Mechanism-Based Phage Display Selection of Active-Site Mutants of Human Glutathione Transferase A1-1 Catalyzing S_NAr Reactions†

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ABSTRACT: A library of active-site mutants has been constructed by targeting selected amino acid residues in human glutathione transferase (GST) A1-1 for random mutagenesis. The mutated residues are suitably positioned for interaction with the second, electrophilic substrate, in particular chloronitrobenzene derivatives undergoing S_NAr reactions. DNA representing the GST A1-1 mutant library was fused with DNA encoding gene III protein, a component of the coat of filamentous phage. Phage display was used for affinity selection of GST A1-1 mutants with altered catalytic properties. The affinity ligand used was the σ-complex of 1,3,5-trinitrobenzene and glutathione immobilized to Sepharose. The complex was designed to mimic the transition state of S_NAr reactions catalyzed by GSTs. The selection system is based on the combination of affinity for the σ-complex as well as the ability to promote its formation, thus mimicking two salient features of the assumed catalytic mechanism for the S_NAr reactions. Many of the GST A1-1 mutants selected and analyzed contained an aromatic amino acid residue in one of the mutated positions, suggesting favorable interactions with the trinitrocyclohexadienate moiety of the affinity ligand. A mutant C36 was selected for more detailed studies. Its catalytic efficiency with several chloronitrobenzene substrates was 20−90-fold lower than that of wild-type GST A1-1, but fully comparable to naturally evolved GSTs of different classes, providing a 10^5-fold rate enhancement over the uncatalyzed reaction. In the conjugation of ethacrynic acid, a Michael addition reaction, mutant C36 was 13-fold more efficient than the wild-type enzyme. Within experimental error, the quotient between the K_F values for wild-type GST A1-1 and mutant C36 is the same as that between the k_{cat}/K_M values determined with 1-chloro-2,4-dinitrobenzene for the two enzyme forms. This result indicates that σ-complex formation is rate-limiting for the catalyzed reaction. Thus, the principle of transition-state stabilization as a component of catalysis has been successfully exploited in affinity selection of catalytically competent GST A1-1 mutants. This mechanism-based procedure also selects for the ability to promote σ-complex formation, and serves as a probe of the catalytic mechanism.

Nucleophilic aromatic substitution, S_NAr,[1] reactions occur by a bimolecular addition−elimination mechanism (Ingold, 1953). The reaction rate is dependent on the chemical nature of the nucleophile, which can be negatively charged or neutral, as well as the electrophilic site of attack on the aromatic compound. The reactivity of the aromatic nucleus is governed by electron withdrawing substituents such as nitro groups in *ortho* and *para* positions to the leaving group. An S_NAr reaction is believed to pass through a high-energy intermediate, a Meisenheimer or σ-complex, in which the carbon undergoing attack in the aromatic ring is no longer sp^2 (planar) but rather sp^3 hybridized (tetrahedral). Such distortion of the aromatic structure may be a rate-limiting process, in which case promotion of σ-complex formation is expected to result in an increased reaction velocity.

This study focuses on the reaction of the glutathione (GSH; γ-L-Glu-L-Cys-Gly) with nitro-substituted benzene derivatives. Figure 1A shows the nucleophilic attack of the GS^- thiolate on 1-chloro-2,4-dinitrobenzene (CDNB). GS^- can also react to form a σ-complex with 1,3,5-trinitrobenzene (TNB) (Figure 1B), but since the hydride ion is a poor leaving group, the reaction with TNB will not proceed to product and results in a dead-end complex. This σ-complex (an equilibrium product) can be viewed as a transition-state analog, since it exhibits steric and electronic features of the postulated transition state in the S_NAr reaction between GSH and CDNB and other aromatic nitro compounds (Ingold, 1953).

The glutathione transferases (GSTs) are dimeric phase II detoxication enzymes in which each of the 25 kDa subunits contains one active site. The active site of GSTs can be divided into a G-site, where specific binding of GSH occurs, and an H-site, where the electrophilic, hydrophobic substrate binds (Mannervik *et al.*, 1978). The latter site is less specific and can accommodate a variety of different compounds. Residues contributing to the H-site have therefore been targeted for mutagenesis in order to elucidate structure−function relationships in their contribution to catalysis of reactions involving different electrophilic compounds.

The enzyme family of GSTs are known to catalyze S_NAr reactions. The reaction between GSH and CDNB (Figure

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[1] Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; oCF_3CDNB, 2,4-dinitro-5-trifluoromethyl-1-chlorobenzene; cfu, colony forming units; DCNB, 1,2-dichloro-4-nitrobenzene; GSH, glutathione; GST, glutathione transferase; NBDC, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; S_NAr, nucleophilic aromatic substitution; TNB, 1,3,5-trinitrobenzene.

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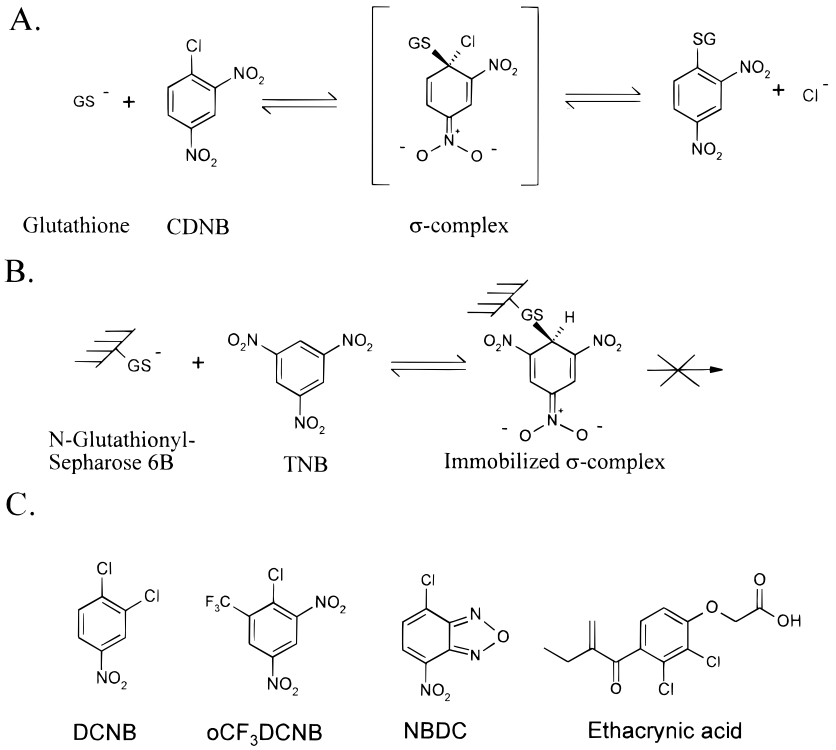

FIGURE 1: (A) Nucleophilic aromatic substitution (S_NAr) reaction: glutathione reacting with 1-chloro-2,4-dinitrobenzene (CDNB). (B) Formation of semistable *σ*-complex of GSH and 1,3,5-trinitrobenzene (TNB) on a Sepharose matrix [*cf.* Graminski *et al.* (1989)]. (C) Structural formulas of other GST substrates: 1,2-dichloro-4-nitrobenzene (DCNB), 2,4-dinitro-5-trifluoromethyl-1-chlorobenzene (oCF_3-CDNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBDC), and ethacrynic acid.

1A) is used as a standard assay in studies of GSTs (Habig *et al.*, 1974) and is therefore well studied. Different techniques have been used to characterize the active site of GSTs in order to explain the catalytic mechanism in the S_N-Ar reactions. Many GST mutants involving residues in the active site have been constructed. Most of the constructs have focused on the binding and the mechanism of deprotonation of GSH, providing the nucleophilic thiolate GS^-, whereas others have revealed the importance of different residues in interactions with the electrophilic substrate. The stabilization of the *σ*-complex of GSH and TNB, 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate, by the enzyme can conveniently be monitored by visible absorption spectroscopy (Graminski *et al.*, 1989), and the structure of an enzyme-bound complex has been determined (Ji *et al.*, 1993).

In the present work, a phage display system was used as an approach to studies of the binding of aromatic compounds to the active site of GSTs. Phage display is a powerful selection tool in biochemistry [*cf.* Choo and Klug (1995), O'Neil and Hoess (1996), and Dunn (1996)]. Libraries of random peptides as well as of mutants of larger functional proteins have been expressed fused to the surface of phage, where each phage carries the variant DNA corresponding to a single peptide sequence. Proteins with high binding affinity and specificity have been obtained by selection based on immobilized ligands. However, the application of the phage display technique to enzymes and their mechanisms of action is largely unexplored. In the present study, a library of GST A1-1 variants, randomly mutated at targeted residues in the H-site, was used for selection of mutants with novel catalytic properties on the basis of their ability to stabilize and bind to an immobilized *σ*-complex of GSH and TNB (Figure 1B). The aim was to generate GST A1-1 mutants with altered catalytic properties and to establish a possible correlation between the extent of *σ*-complex stabilization and the ability to catalyze corresponding S_NAr reactions, such as the conjugation of GSH with CDNB (Figure 1A).

EXPERIMENTAL PROCEDURES

Construction of Phagemid Library. The library was constructed and phagemid was prepared essentially as described for production of recombinant "Phab" fragments using the vector pComb3 (Barbas *et al.*, 1991) with modifications as follows. cDNA encoding a two-histidine mutant of human GST A1-1 (Yilmaz *et al.*, 1995) was used as template in PCR with primers RAN11 (5′-CGG GAT CCC TCG AGA TGG CAG AGA AGC CCA AGC TCC ACT ACN NSA ATN NSC GGG GCA GAA TGG AG) and RAN14 (5′-TTT TTT AAG CTT GGC ATC TTT TTC CTC AGG TGG ACA TAC GGG CAG SNN SNN GAT CAT TTC ACC CAA) (S = G/C). The product was digested with *Xho*I and *Hin*dIII and ligated into a derivative of pComb3 [derivative described in Widersten and Mannervik, (1995)]. The phagemid vector contained the cDNA of wild-type human GST A1-1 (Stenberg *et al.*, 1992) subcloned into the *Xho*I and *Spe*I sites. The wild-type *Xho*I/*Hin*dIII fragment had been removed from the vector by digestion and separation on agarose gel. The ligation was run 16 h at room temperature with a molar ratio of vector to fragment of 1:5. The ligase was then heat inactivated for 10 min at 70 °C, and DNA in the ligation mixture was precipitated with sodium acetate/ethanol two times prior to transformation of electrocompetent *E. coli* XL1-Blue (Stratagene, La Jolla, CA) by electroporation. Further steps in the preparation of phage have been described elsewhere (Widersten & Mannervik, 1995).

Characterization of Phage Preparations. Phages were reprecipitated in order to decrease the large amount of bovine

serum albumin (10 mg/mL BSA) in the preparation, which would otherwise interfere with analyses such as SDS−PAGE. Precipitation was performed by addition of 4% (w/v) PEG 8000 and 3% (w/v) NaCl and incubation on ice for 30 min. Phages were pelleted by centrifugation at 16000*g* for 20 min. Precipitated phages were suspended in PBS (140 mM NaCl, 3.7 mM KH_2PO_4, and 11.2 mM Na_2HPO_4, pH 7.2). Protein quantification was performed as described by Peterson (1977). Protein (approximately 50 μg) obtained from phages was subjected to SDS−PAGE and western blot analysis using affinity-purified rabbit anti-human GST A1-1 antibodies (Hao *et al.*, 1994) and enhanced chemiluminescence detection (Amersham International, Amersham, Buckinghamshire, U.K.).

The GST activity with CDNB of phage preparations from different rounds of affinity selection was determined by addition of 50 μL of phage preparation to a reaction mixture containing 1 mM GSH and 1 mM CDNB in 0.1 M sodium phosphate, pH 6.0, and incubation at room temperature (22 °C) for 30 min. The absorbance at 340 nm was determined every 10 min in triplicate. The nonenzymatic reaction was monitored in parallel.

Immobilization of Ligand. Glutathione disulfide (GSSG, 0.30 g) was incubated with 9 mL of a slurry of swollen epoxy-activated Sepharose 6B (Pharmacia Biotech, Uppsala, Sweden) in 0.1 M sodium carbonate, pH 10, for 40 h at 37 °C. After unreacted GSSG was washed off with 5 gel bed volumes of carbonate buffer, unreacted epoxy groups were blocked with 1 M ethanolamine, pH 9. Prior to use, the immobilized GSSG was reduced by addition of 500 μL of 15 mM dithiothreitol to a 200 μL gel aliquot and incubation for 30 min at 37 °C. The gel was washed with deionized water, and the amount of immobilized GSH thiol groups was determined by reaction with 5,5′-dithiobis(2-nitrobenzoic acid) in 0.1 M sodium phosphate, pH 7.4, $\epsilon_{412} = 13.6$ mM^{-1} cm^{-1} (Ellman, 1959).

Affinity Selection by Phage Display. Four successive selection rounds were performed, starting with the described phage display library and using the σ-complex transition-state analog as affinity ligand. A 30 μL amount of phage library (10^{12} cfu/mL) was incubated with 50 μL of GSH-Sepharose and 0.1 mM TNB in 0.1 M sodium phosphate, pH 7.0, for 3 h at 37 °C. The gel was centrifuged at 7000*g* for 15 s and washed 3 times with the same buffer containing 4 mM TNB and 0.5% (v/v) Tween-20. Phages remaining bound to the Sepharose matrix were eluted by incubation with 100 μL of 0.1 M glycine-HCl, pH 2.2, for 10 min at room temperature (22 °C). The eluate was neutralized with 6 μL of 2 M Tris base and subsequently used for infection of 3 mL of *E. coli* XL1-Blue ($OD_{600} = 1$) for 15 min. After infection, the bacteria were transferred to 10 mL of 2TY medium [1.6% (w/v) tryptone, 1% (w/v) yeast extract, and 0.5% (w/v) NaCl, pH 7.2] containing 10 μg/mL tetracycline and 20 μg/mL ampicillin. After 1 h of incubation the ampicillin concentration was increased to 50 μg/mL. One hour later, the culture was transferred to 100 mL of 2TY with the same antibiotics and approximately 10^{11} plaque forming units of M13K07 helper phage were added. After 2 h of incubation, kanamycin was added to 70 μg/mL and incubation was continued overnight. The phage particles were precipitated with 3% (w/v) NaCl, 4% (w/v) polyethylene glycol 8000, and were redissolved in 1% (w/v) BSA in PBS containing 0.02% (w/v) NaN_3. The ratio of the total amount of phage particles added and eluted during the selection was titrated by plating infected *E. coli* XL1-Blue on LB-ampicillin plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) bacto-agar, 50 μg/mL ampicillin]. To further monitor the selection process, plasmid DNA was prepared from 10 randomly picked clones from the third and fourth rounds of selection (clones C1−C10) and the sequences of the cDNA inserts were determined.

Screening for Enzymatic Activity in Bacterial Cell Lysate of Sampled Clones. In the screening for catalytically active mutants in the output population from the fourth round of selection, 40 randomly picked clones were each grown in 2 mL of 2TY supplemented with 50 μg/mL ampicillin until log phase was reached. Transcription from the *lac* promoter was induced with 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG), and the bacteria were grown overnight. Cells were harvested by centrifugation at 5000*g*, resuspended in 250 μL of PBS, and lysed by ultrasonication. Cell debris were removed by centrifugation at 14000*g* for 10 min. The enzymatic activity with GSH and CDNB was measured at 340 nm in a microtiter plate reader (Molecular Devices, Menlo Park, CA), each well containing 50 μL of bacterial cell lysate, 1 mM GSH, and 1 mM CDNB in a total volume of 300 μL in 0.1 M sodium phosphate, pH 6.0. Lysate containing GST A1-1 wild-type enzyme expressed from the same pComb3 vector and cell lysate of cells containing no plasmid (grown without ampicillin) were run in parallel as controls. In order to estimate the specific activity with CDNB, clones C4, C5, C8, and C9 were expressed in 100 mL of 2TY medium. In addition, mutant C36, as a representative of clones identified as positive in the screening for catalytic activity, was expressed in 500 mL of medium. In all cases 50 μg/mL ampicillin was included in the medium. The cultures were grown to log phase and induced with IPTG, and cell lysates were prepared as above. The lysates were desalted on Sephadex G-25 PD-10 columns (Pharmacia Biotech), and the amount of GST A1-1 protein was determined by ELISA (Hao *et al.*, 1994).

Subcloning, Expression, and Purification of Mutant C36. The cDNA encoding mutant C36 was subjected to PCR using primers A1C-TERM (5′-TTT TTT TCT AGA TTA TTA AAA CCT GAA AAT CTT CCT TG) and C3A15′ (Widersten & Mannervik, 1995) in order to copy the DNA from the pComb3 vector. The PCR product was digested with *Eco*RI and *Xba*I, purified on agarose gel, and ligated into pGΔETac digested with the same enzymes. *E. coli* XL1-Blue cells were transformed with the ligation mixture and the cDNA was subsequently sequenced in order to rule out PCR artifacts. The mutant enzyme was expressed in *E. coli* XL1-Blue and cell lysate was obtained essentially as described (Widersten *et al.*, 1991) with the difference that the buffer used throughout the procedure was 20 mM sodium phosphate, pH 7.0. The cell lysate was desalted on Sephadex G-25 equilibrated with the same phosphate buffer and the enzyme was purified by *S*-hexylglutathione Sepharose affinity chromatography (Mannervik & Guthenberg, 1981). The column was washed with 20 mM sodium phosphate containing 0.2 M NaCl. Bound protein was eluted by 50 mM glycine-NaOH (pH 10) and neutralized by addition of 1/10 volume 2 M Tris-HCl (pH 7.4). The purified enzyme was concentrated by ultrafiltration and dialyzed against 2 × 4 L of 25 mM sodium phosphate, pH 7.8. The concentration of

the mutant enzyme was determined spectrophotometrically using $\epsilon_{280} = 30\,000\ M^{-1}\ cm^{-1}$ (determined for GST A1-1 mutant M208W by amino acid analysis; Widersten *et al.*, 1994).

Activity Measurements. Steady-state kinetics measurements were carried out on Varian 2290 and Shimadsu UV-2501PC spectrophotometers at 30 °C. The assays of the S_NAr reactions (Figure 1) were performed according to references: CDNB (Habig *et al.*, 1974), DCNB (Booth *et al.*, 1961), oCF_3CDNB (Widersten *et al.*, 1996), and NBDC (Ricci *et al.*, 1994). The assays for the reactions of GSH with ethacrynic acid (Habig *et al.*, 1974), cumene hydroperoxide (Lawrence & Burke, 1976), and Δ^5-androstene-3,-17-dione (Benson *et al.*, 1977) were carried out as described. In the determinations of the kinetic parameters, k_{cat}, K_M and k_{cat}/K_M, the GSH concentration was kept constant at a saturating value of 2.5 mM, except for ethacrynic acid as second substrate, for which 1.0 mM of GSH was used. The concentrations of the following electrophiles were varied: CDNB, 0.10−2.0 mM; DCNB, 0.50−1.0 mM; oCF_3CDNB, 2.5−500 μM; NBDC, 2.5−500 μM; and ethacrynic acid, 10−200 μM. The nonenzymatic second-order rate constants, k_2, were calculated using linear regression analysis. The Michaelis−Menten equation was fitted to experimental data with the SIMFIT program package (Bardsley *et al.*, 1989). When k_{cat} and K_M could not be determined separately due to a high K_M value in comparison to the experimentally attainable substrate concentration, k_{cat}/K_M was calculated from initial rates determined at low levels of saturation with the substrate [*cf.* Danielson and Mannervik (1985)]. The formation constant of σ-complex in the active site, K_F, was calculated as described by Graminski *et al.* (1989) based on difference spectroscopy measurements with 2.5 mM GSH and 0.070−1.2 mM TNB in 0.1 M sodium phosphate, pH 6.5. Mutant C36 (16 μM) was included in the sample cuvette. TNB was kindly provided by Mr. Göran Karlsson, Nobel Chemicals AB, Karlskoga, Sweden.

Computer Modeling. The amino acid residues in available models of three-dimensional structures of wild-type GST A1-1 (Sinning *et al.*, 1993; Cameron *et al.*, 1995) were replaced by those of mutant C36 using the program O (Jones *et al.*, 1991). The structure of the σ-complex of GSH and TNB in the active site of rat GST M1-1 isoenzyme (Ji *et al.*, 1993) was modeled in the active site of GST A1-1 using the InsightII package (Biosym/MSI, San Diego, CA). The cysteinyl residues of the different glutathione conjugates (σ-complex, ethacrynic acid conjugate, and *S*-benzylglutathione) in the active site were superimposed in order to probe the spatial differences of their *S*-substituents in the context of the active site of the mutant. Modeling was performed on Silicon Graphics Indy or Indigo2 Extreme workstations.

RESULTS

Characterization of the GST A1-1 Fusion Protein. Phage particles carrying the plasmid pComb3A1, designed to express wild-type GST A1-1 linked to the phage gene III coat protein, contained an immunodetectable component with the electrophoretic mobility expected for the GST A1-1 fusion protein. The same component was not found in protein from phage particles not containing the GST A1-1 cDNA, pComb3ΔNX (data not shown). Freshly prepared filamentous phage displaying wild-type GST A1-1 exhibited detectable enzymatic activity with CDNB (data not shown). The activity determined with the GST A1-1 fusion protein appeared not to be due to free soluble enzyme liberated by hydrolysis of the linker region between the GST A1-1 subunit and the gene III protein, since no detectable amounts of free GST A1-1 subunits were present in preparations of phages carrying the fusion protein.

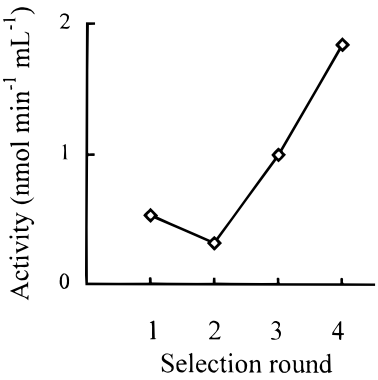

FIGURE 2: GST activity of phage preparations from each successive round of selection. Assays were made in triplicate with CDNB and GSH at pH 6.0, 22 ° C, in the crude phage preparations as described in the Experimental Procedures. Symbols represent means (SD ≤ 21% of the measured values) after subtraction of nonenzymatic reaction rates for each of the four successive rounds.

Construction of Phagemid Library. The library of GST A1-1 active-site mutants was constructed by randomization of residues 10, 12, 107, and 108 and the number of individual clones was determined. The library contained approximately 5×10^6 individuals as estimated by counting the number of cfu from dilutions of the transformed bacterial culture, after subtracting the estimated background of phagemid vectors not containing any cDNA. Sequence analysis demonstrated substitutions in the four targeted codons in DNA from 13 out of 16 randomly picked clones from the library. One clone contained the wild-type sequence and two consisted of vector without cDNA, making up slightly more than 10% of the total number of clones.

Selection by Phage Display and Screening for CDNB Activity in Cell Lysates. The phage library of GST A1-1 active-site mutants was subjected to affinity selection by use of the immobilized σ-complex of GSH and TNB. Bound phages were eluted with acid and propagated in bacteria. The selection round was run four times in total. The enzymatic activity determined with GSH and CDNB increased in the phage preparations as a function of the number of selection rounds (Figure 2). Table 1 presents cDNA sequences of clones isolated after three and four rounds of affinity selection. A number of clones were expressed at a larger scale, making GST quantification by ELISA possible. The specific activity measured in lysates of bacteria harboring mutants C4, C5, C8, C9, and C36 ranged between 10% and 30% of that of the GST wild-type A1-1. The most active mutant, C4, proved unstable upon purification, and more detailed studies were restricted to mutant C36 showing 19% of the wild-type activity in crude form.

Characterization of Mutant C36. Mutant C36 (GST A1-1: F10P, A12W, L107F, L108R) was expressed at a larger scale and purified to homogeneity, as judged by SDS−PAGE and silver-staining (data not shown). From a three liter bacterial batch culture, 24 mg of pure enzyme was obtained. The specific activity with CDNB was determined to be 10

Table 1: Sequences of GST A1-1 Mutant Clones Obtained by Mechanism-Based Affinity Selection

	DNA sequence *(codon position)*				Deduced amino acid			
	10	*12*	*107*	*108*	*10*	*12*	*107*	*108*
wild-type	T T C	G C A	C T C	C T T	F	A	L	L
clones isolated from panning[a]								
C1	C C C	A T G	C T G	T G C	P	M	L	C
C2	G T G	T A C	T C C	C T G	V	Y	S	L
C3	T A G	G T G	T G C	C C C	Q	V	C	P
C4	G C C	G G G	T G C	G G C	A	G	C	G
C5	A T G	C C C	T G G	T A G	M	P	W	Q
C6	T T G	A G G	C C G	T G G	L	R	P	W
C7	G T G	T A C	A C G	C T G	V	Y	T	L
C8	T G G	G A C	C T G	A C G	W	D	L	T
C9	G C C	T G G	A C C	C A C	A	W	T	H
C10	G A C	C A G	C G G	G T C	D	Q	R	V
C36	C C G	T G G	T T C	A G G	P	W	F	R

[a] Clones C1−C5 isolated after three rounds and C6−C36 after four rounds of panning.

Table 2: Specific Activities (μmol min^{-1} mg^{-1}) of Mutant C36 and Wild-Type GST A1-1

substrate	mutant C36	wild-type
CDNB	10 ± 0.6	80
DCNB	0.006 ± 0.001	0.2
Δ^5-androstene-3,17-dione	0.47	22
ethacrynic acid	1.8 ± 0.1	0.24 ± 0.03
cumene hydroperoxide	1.5 ± 0.1	10 ± 0.3

μmol min^{-1} mg^{-1} at pH 6.5. The specific activities determined with other commonly used GST substrates (Figure 1) as well as steady-state kinetic parameters for S_NAr reactions were also determined (Tables 2 and 3). C36 enhances the rate of conjugation (k_{cat}/K_M) of GSH with each of the four nitrosubstituted chlorobenzenes tested by about 10^5-fold at pH 6.5 as compared to the uncatalyzed reaction (k_2).

Mutant C36 facilitated the formation of the σ-complex between GSH and TNB in the active site (Figure 3, Table 3). The k_{cat}/K_M for the Michael addition of GSH to ethacrynic acid was determined to be 5.6×10^4 M^{-1} s^{-1}, about 13-fold higher than that of the wild-type enzyme. The I_{50} value of ethacrynic acid used as an inhibitor for mutant C36 (35 μM) in the CDNB assay was 3 times higher as compared to that of GST A1-1 (11 μM).

DISCUSSION

Application of Phage Display to Enzymology. Previous applications of phage display to proteins have primarily involved studies related to the binding properties of antibody fragments, such as Fabs or single-chain Fv fragments. In studies more focused on the action of enzymes, the phage display methodology has been used mainly for expression of enzyme inhibitors rather than the enzymes themselves (Wang *et al.*, 1995; Markland *et al.*, 1996) in order to isolate inhibitors of altered specificities or binding strength. Further, the substrate specificities of enzymes such as proteases (Matthews & Wells, 1993; Matthews *et al.*, 1994) and protein kinases (Schmitz *et al.*, 1996) have been explored by phage display of peptide substrate libraries. However, actual expression of enzymes or catalytic Fabs has been reported only in a few cases (McCafferty *et al.*, 1991; Soumillion *et al.*, 1994; Janda *et al.*, 1994; Widersten & Mannervik, 1995; Maenaka *et al.*, 1996), and in even fewer cases has the displayed enzyme or catalytic antibody been proven functional on the phage particle. In only one study, to our knowledge, has the selective power of monovalent phage display been applied in the design of enzymes with new properties from libraries of mutants randomly altered structurally in the enzyme active site (Widersten & Mannervik, 1995).

In general, significant contribution to the catalytic power of enzymes is afforded by their ability to selectively stabilize the transition state (Pauling, 1946). Support for this contention is provided by the successful design of catalytic antibodies (Schultz & Lerner, 1995) by selecting for immunoglobulins with affinity for transition-state analog haptens. The S_NAr reaction studied here includes an unstable σ-complex close to the transition state on the reaction coordinate. This σ-complex has now been exploited for mechanism-based selection of catalytically competent variants of GST A1-1 from a library of active-site mutants.

Rationale for the Choice of Transition State Ligand. The ligand used for selection derives from an equilibrium mixture of immobilized GSH and soluble TNB forming an immobilized σ-complex, 1-(*S*-glutathionyl)-2,4,6-trinitrocyclohexadienate. This complex is thought to mimic an intermediate in corresponding S_NAr reactions with chloronitrobenzenes (Figure 1) and resemble the postulated transition state on the reaction coordinate. Earlier attempts to select active GST A1-1 mutants using phage display with aromatic nitrocompounds as affinity ligands have met with some success (Widersten & Mannervik, 1995), but the ligands did not display the distorted ring structure characterizing a proper σ-complex. The present study has, therefore, carried the probing of the catalytic mechanism of GSTs a significant step further. The formation of a σ-complex has been proposed to be rate-determining step in the uncatalyzed reaction (Ingold, 1953). The σ-complex of TNB was first shown to be formed in the active site of rat GSTs M1-1 and M2-2 (earlier called GSTs 3−3 and 4−4; Graminski *et al.*, 1989) and has later also been demonstrated in human GST P1−1 (Bico *et al.*, 1994) and A1-1 (Widersten *et al.*, 1996).

It is noteworthy that the spontaneous reaction between GSH and TNB occurs only to a limited extent ($K_{eq} = 28$ M^{-1}; Gan, 1977), whereas binding to the GST protein promotes the formation of the σ-complex (*cf.* Table 3). Thus, the procedure used for selection of GST mutants is based both on high affinity for the immobilized σ-complex and the ability to promote its formation. These combined features are expected to facilitate the identification of mutants with the ability to catalyze the studied S_NAr reactions.

Support for the Use of σ-Complex as Transition State Analog. It has been shown that a correlation exists between the propensity of zwitterionic detergent molecules (some of which catalyze S_NAr reactions) to stabilize the σ-complex between TNB and GSH and their catalytic efficiency in the reaction involving GSH and CDNB (Lindkvist *et al.*, 1997). The detergent cetyl trimethylammonium bromide, which stabilizes the GSH-TNB complex 7100-fold more strongly than does *N*,*N*-dimethyllaurylamine-*N*-oxide, also catalyzes the conjugation of GSH to CDNB 100-fold more efficiently than the latter.

Construction of the Library of Active-Site Mutants. In GST A1-1, 15 amino acid residues make up the surface in contact with the ligand bound in the H-site (Sinning *et al.*,

Table 3: Kinetic Parameters for Mutant C36 and Wild-Type GST A1-1 and Binding Parameters for σ-Complex Binding

substrate/enzyme	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (s^{-1} M^{-1})
CDNB, pH 6.5			
wild-type[a]	88 ± 3	0.56 ± 0.04	$(1.6 \pm 0.08) \times 10^5$
mutant C36	*b*	*b*	$(6.7 \pm 0.2) \times 10^3$
DCNB, pH 8.0			
wild-type	*b*	*b*	69 ± 10
mutant C36	*b*	*b*	3.7 ± 0.3
oCF_3CDNB, pH 6.5			
wild-type[a]	69 ± 2	0.084 ± 0.008	$(8.2 \pm 0.6) \times 10^5$
mutant C36	9.8 ± 1.9	1.1 ± 0.3	$(9.1 \pm 3.1) \times 10^3$
NBDC, pH 5.0			
wild-type	17 ± 0.78	$(2.9 \pm 0.9) \times 10^{-3}$	$(5.8 \pm 2) \times 10^6$
mutant C36	10 ± 0.36	0.13 ± 0.01	$(7.9 \pm 0.8) \times 10^4$
ethacrynic acid, pH 6.5			
wild-type	0.19 ± 0.02	0.048 ± 0.016	$(3.9 \pm 1.4) \times 10^3$
mutant C36	0.83 ± 0.04	0.016 ± 0.004	$(5.3 \pm 1.2) \times 10^4$
TNB; σ-complex stabilization, pH 6.5			
wild-type [a]	$K_F = (8.9 \pm 1.4) \times 10^3$ M^{-1}	$\epsilon_{450} = (8.5 \pm 0.3) \times 10^3$ M^{-1} cm^{-1}	
mutant C36	$K_F = (4.1 \pm 1.1) \times 10^2$ M^{-1}	$\epsilon_{450} = (7.1 \pm 0.8) \times 10^3$ M^{-1} cm^{-1}	

[a] From Widersten *et al.* (1996). [b] Not possible to determine due to low degree of saturation. The second-order rate constant for the nonenzymatic reactions (s^{-1} M^{-1}) measured under the same experimental conditions were: CDNB, 0.015; oCF_3CDNB, 0.12; NBDC, 0.24; and ethacrynic acid, 0.80.

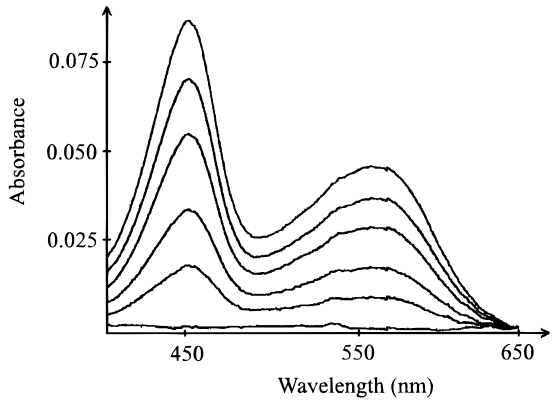

FIGURE 3: Absorption spectra of σ-complex in the active site of GST A1-1 mutant C36. The spectra were measured in the presence of 2.5 mM GSH, 16 μM enzyme, and (from bottom to top) 0.0, 0.28, 0.48, 0.69, 0.96, and 1.2 mM 1,3,5-trinitrobenzene.

1993). In order to increase the variability of the hydrophobic pocket of the active site, the H-site, four of these residues were randomly mutated (Figure 4). Two of the amino-acid side chains are located in a loop close to the N-terminus (Phe10 and Ala12), whereas the other two residues are situated in α-helix 4 (Leu107 and Leu108). All four residues are located in proximity to the benzene ring of an active-site ligand, S-benzylglutathione (Sinning *et al.*, 1993), which is expected to define the binding site of the aromatic electrophilic substrate. Other GSTs, belonging to the same structural class as GST A1-1, have different substrate specificity profiles, presumable mainly due to the structural differences in their H-site. Consequently, it should be possible to find H-site mutants with altered catalytic efficiencies for a particular reaction, such as the S_NAr reaction.

In the design of the mutant enzyme library, the number of residues targeted for mutagenesis was restricted to four in order for all possible variants to be included in the library. Approximately 5×10^6 individuals were scored in the constructed library, a number well exceeding the theoretically calculated figure of possible combinations of four mutated codons ($20^4 = 1.6 \times 10^5$ combinations at the protein level, or $32^4 \approx 1.0 \times 10^6$ combinations at the DNA level), which should ensure good coverage of all possible variants in the library. The catalytic activity of a randomly chosen mutant in the library is assumed to be low. In general, introduction of a few random mutations of residues in the active site of an enzyme is expected to cause a loss of function [*cf.* Delagrave and Youvan (1993)]. Experience from previous mutagenesis of the H-site in GST A1-1 in our laboratory supports this assumption (data not shown).

Results of Selection. The GST activity with GSH and CDNB increased as a function of selection round (Figure 3), but the absolute amount of GST fusion protein could not be determined due to interfering components in the phage preparation. The average specific activity could therefore not be estimated. The study of mutations found after affinity selection (Table 1) shows that more than half of the clones analyzed contain aromatic residues. Statistically, Trp is represented by one of 32 possible codons obtained by use of the the base combination NNS (N = any base; S = C or G) in the library construction, but five out of the eleven mutants isolated were found to contain Trp. This apparent overrepresentation may be due to favorable interactions of Trp with the trinitrohexadienate moiety of the ligand used in the affinity selection. A comparison with corresponding amino acid positions in other GST isoenzymes (Table 4) reveals the frequent presence of aromatic residues in the active site, predominately in positions 10 and 111 (residue 111 is also contributing to the H-site). Structural analyses of antibodies selected for binding of dinitrophenyl-derivatives have shown hydrophobic binding pockets with π-π interactions between Trp residues and the bound hapten [NMR studies by Anglister *et al.* (1987) and Gettins *et al.* (1978); X-ray diffraction analysis by Mitzutani *et al.* (1995)]. Also Phe and Tyr were found in van der Waals contact with dinitrophenyl groups in the antibody structures. Aromatic amino acids consequently are prime candidates for the generation of protein binding sites for nitrosubstituted aromatic ligands.

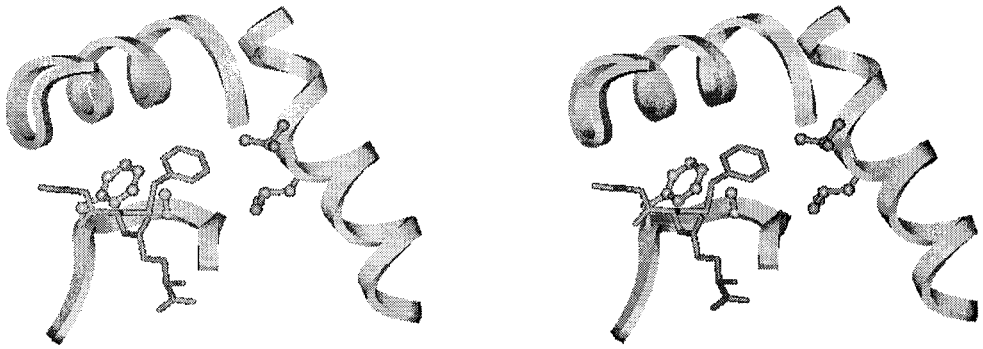

FIGURE 4: Schematic view of *S*-benzylglutathione bound to the active site of wild-type GST A1-1 (Sinning *et al.*, 1993). Four residues contributing to the hydrophobic pocket are outlined in ball-and-stick representation. Phe 10 and Ala 12 are situated in a loop close to the N-terminus (lower left), while Leu 107 and Leu 108 are found in α-helix 4 (right). The C-terminal α-helix (upper left), a third element of secondary structure contributing to the hydrophobic pocket (H-site), is also displayed.

Table 4: Comparison of GST A1-1 Mutant C36 with Various Wild-Type GST Sequences and Their CDNB Activities

amino acid[a] in GST	9	10	11	12	13	106	107	108	109	110	111	specific activity (μmol min^{-1} mg^{-1})	k_{cat}/K_m (mM^{-1}s^{-1})
hA1-1	Y	F	N	A	R	I	L	L	L	P	V	80	160[b]
mutant C36	.	P	.	W	.	.	F	R	.	.	.	10	6.7
hA2-2	.	S	.	I	.	.	.	.	.	.	F	80	
rA1-1(1-1)	.	.	.	.	.	.	I	Q	.	V	I	45	126[c]
rA4-4(8-8)	.	.	Q	G	.	M	I	G	P	F	P	10	8[c]
hM1-1	.	W	D	I	.	L	G	M	I	C	Y	180	240[d]
hM2-2	.	W	.	I	.	L	A	K	.	C	Y	220	
hM3-3	.	W	D	I	.	L	I	R	.	C	Y	7	
hM4-4	.	W	D	I	.	L	A	R	V	C	Y	1.4	3.2[e]
rM1-1(3-3)	.	W	.	V	.	L	I	M	.	C	Y	58	430[f]
rM2-2(4-4)	.	W	D	I	.	L	A	M	V	C	Y	17	21[f]
hP1-1	.	.	P	V	.	Y	I	S	.	I	Y	110[g]	440[g]

[a] Amino acids are numbered according to the GST A1-1 sequence. Sequences and specific activities from compilations by Hayes and Pulford (1995) and Mannervik and Widersten (1995); "h" and "r" denote human and rat GSTs, respectively, designations within parentheses represent earlier nomenclature, and dots denote residues identical to wild-type GST A1-1. [b] Widersten *et al.* (1994). [c] Björnestedt *et al.* (1995). [d] Widersten and Mannervik (1992). [e] Comstock *et al.* (1994). [f] Graminski *et al.* (1989). [g] Widersten *et al.* (1992).

Structural Characterization of Mutant C36. Mutant C36, displaying a comparatively high enzymatic activity with CDNB in a bacterial cell lysate, contains residues in the mutated positions that are also found in other GSTs. As shown in Table 4, most of the naturally evolved Mu class isoenzymes contain a Trp in the N-terminal loop, denoted as residue 10 (based on the GSTA1-1 sequence), while mutant C36 presents an Ala12 → Trp mutation. The positively charged Arg in position 108 of C36 has counterparts in a few Mu class isoenzymes, where both Arg and Lys can be found in the corresponding position. In the three-dimensional structure of human Mu class GST M2-2, the corresponding Lys residue extends its ammonium group into the surrounding medium, while the methylene groups of the side chain contribute to the H-site of GST M2-2 (Raghunathan *et al.*, 1994). Phe 107 in mutant C36 fills a larger space in the active-site cavity than do the smaller hydrophobic residues found in other GSTs. The distance between Phe 107 and the trinitrocyclohexadienate of the σ-complex of TNB and GSH appears from modeling to be too large for any close $\pi-\pi$ interaction. Table 4 displays only a fraction of the sequence differences between the various GSTs and does not take into account the minor differences in tertiary structure that do exist. Still, the residues discussed all contribute to the H-site of the enzymes and therefore may influence the catalytic properties.

Functional Characterization of Mutant C36. The four substitutions in the active site of mutant C36 have significantly altered the substrate specificity profile as compared to wild-type GST A1-1. The ability to catalyze the reduction of an organic hydroperoxide (cumene hydroperoxide), was decreased by 7-fold, as measured by specific activity (Table 2). Further, the β-keto steroid isomerase activity assayed with Δ^5-androstene-3,17-dione was reduced by a factor of 50 as compared to the wild-type enzyme. The high isomerase activity is a characteristic of wild-type GST A1-1 among the human GST isoenzymes (Mannervik & Widersten, 1995), but the mechanism of this particular reaction has not been elucidated.

In contrast, the catalytic efficiencies in the nucleophilic aromatic substitution reactions (Figure 1) were generally more similar to those of wild-type GST A1-1, as would be expected on the basis of the principle used for mutant selection. Mutant C36 shows a rate enhancement of 10^5-

fold in the S_NAr reaction with CDNB as judged from comparison of the values of k_{cat}/K_M and the second-order rate constant for the uncatalyzed reaction (Table 3). The specific activity with CDNB of the mutant is of the same order of magnitude as naturally evolved GSTs (Table 4) and the catalytic efficiency (k_{cat}/K_M) is not significantly different from that of the homologous rat isoenzyme A1-1, which is structurally closely related to human isoenzyme A1-1. The lower catalytic efficiency of mutant C36 in comparison with the wild-type human GST A1-1 is mainly due to an increased K_M value (one order of magnitude), whereas the k_{cat} values determined are similar to those of wild-type GST A1-1. Also the S_NAr reactions involving DCNB, oCF_3CDNB, and NBDC showed similar catalytic efficiencies as CDNB even though their structures and reactivities vary to different extents (Table 3).

In the study of σ-complex stabilization in the active site, mutant C36 exhibited a 1 order of magnitude lower formation constant for the TNB−GSH complex as compared to the wild-type enzyme, while the maximal molar absorptivity per active site was not significantly different from that of wild-type GST A1-1 (Table 3). Within experimental error, the quotient between the K_F values for wild-type GST A1-1 and mutant C36 (22 ± 6) is the same as that between the k_{cat}/K_M values determined with CDNB (24 ± 1) for the two enzyme forms. The k_{cat}/K_M quotients for the other nitrobenzene substrates are also of the same magnitude. This strongly suggests that σ-complex formation is rate limiting for approach to the transition state in these S_NAr reactions catalyzed by the GST A1-1 enzyme. Further support for this conclusion derives from the positive correlation between the inverse of the K_M values for the electrophilic substrate and the K_F values for the σ-complex of GSH and TNB previously observed with point-mutated active-site variants of GST A1-1 (Widersten *et al.*, 1996). Van der Aar *et al.* (1996) have proposed that the initial phase of σ-complex formation is the rate-limiting step in S_NAr reactions catalyzed by Alpha and Pi class GSTs. In contrast, the kinetics of a Mu class isoenzyme is limited by product release, a step following the σ-complex formation (Johnson *et al.*, 1993).

It is noteworthy that the catalytic efficiency in the reaction between GSH and ethacrynic acid (a Michael addition) was significantly increased in mutant C36 as compared to the wild-type enzyme (Table 3). The specific activity was improved 7-fold and the k_{cat}/K_M value was increased 13-fold. The wild-type GST A1-1 has the ability to bind free ethacrynic acid in an unproductive manner, according to the available models of the three-dimensional structure of the complex (Cameron *et al.*, 1995). The ethacrynic acid moiety of the corresponding glutathione conjugate can also bind in alternative conformations in the active site. In comparison with the wild-type enzyme, mutant C36 exhibits a lower K_M value, but a higher I_{50} value for ethacrynic acid, as determined by steady-state kinetics and inhibition experiment, respectively (Table 3). Computer modeling demonstrates a topologically restricted binding pocket for the substrate in mutant C36 due to introduction of more voluminous residues and suggests that Trp 12 is in a position making possible $\pi-\pi$ interactions with the aromatic ring of ethacrynic acid. A full explanation of the kinetic behavior should account for the physical restriction of the possible binding modes for ethacrynic acid in the active-site cavity.

Conclusion. The present study exploits the principle of transition-state stabilization for construction of a catalytically active protein binding site (Pauling, 1946; Jencks, 1969) for the redesign of human GST A1-1. The assumption that active-site mutants with affinity for the σ-complex of GSH and TNB would catalyze the S_NAr reaction between GSH and related chloronitrobenzene compounds has been verified and thus provided support for transition-state stabilization as a rate-limiting step in catalysis. The selection system used is partly based on binding to a preformed transition-state analog but also includes the important feature of promoting the actual formation of the σ-complex from a low concentration in the equilibrium mixture of GSH and TNB. The promotion of an increased concentration of σ-complex as a contributing factor in the affinity selection affords a mechanism-based design mimicking the addition step of the S_NAr reaction. Thereby, the principle of transition-state binding, previously used for the generation of catalytic antibodies, has been extended to include the preceding formation step and allowed a component of the dynamics characterizing enzyme catalysis to operate in the *in vitro* evolution of the active-site properties. Thus, probing the mechanism of GST A1-1 by exploiting the combination of transition-state stabilization and phage affinity selection contributes both to the understanding of this particular enzyme and introduces a novel approach to selection of catalytically competent variants from a library of mutant proteins.

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