strongly indicated by the finding that the decarboxy analog of GSH (4-aminobutyryl-L-Cys-Gly) is completely inactive as a nucleophilic substrate for the GSTs analyzed (Adang *et al.*, 1988). This result with the class Alpha GSTs 1-1 and 2-2 from the rat is particularly relevant, since these isoenzymes are structurally and functionally closely related to human GST A1-1 used in the present study.

Crystal structures of GSTs show that the Glu carboxylate is hydrogen-bonded through one of its oxygens to a hydroxyl group of the protein, in GST A1-1 Thr68 (Sinning *et al.*, 1993); in other GSTs this residue is either a Thr or a Ser. The second carboxylate oxygen is hydrogen-bonded to the main-chain NH of Thr68. The second oxygen is also

Table 2: Formation of the σ-Complex Involving 1,3,5-Trinitrobenzene and GSH Derivatives[a]

enzyme	A_{max}[b] (M^{-1} cm^{-1})	K_F (M^{-1})	$1/K_M^{o-CF_3CDNB}$ (M^{-1})
	Varied [TNB] + 5 mM GSH		
wild type	12300 ± 400	8900 ± 1400	12000 ± 1000
Y132F	7100 ± 200	5700 ± 660	6700 ± 1100
Y132L	5500 ± 100	4100 ± 310	
M208A	>1300[c]	<1300[c]	1500 ± 180
M208W	8900 ± 300	12300 ± 1700	50000 ± 8300
	Varied [TNB] + 5 mM GSHOEt		
wild type	2300 ± 600	3400 ± 260	24000 ± 3200
	Varied [TNB] + 20 mM γ-L-Glu-L-Cys		
wild type	8300 ± 300	860 ± 60	910 ± 240
M208A	>340[c]	<500[c]	

[a] The noncatalyzed formation of the GSH−TNB σ-complex at pH 6.5 was determined to be 0.034 ± 0.002 M^{-1}. [b] Absorbance at 450 nm at saturating concentrations of TNB and thiol. [c] No saturation was observed within the concentration range used.

Table 3: Effect on k_{cat}^{CDNB} by Changes in Medium Viscosity

	η_{rel}	[sucrose] (% w/v)	k_{cat}^0/k_{cat}[a]
5 mM GSH, pH 6.5	1.6	15	0.96
	3.9	37.5	1.06
5 mM GSH, pH 8.0	3.2	30	1.43
5 mM GSH, pH 8.5	3.2	30	1.18
5 mM GSHOEt, pH 6.5	3.2	30	0.63

[a] k_{cat}^0 denotes determined maximum turnover in the absence of sucrose ($\eta_{rel} = 1$).

Table 4: Dissociation Constant for the Products of the Reactions Involving GSH and either CDNB or *o*-CF_3CDNB

enzyme	K_D (μM)
S-(2,4-Dinitrophenyl)glutathione	
wild type	29 ± 2[a]
M208W	28 ± 6[a]
S-[2,4-Dinitro-6-(trifluoromethyl)phenyl]glutathione	
wild type	24 ± 2
M208A	76 ± 16
M208W	43 ± 5

[a] From Widersten *et al.* (1994).

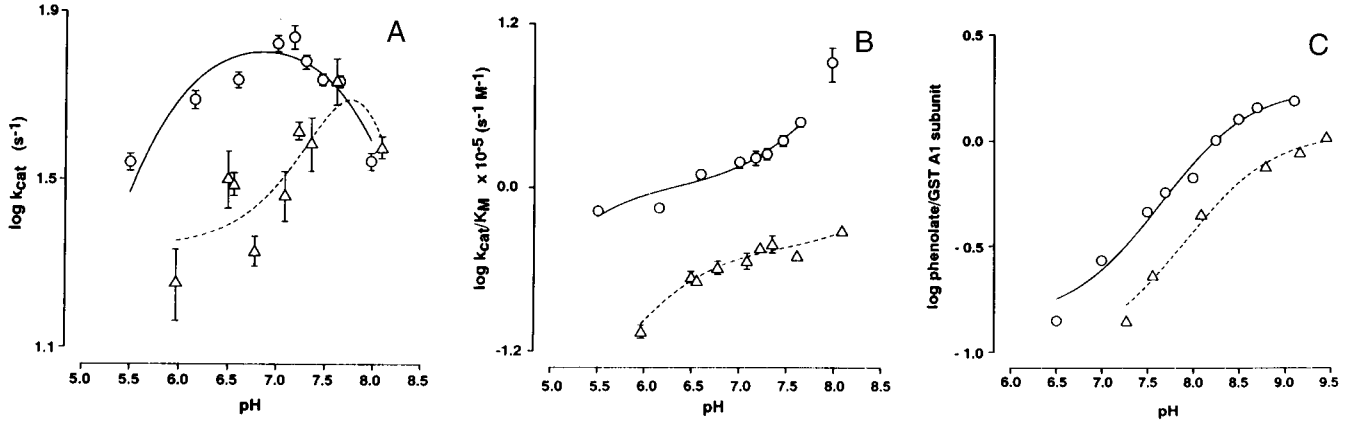


FIGURE 4: pH dependence of kinetic parameters of wild-type and mutant T68V GST A1-1 (A and B) and ionization of the phenolic group of Tyr9 (C): (○) wild-type; (△) mutant T68V. (A) log k_{cat}^{CDNB} versus pH. (B) log k_{cat}/K_M^{CDNB} versus pH. Error bars represent the standard deviation as given by the regression analysis. The curve for the ionization of wild-type GST A1-1 (Björnestedt *et al.*, 1995) has been included in (C) for comparison.

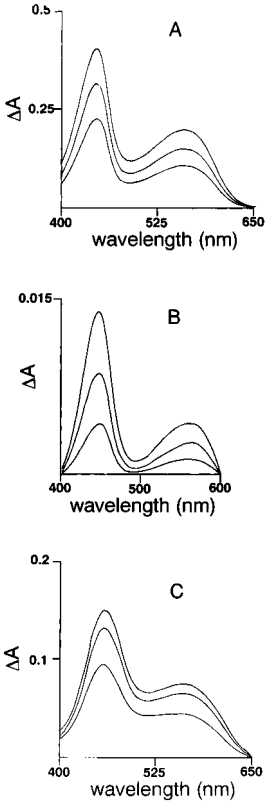


FIGURE 5: Spectra of the σ-complex of TNB formed with (A) 5 mM GSH, (B) 5 mM GSHOEt, and (C) 20 mM γ-L-Glu-L-Cys in the presence of 20 μM GST A1-1 in 0.1 M sodium phosphate, pH 6.5. Spectra were recorded after additions of 100−1200 μM TNB. The absorbance contribution from the σ-complex formed in the absence of enzyme has been subtracted.

hydrogen-bonded, in this case to a water molecule bound in the active site (Figure 6). Both hydrogen bonds involving the second carboxylate oxygen are shielded from solvent molecules that enter the nearby cleft between the two protein subunits (Figure 6). It is noteworthy that there is no positively charged protein side chain that could match the negative charge of the Glu carboxylate of GSH; the α-ammonium group of the GSH Glu residue is bound to the side-chain β-carboxylate of Asp101, which extends to GSH from the neighboring protein subunit. An unbalanced negative charge of the Glu carboxylate and the ionic bond with an Asp from the neighboring subunit are conserved features among the known GST structures (Reinemer *et al.*, 1991, 1992; Ji *et al.*, 1992; Sinning *et al.*, 1993; Dirr *et al.*, 1994; García-Sáez *et al.*, 1994; Raghunathan *et al.*, 1994; Cameron *et al.*, 1995).

The k_{cat}^{CDNB} versus pH profile of GST A1-1 is bell-shaped with apparent pK_a values of 5.6 and 8.1 (Figure 4). The mutant T68V has a catalytic activity somewhat lower than that of the wild-type enzyme, but the ascending limb of the pH−activity profile is shifted >1 pH unit to higher values. Direct determination of the pK_a value of the Glu carboxylate of GSH has not been made, but assignment of the lower pK_a value of the pH profiles of k_{cat}^{CDNB} and k_{cat}/K_M^{CDNB} to the Glu carboxylate has support from the perturbation of the pH profile by the T68V mutation. The positioning of the Glu carboxylate of GSH in the active site is expected to raise its pK_a value above the value in aqueous solution. No other ionizable groups in the active site are likely candidates for the acidic limb of the pH profile. The β-carboxylate of

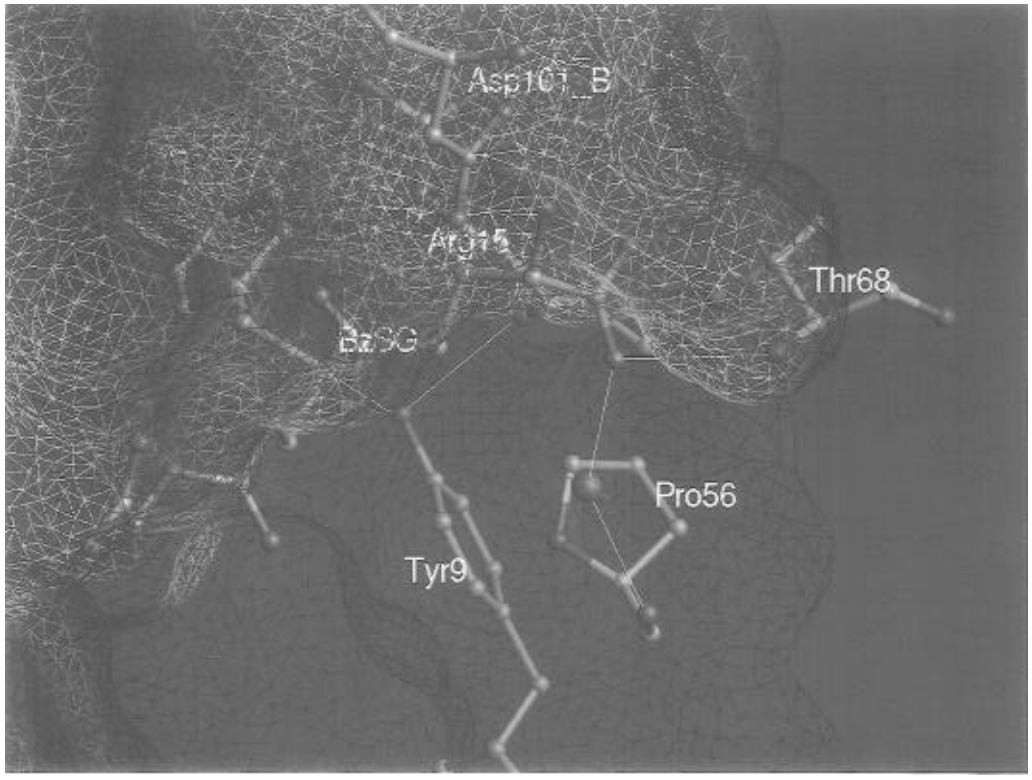


FIGURE 6: Hydrogen bond interactions in the G-site of GST A1-1. The interactions made by the the α-carboxylate of the Glu residue of GSH with the side-chain OH, the main-chain NH of Thr68, and a water molecule bound to the enzyme are shown. The same water molecule is also linked to the carbonyl oxygen of *cis*-Pro56. Carbons of residue Asp101 from the neighboring protein subunit are colored orange. Carbons of the active site ligand *S*-benzylglutathione are colored gray. The solvent accessibility, calculated with a diameter of the model solvent molecule of 1.4 Å, is shown as a blue surface. Image made with InsightII from atomic coordinates 1GUH (Sinning *et al.*, 1993) supplemented with coordinates for crystal water molecules (T. A. Jones and A. D. Cameron, Department of Molecular Biology, Uppsala University, personal communication).

Asp101 has been mutated without loss of the acidic branch of the pH profile (Wang *et al.*, 1992; Widersten *et al*., 1992). Tyr9 and the sulfhydryl group of GSH in the active site have pK_a values estimated at 8.1 and 6.6, respectively (Björnestedt *et al.*, 1995). Further, the Y9F mutation primarily affects the basic limb of the pH−activity profile.

Work by Adang *et al.* (1988) shows that the α-ammonium group of GSH is not necessary for enzymatic activity, since a derivative lacking this group (glutaryl-Cys-Gly) functions as a substrate. This result appears to exclude involvement of this group for proton transfer in catalysis.

If the Glu carboxylate of GSH is to be functional as a catalytic base in the active site of the enzyme, the question remains how the cysteinyl thiol proton is transported from the sulfur to the Glu carboxylate. From the X-ray structures of GST A1-1 it is clear that GSH has an extended conformation with the two groups approximately 7 Å apart, and no functional groups of the protein appear to be available for such proton transport when the G-site is occupied by glutathione or glutathione derivatives. However, in the structure of the apoenzyme (Cameron *et al.*, 1995) water molecules have replaced both the GSH thiolate and the Glu carboxylate as ligands to the phenolic hydroxyl of Tyr9 and the hydroxyl and amide groups of Thr68, respectively. It is plausible that, upon binding of GSH to the G-site, one of these water molecules relays the thiol proton of GSH to the Glu carboxylate of the same molecule as they are being expelled from the active site. If not released earlier by other means, the proton may leave the active site with the GSH conjugate formed in the catalyzed reaction (Figure 2).

The water molecule that is hydrogen-bonded to the Glu carboxylate is also liganded to the backbone carbonyl of the *cis*-proline at position 56 in GST A1-1 (Figure 6). Mutational studies on the homologous rat class Alpha GST 1-1 replaced Pro56 by Ala, a residue which does not adopt a *cis* configuration of the peptide bond. The mutation decreased the k_{cat}^{CDNB} value 25-fold (Wang *et al.*, 1993). A Pro residue in a *cis* configuration is another structural feature conserved in all known GSTs as well as in other GSH binding proteins such as the glutaredoxins from phage T4 (Söderberg *et al.*, 1978) and *E. coli* (Bushweller *et al.*, 1994).

The T68V mutant of GST A1-1 also shows increased K_M values for the electrophiles tested (3−5-fold), rather than an increase in K_M^{GSH} (<2-fold; Table 1), as might have been expected from removing a hydrogen bond interaction between the enzyme and GSH. The observed increase in the K_M values for the electrophiles tested is likely to reflect impaired catalytic function regarding stabilization of reaction intermediates, since the Thr→Val substitution is not likely to affect the H-site structure as such. Computer models of the T68V mutant were subjected to energy minimization and molecular dynamics studies to confirm that no adverse sterical effects were to be expected by mutating the γ-hydroxyl into a methyl group.

Another, rather surprising, effect of replacing the hydroxyl group by a methyl group in the T68V mutant was observed in the ionization of the Tyr9 phenol group (Figure 4C), which is situated almost 8 Å from the Thr68 hydroxyl group (Figure 6). The pK_a value determined for the phenol group was raised approximately 0.3 pH unit as compared to the wild-type enzyme. This result may be due to a distorsion of the electrostatic field in the G-site by the Thr68 mutation.

Potential Role of Other Water Molecules Bound to GST A1-1. In addition to the Glu carboxylate of GSH, water molecules with low temperature factors were investigated as possible proton acceptor candidates.

In the GST A1-1 structure Tyr132 reaches into the G-site from the neighboring subunit. This is a residue conserved in all known class Alpha GSTs. The deduced interactions of Tyr132 are limited to those of its hydroxyl group with water molecules having low mobility (Figure 7). A bridge of possible hydrogen bond interactions from the thiolate of GSH to the peptide amide of the Cys residue of GSH, to the γ-carbonyl oxygen of Gln54, and further to a water molecule interacting with Tyr132 can be deduced from the three-dimensional structure (Figure 7). The atomic distances are well within the range for hydrogen bonding, except for the distance between the NH of the cysteinyl residue of GSH and the side-chain carbonyl of Gln54, which exceeds 4 Å. The removal of the hydroxyl of Tyr132 in the Y132F and Y132L mutants decreased only marginally k_{cat} for the S_NAr reactions tested. This shows that no significant contribution to catalysis is made by an interaction of Tyr132 with these water molecules. A plausible role for the conserved Tyr132 could be as a participant of a hydrogen-bonding network in the active site needed for optimal catalytic function, where the hydroxyl group of Tyr132 would be important as a member fixed in space by virtue of the relative rigidity of its aromatic side chain.

Effects of GSH Binding on K_M for the Electrophile. The C-terminal helix 9 in GST A1-1 forms a lid covering the hydrophobic electrophile binding subsite (H-site) of the active site (Figure 8). Earlier mutagenesis studies demonstrated an increased K_M^{CDNB} value as a result of replacing Met208, a residue immediately preceding the C-terminal helix (Figure 8), with potentially charged residues or residues of lesser molecular volume (Widersten *et al.*, 1994). Structural data from both X-ray crystallography (Cameron *et al.*, 1995) and NMR studies (Allardyce *et al.*, 1995) have provided proof for an increased mobility of helix 9 in the absence of GSH; in the crystal structure no distinct electron density for helix 9 was observed in the apoenzyme, and the NMR data indicate increased mobility of phenylalanines 220 and 222, which are situated in the C-terminal region of the structure.

Several interactions between GST A1-1 and bound GSH have been identified (Sinning *et al.*, 1993). The interaction between the GSH Gly carboxylate and the enzyme is mainly made by two Arg residues, Arg131 from the neighboring subunit and Arg45 (Figure 8). The guanidinium group of Arg45 is also within ionic-bond distance of the carboxylate of Asp42. Asp42 is further linked to the NH_2^{ω} of Arg221. The electrostatic bond made by Asp42 and Arg221 is the only obvious polar interaction present in the enzyme that could contribute to a structural stabilization of the C-terminus.

The Gly carboxyl group of GSH is important for maintaining a low K_M for the electrophilic substrate. This is demonstrated by the elevation of $K_M^{o\text{-}CF_3CDNB}$ when using γ-L-Glu-L-Cys as the thiol substrate. However, electrostatic interactions involving the negative charge on the Gly carboxylate appear not to be essential for maintaining a low K_M for the electrophile, as illustrated by the $K_M^{o\text{-}CF_3CDNB}$ determined with GSH esterified on its Gly carboxyl group (Table 1). It should also be noted that γ-L-Glu-L-Cys has a

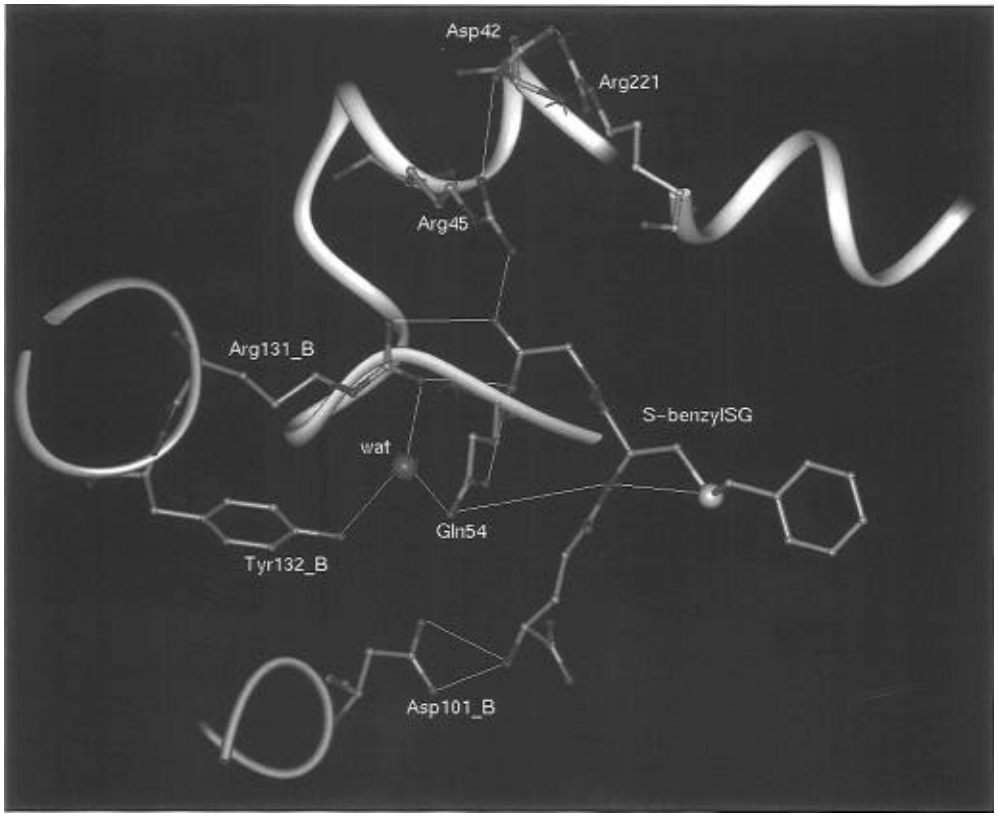


FIGURE 7: Hydrogen-bonding network involving Tyr132 and water molecules at the active site. The side-chain OH of Tyr132 is hydrogen-bonded to a crystal water molecule, which is further linked to residues interacting with GSH. Putative interactions are given as yellow lines. The atoms linked by straight lines are within hydrogen-bonding distance except for the side-chain carbonyl of Gln54 and the backbone NH of the Cys residue of GSH. Carbons of side chains from protein subunit A are in pink, and those from subunit B are in orange. The active site ligand *S*-benzylglutathione is in gray. Image made with InsightII from atomic coordinates 1GUH (Sinning *et al.*, 1993) supplemented with coordinates for crystal water molecules (T. A. Jones and A. D. Cameron, Department of Molecular Biology, Uppsala University, personal communication).

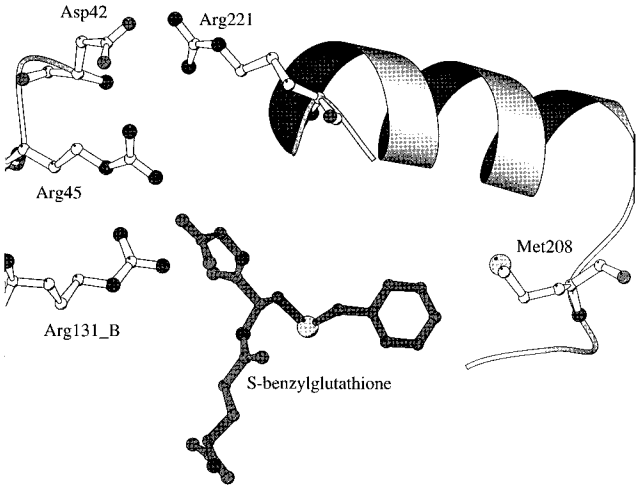


FIGURE 8: Interactions made by the Gly carboxylate of GSH and amino acid residues in the G-site. The Gly carboxylate is bound to arginines 45 and 131–B, entering the G-site from the neighboring subunit B. Arg45 is also interacting with the carboxylate of Asp42, which in turn is making contact with Arg221. The H-site residue Met208 is also shown. The active site ligand *S*-benzylglutathione is shown in gray. The atomic coordinates (1GUH; Sinning *et al.*, 1993) were obtained from the Brookhaven Protein Data Bank. The image was made with MolScript (Kraulis, 1991).

negatively charged C-terminus similar to that of GSH, since the Cys residue has a free carboxylate group.

The ionic interaction Asp42−Arg221 is structurally conserved in class Alpha GSTs, as judged by comparison of available amino acid sequences (Mannervik & Danielson, 1988). In some cases where these residues are not strictly conserved, they have been replaced by Glu and Lys, respectively, which represent compensating substitutions that should maintain a proper distance between the ionizable groups of the side chains. Such a possible Glu42−Lys221 interaction can be deduced from the primary structure of rat GST 1-1 (Pickett *et al.*, 1984). Rat GST 1-1 also carries a compensating mutation in position 45 (Arg→Lys) which would enable the ϵ-amino group to be correctly spaced for interaction with Glu42. Lys45 in rat GST 1-1 was mutated (Wang *et al.*, 1993), and the mutant enzyme maintained the K_M^{GSH} value of the wild-type enzyme. However, it showed a decrease in K_M^{CDNB}, which agrees with the notion that if the interaction between residue 42 (Asp/Glu) and residue 45 (Arg/Lys) is absent, the interaction made by residues 42 and 221 tightens, resulting in a decrease in K_M for the electrophile.

Notably, the GSH binding function of Arg131 is a structural feature which appears to be unique to the Alpha class GSTs. This residue is "inserted" into helix 5, which as a consequence is kinked (Sinning *et al.*, 1993). In classes Mu and Pi the only ionic interaction made by the Gly carboxylate is to a basic residue in the region of positions 40−50, which in the Alpha class most probably is represented by residue 45. The fact that mutation of Lys45 in rat GST 1-1 did not affect the K_M^{GSH} value (Wang *et al.*, 1993) strongly indicates that Arg131 contributes most of the binding interactions required for efficient GSH binding. The role of Arg45 in GST A1-1 could therefore be restricted to

regulating the binding of electrophilic substrate by acting as a sensor of GSH binding to the active site via its interactions with the Gly carboxylate of GSH and Asp42.

In evaluating K_M effects resulting from modifications of the Gly carboxylate of GSH structure, it should be noted that Tyr132 is involved in a chain of hydrogen bonds reaching from the phenolic hydroxyl of Tyr132 to the Gly carboxylate of GSH either via Gln54 or Arg131 (Figure 7); such interactions might also contribute to the structural stabilization of the C-terminal helix as discussed above. This putative chain of hydrogen bonds is expected to be broken in mutants Y132F and Y132L, which may explain their increased K_M values for the electrophilic substrates (Table 1).

Mutant M208K had the feature that the elevated K_M^{CDNB} caused by the mutation could be restored (lowered) by addition of *S*-(*p*-carboxybenzyl)glutathione (Widersten *et al.*, 1994). This effect may be related to the interaction described between Asp42 and Arg221, which could provide a structural basis for a memory function of the active site. The binding of the activator [*S*-(*p*-carboxybenzyl)glutathione] could enable the C-terminal helix to return to a conformation in which Asp42 and Arg221 are within a distance that makes an electrostatic interaction possible. Dissociation of the activator from the active site leaves an enzyme with an H-site in a favorable conformation which would be reflected in a decrease in K_M^{CDNB}.

In the interpretation of the effects of point mutations in the enzyme as well as of modifications of the thiol substrate, consideration must be given to possible indirect effects that might occur due to possible conformational rearrangements of the protein and an altered binding mode of the substrate. Such indirect effects may be diagnosed by the lack of additivity when the effects of single modifications are compared with the effect of more than one modification occurring simultanously. In the present case, the tested combinations of site-directed mutagenesis of GST A1-1 and variations of the structure of the thiol substrate are additive, within the limits of experimental error (Figure 3). Therefore, it is reasonable to neglect possible indirect effects and interpret the data as being caused by discrete localized effects.

σ-Complex Formation in Relation to K_M for the Electrophile. The S_NAr reaction is postulated to involve the formation of a labile σ-complex which on the reaction coordinate is close to the transition state of the reaction. Thus, analysis of the enzyme-promoted formation of the 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate σ-complex from TNB and GSH provides information about the contribution to catalysis afforded by transition state stabilization in the active site (Graminski *et al.*, 1989a).

Decreased values of formation constants for the *S*-1-thionyl-2,4,6-trinitrocyclohexyldienate σ-complex upon alteration of the GSH molecule or the enzyme structures should be reflected in increased K_M values for structurally related electrophilic substrates such as *o*-CF_3CDNB, since the interactions made by the enzyme and the σ-complex of TNB are expected to be similar to the interactions realized between the enzyme and certain intermediates along the pathway of the catalyzed reaction. The formation constants determined indeed appear to (inversely) reflect alterations in K_M values in the cases when GSH or γ-L-Glu-L-Cys was used as thiol substrates (Tables 1 and 2).

K_M values for highly reactive electrophilic substrates are composites of several elementary rate constants, in which the binding interactions between the enzyme−GSH complex and the electrophile as such may play a minor role. The K_M parameter is therefore sensitive to the reactivity of the substrate which will affect rate constants along the catalytic pathway. This is obvious in the comparison of K_M values obtained with CDNB and *o*-CF_3CDNB. The latter electrophile, which is approximately 8-fold more reactive than CDNB, gives a K_M value 7-fold lower than that obtained with CDNB (Table 1).

An observed decrease in K_M^{CDNB} effected by the Met208→Trp mutation was earlier proposed as being possibly due to direct hydrogen bond interactions between the *p*-nitro group of a negatively charged transition state structure and the $N^{\delta}H$ of the indole group (Widersten *et al.*, 1994). The kinetic results obtained with the substrate analogs *o*-CF_3-CDNB and *p*-CF_3CDNB in the present study (Figure 1B; Table 1) rather suggest that the lowered K_M^{CDNB} is due to other interactions, since the *para*-positioned trifluoromethyl group of *p*-CF_3CDNB would be unable to participate in hydrogen bond interactions in a manner similar to that of a nitro group. This mutant, however, appears to be more efficient (1.4-fold) in stabilizing σ-complex structures when measured as the formation constant of the TNB complex (Table 2).

Rate-Limiting Step(s) in the Catalytic Mechanism. (A) GSH Binding and Ionization. The values for the dissociation constant, K_D, determined for GSH for GSTs from classes Mu (7 μM, Jakobson *et al.*, 1979; 22 μM, Graminski *et al.*, 1989b), Pi (78 μM, Widersten *et al.*, 1992), and Alpha (180−230 μM, this work) suggest that the enzyme is essentially fully saturated under normal cellular conditions, where intracellular GSH concentrations often reach millimolar values. Consequently, GSH is the first substrate to bind in the catalytic cycle, and in its physiological state the "idle" enzyme contains the thiolate of GSH ready to react with incoming electrophiles. Since association rates by far exceed the turnover rate of the catalyzed reaction, GSH binding is not rate-limiting for catalysis. This conclusion is also confirmed by the kinetic results performed at different viscosities described below.

The deprotonation of the sulfhydryl group of GSH has been proposed to be a key function of GSTs in the catalysis. In rat class Mu GST 4-4, the enzyme lowers the pK_a of the thiol from a value of 9.2 in solution to approximately 6.6 in the active site (Graminski *et al.*, 1989b). The same pK_a value for the thiol of GSH bound to GST A1-1 has been obtained indirectly from titration studies of the active site Tyr9 (Björnestedt *et al.*, 1995). The estimate of 6.6 for the thiol pK_a is supported by studies of the enzyme-bound σ-complex of GSH and TNB at pH 6.5 (Table 2). The value of the limiting absorbance at saturation is approximately 10 000 M^{-1} cm^{-1}, which corresponds to 40% complex formed at the active site of GST A1-1 at pH 6.5, assuming that the molar extinction of the σ-complex bound to the active site of GST A1-1 is similar to that in solution (Crampton, 1968). The low pK_a value rules out the ionization of the sulfhydryl group of GSH as a rate-limiting process.

(B) Stabilization of the σ-Complex. GST A1-1 increases the formation constant for the enzyme-bound σ-complex between GSH and TNB by 340-fold (K_F = 8900 M^{-1}) at pH 6.5, as compared to that for aquated GSH and TNB (K_F

$= 26\ M^{-1}$; Gan, 1977). However, the increase in reaction rate afforded by GST A1-1, calculated either as effective molarity (k_{cat}/k_2,) or as rate enhancement ($k_{cat}/K_M k_2$), is higher than what can be explained by σ-complex stabilization. This suggests that the contribution of this stabilizing effect to the catalytic rate is not a single major factor for GST A1-1.

(C) Product Release. Product release has been proven to be the rate-limiting step of the reaction between GSH and CDNB in rat GST 3-3 (Johnson *et al.*, 1993). However, in the case of human GST A1-1, we conclude that release of the glutathione conjugate is not a major rate-limiting process.

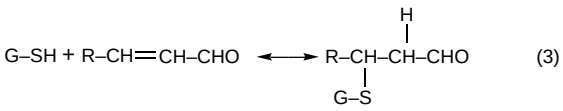
The diffusion rate of reaction products and substrates is inversely proportional to the medium viscosity and is expected to affect k_{cat}, if product release or diffusion of substrate into the active site is rate determining. In the case of the GST-catalyzed reaction with GSH and CDNB, the value of k_{cat}/K_M ($10^5\ s^{-1}\ M^{-1}$) is well below the value beyond which substrate binding is considered to be rate limiting ($10^9\ s^{-1}\ M^{-1}$). Therefore, any observed effect is expected to be related to product release.

The k_{cat}^{CDNB} values measured in the presence of GSH were not affected by the medium viscosity at pH 6.5, but at pH values of 8.0 or 8.5 a decrease in k_{cat} was observed at increased viscosities (Table 3). This finding indicates a shift in rate-determining step of the catalysis as pH increases from 6.5 to 8. However, if the value of k_{cat} is only determined by parameters sensitive to the medium viscosity, k_{cat}^0/k_{cat} (Table 3) and the relative viscosity should be linearily dependent with a slope of 1 (Brouwer & Kirsch, 1982; Johnson *et al.*, 1993), which was not observed. This indicates that also at these higher pH values product release is not the sole rate-limiting process.

Viscosity effects on the reaction with GSHOEt were also examined, due to the decreased $k_{cat}^{o\text{-}CF_3CDNB}$ observed with this thiol substrate (Table 1). The data suggest that the lowered enzymatic rate measured with GSHOEt is not an effect of rate-limiting product release. The turnover rate with this thiol substrate rather increases, for unknown reasons, with increasing viscosity (Table 3).

Further, the conclusion that product release has a minor influence on k_{cat} is supported by the finding that dissociation constants, K_D, for the reaction products of the CDNB and the *o*-CF_3CDNB reactions did not correlate with values of k_{cat} [this study and Widersten *et al.* (1994)]. However, mutant M208A, which shows the highest k_{cat} with *o*-CF_3-CDNB, also binds the reaction product with weakest affinity.

(D) Comparing the S_NAr and Michael Addition Reactions. To investigate how the mutations introduced in GST A1-1 affect a mechanistically distinct reaction, the electrophile NCA (Figure 1B) was also tested as substrate. This α,β-unsaturated carbonyl compound undergoes a Michael addition reaction with nucleophiles such as GSH (eq 3). In distinction from the S_NAr reaction, the Michael addition does not yield a net release of a proton.



(3)

The T68V mutant, which shows 3−6-fold decreased k_{cat} values for the aromatic substitution reactions, is apparently unaffected regarding k_{cat}^{NCA} as compared to the wild-type enzyme. The insensitivity of k_{cat}^{NCA} to the T68V mutation, in contrast to the S_NAr reactions analyzed, suggests that the Michael addition reaction relies on different rate-limiting steps as compared to the S_NAr type of reactions.

CONCLUSION

In summary, the combination of structural and functional studies of GST A1-1 provides evidence for direct participation in the catalytic mechanism of several functional groups of the GSH molecule. The sulfhydryl group of GSH has previously been recognized as an obvious participant in the catalyzed reaction. The present work shows that both α-carboxylates of GSH have functions in addition to being involved in the binding of GSH to the G-site; the Glu carboxylate may serve as a base in the ionization of the sulfhydryl group, and the Gly carboxylate may modulate the affinity for the second substrate via indirect interactions with the C-terminal α-helix of the H-site. Thus, GSH not only is a reactant in the catalyzed reaction but also contributes functional groups to the catalytic apparatus. In this manner GSH simultanously serves as both a substrate and a cofactor for the enzyme.

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