Site-directed Mutagenesis of Tyrosine 108 Residue in Human Glutathione *S*-Transferase P1-1

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In order to study the role of residue in the active site of glutathione *S*-transferase (GST), Tyr 108 residue in human GST P1-1 was replaced with alanine, phenylalanine and tryptophan by site-directed mutagenesis to obtain mutants Y108A, Y108F and Y108W. These three mutant enzymes were expressed in *Escherichia coli* and purified to electrophoretic homogeneity by affinity chromatography on immobilized GSH. The substitutions of Tyr108 significantly affected K_m^{CDNB} and K_m^{ETA}, whereas scarcely affected K_m^{GSH}. The substitutions of Tyr108 also significantly affected I_{50} of ETA, an electrophilic substrate-like compound. The effect of these substitutions on kinetic parameters and the response to inhibition suggests that tyrosine 108 in hGST P1-1 contributes to the binding of the electrophilic substrate and a major determinant in the binding of CDNB is the aromatic ring of Tyr108, not its hydroxyl group.

Key Words : Tyrosinase 108 residue, Enzymatic properties, Glutathione *S*-transferase, Inhibition characteristics, Substrate specificity

Introduction

Glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of multifunctional proteins that participate in the biotransformation of xenobiotics by catalyzing the formation of conjugates between glutathione (GSH) and various electrophilic compounds.[1,2] Certain GSTs can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity.[2] Others catalyze the isomerization of certain steroids and play an important role in the intracellular transport of numerous hydrophobic nonsubstrate ligands such as bile acids, billirubin and a number of drugs.[1]

GSTs are distributed in a wide range of organisms from mammals to *E. coli*.[3] Mammalian cytosolic GSTs, which can exist as homo- or heterodimers, are grouped into at least five distinct classes, alpha, mu, pi, sigma, and theta according to based on studies of substrate specificity and primary structures.[1,4] The GSH-binding site (G-site) and the catalytic mechanism of these enzymes have been the targets of many investigations involving chemical modification,[5-9] site-directed mutagenesis,[10-16] and X-ray crystallographic analysis.[17-21] The extent of the information concerning the precise enzyme-GSH interactions responsible for the catalytic properties has been greatly increased by these studies. On the contrary, the electrophilic substrate-binding site (H-site) of GSTs has remained unclear for a long time. Barycki and Colman provided the first evidence that Tyr 115 contributes to xenobiotic substrate binding of mu class isoenzymes by showing that this residue in isoenzyme 4-4 was modified by 4-(fluorosulfonyl)benzoic acid, a xenobiotic substrate analogue.[22] Pettigrew *et al.* also provided another evidence by affinity labeling study using 4-(fluorosulfonyl)benzoic acid that Tyr 106 of the pig lung class pi GST is located at or close to the substrate binding site of the enzyme.[23] Subsequent crystallographic studies of all five GST classes show that the H-site is quite different among them and very little is known about the key determinants of xenobiotic substrate specificity.[17-21,24] Only a few amino acid residues have been identified as key determinants of the H-site: Tyr 115 in rat mu GST (isoenzymes 3-3 and 4-4)[25,26]; Met 208 in GST A1-1[27]; Ile 104 in GST P1-1[28]; and Val 10, Arg 11, and Val 104 in the murine class pi GST.[29] Ji *et al.* reported Tyr 115 in mu GST locates in domain II on the face of the4-helix that forms one wall of the xenobiotic substrate binding site, the hydroxyl oxygen being about 7.5 from the sulfur of GSH.[26] Tyr 108 of hGST P1-1, the equivalent to Tyr 115 in class mu, is positioned in close contact to the xenobiotic substrate and is a possible candidate for involvement in H-site.

Therefore, in order to examine the importance of Tyr 108 in hGST P1-1, it was substituted with alanine, phenylalanine, and tryptophan by using site-directed mutagenesis, and effects of these substitutions on enzymatic activities were examined in this study.

Materials and Methods

Materials. GSH and 1-chloro-2,4-dinitrobenzene were purchased from Kohjin Co. and Wako Pure Chem. Ind. (Osaka, Japan), respectively. Ethacrynic acid and *S*-hexylGSH were obtained from Sigma (St. Louis, USA). Glutathione Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). All other reagents used were of the highest grade commercially available.

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**Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; ETA, ethacrynic acid; GSH, glutathione; G-site, glutathione-binding site; GST, glutathione *S*-transferase; hGST, human GST; H-site, electrophilic substrate-binding site; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Table 1. Oligonucleotides used for site-directed mutagenesis

Mutant	Sequence of primer[a]	Sense
Y108A	5'-AAATACATCTCCCTCATCGCCACCAA-CTATGAG-3'	Forward
Y108F	5'-AAATACATCTCCCTCATCTTCACCAA-CTATGAG-3'	Forward
Y108W	5'-AAATACATCTCCCTCATCTGGACCAA-CTATGAG-3'	Forward

[a]Changed bases are shown by underlines.

Preparation of mutant enzymes. Wild-type hGST P1-1 was obtained by expression of a cloned cDNA[30] in *E. coli* as described in the previous paper.[16] The oligonucleotide primers used for site-directed mutagenesis of Tyr 108 into Ala, Phe, and Trp are shown in Table 1. Mutagenesis was performed according to the procedure of Kunkel[31] using a Mutant^{TM}-Super Express Km kit (Takara Shuzo Co.). Construction of single-stranded DNA template for mutagenesis, confirmation of mutation, construction of the expression plasmid, and expression of the mutant enzymes were performed as described in the previous paper.[16] The mutant enzymes were expressed in *E. coli* under the control of the *tac* promoter. Cultured cells were lysed, followed by centrifugation. The dialyzed supernatant of the cell lysate was loaded directly onto a 20 mL column of GSH-Sepharose equilibrated with 20 mM potassium phosphate buffer (pH 7.0) (buffer A). The column was extensively washed with the same buffer. The enzyme was eluted with a 50 mM Tris-HCl buffer (pH 8.5) containing 10 mM GSH and dialyzed against buffer A. The dialyzed purified enzyme was used for next experiment.

Determination of protein concentration. Protein concentration of the wild type enzyme was determined by measuring the absorbance at 280 nm as described by Parker[32] and protein concentration of the mutant was determined by using protein assay reagent (Bio-Rad Lab.) and the wild-type enzyme as a standard protein.

Enzyme activity. The specific activities were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) or ethacrynic acid (ETA) as described by Habig and Jakoby.[33] Assays were performed in a HITACHI U-2000 double-beam spectrophotometer (Hitachi CO., Tokyo, Japan) at 30 °C using cuvettes of 1 cm pathlength. Initial rates were measured for 5 min, commencing 10 sec after initial mixing. The reaction was initiated by 20 μL of 50 mM CDNB or ETA to 860 μL of 100 mM potassium phosphate (pH 7.5) containing, in order of addition, 100 μL of 50 mM GSH and 20 μL of the enzyme. Nonenzymatic reaction rates served as controls, and were subtracted from enzymatic rates. CDNB and ETA were dissolved in ethanol. The concentration of ethanol in the reaction mixture (1 mL) was constant at 2% (v/v). GSH was dissolved in H_2O immediately before use and kept in an ice-bath to prevent oxidation. The enzymes were diluted in 20 mM potassium phosphate buffer (pH 7.0) containing 3 mM EDTA, 3 mM 2-mercaptoethanol, and 20% (v/v) glycerol to a concentration that the enzymatic reaction rate was linear with time for up to 60 sec after initiation, and up to a ΔA/min of 0.15. All assays were done in the presence of 0.4% (v/v) glycerol. Conditions were: (a) 1 mM CDNB, 1 mM GSH, 340 nm ($\Delta\varepsilon$ = 9.8 mM^{-1} cm^{-1}) and (b) 0.2 mM ETA, 0.25 mM GSH, 270 nm ($\Delta\varepsilon$ = 5 mM^{-1} cm^{-1}). A unit of activity is defined as the amount of enzyme catalyzing the formation of 1 μmole of product per min under the conditions of the specific assay. Specific activity is defined as the units of enzyme activity per mg of protein.

Kinetic studies. Kinetic studies with GSH and electrophilic substrates were carried out at 30 °C as described by Chen *et al.*[10] Kinetic parameter K_m values were determined under first order conditions at low substrate concentration with respect to the varied substrate: for GSH with a fixed concentration of 1 mM CDNB, and for CDNB with a fixed concentration of 1 mM GSH. The k_{cat} values were calculated on the basis of mol dimeric enzyme using a Mr of 45,000. Other experimental conditions were the same as for determination of specific activities.

Inhibition studies. The inhibitory effects on the activity of the enzyme were measured by preincubating the enzyme with 1 mM GSH and the inhibitor for 2 min and initiating the reaction by addition of 20 μL of 50 mM CDNB (final concentration, 1 mM). The concentration of inhibitor giving 50 % inhibition (I_{50}) was determined from plot of residual activity against inhibitor concentration.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Denaturing SDS-PAGE was carried out by the method of Laemmli[34] in 12.5% gels. Gels were stained with coomassie blue R-250. The molecular-mass markers used were SDS molecule weight standard markers (Bio-Rad) that contained phosphorylase B (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa).

Results

Purification and PAGE of mutant enzymes. To investigate the role of tyrosine 108 in hGST P1-1, it was substituted with alanine (Y108A), phenylalanine (Y108F), and tryptophan (Y108W) by oligonucleotide-directed mutagenesis. The mutant enzymes were expressed in *E. coli* under the control of *tac* promoter. The expressed mutant enzymes were isolated and purified by affinity chromatography on immobilized GSH. The mutants Y108F and Y108W were isolated in a yield of approximately 2 mg per liter of cultures as in the case of the wild type. However, in the case of Y108A, the amount of the isolated enzymes were 1 mg per liter of culture. As compared with the total activity in the crude extract of *E. coli* cell lysate, the activity recoveries for the wild type, Y108F and Y108W were more than 70%, but that for Y108A was less than 30%. Thus, the binding abilities Y108F and Y108W to GSH-Sepharose were not so much different from that of the wild type, but Y108A had low affinities for GSH-Sepharose. The purified wild type and mutants give a single band on SDS-PAGE with an apparent

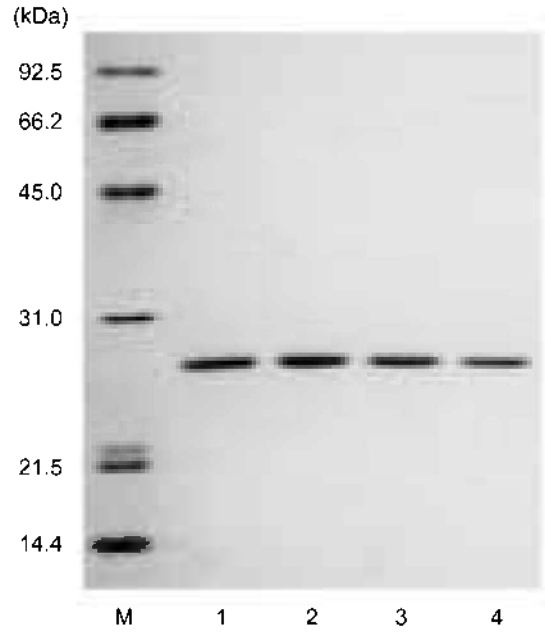

Figure 1. Electrophoresis of recombinant hGSTP1-1 and its mutants under denaturing conditions. Denaturing SDS-PAGE was carried out using the method of Laemmli (Laemmli, 1979) in 12.5% gels. Coomassie blue R-250 was used for staining. Lane M, molecular mass marker; lane 1, wild-type; lane 2, Y108A; lane 3, Y108F; lane 4, Y108W.

Mr of 25 kDa equivalent with that of the wild type (Figure 1).

Substrate specificity. The specific activities of the mutant enzymes for GSH conjugations of CDNA and ETA are shown in Table 2. Neither the substitution of Tyr 108 with tryptophan significantly affected the specific activities. On the other hand, the substitution of Tyr 108 with alanine resulted in a decrease of the specific activities to approximately 35-40% of those of the wild type. The substitution of Tyr 108 with phenylalanine had negligible effect on the specific activity toward CDNB, but it resulted in approximately 90% decrease of the specific activity toward ETA. These results suggest that the contribution of tyrosine 108 in binding is very dependent on the nature of the electrophilic substrates.

Table 2. Specific activity of the wild type and mutants for GSH-conjugation reaction towards 1-chloro-2,4-dinitrobenzene and ethacrynic acid

Enzyme	1-Chloro-2,4-dinitrobenzene		Ethacrynic acid	
	Specific activity (μmol/min/mg)	Relative activity (%)	Specific activity (μmol/min/mg)	Relative activity (%)
Wild type	76.6 ± 1.5	100	1.54 ± 0.12	100
Y108A	31.2 ± 0.2	41	0.56 ± 0.05	36
Y108F	70.1 ± 2.1	92	0.16 ± 0.01	10
Y108W	100.0 ± 8.5	131	1.50 ± 0.02	98

Values are Means ± S.D., generally based on $n \geq 5$.

Kinetic studies. The catalytic mechanism of CDNB conjugation has been the subject of many studies, since this reaction is the most commonly used assay for GST activity.[2] Table 3 summarizes the kinetic parameters of the mutants for GSH-CDNB conjugation. The substitution of Tyr 108 with alanine resulted in a 2.6-fold increase of K_m^{CDNB}, whereas the K_m^{GSH} was similar to that of the wild type. On the other hand, the substitution of Tyr 108 with tryptophan resulted in a 1.7-fold decrease of K_m^{CDNB}, whereas the K_m^{GSH} was similar to that of the wild type. The substitution of Tyr 108 with phenylalanine scarcely affected the kinetic parameters. Table 4 summarizes the kinetic parameters of the mutants for GSH-ETA conjugation. The substitutions of Tyr 108 with

Table 3. Enzymatic kinetic parameters for GSH-[1-Chloro-2,4-dinitrobenzene] conjugation

Enzyme	GSH			1-Chloro-2.4-dinitrobenzene		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	0.16 ± 0.02	6.3 ± 0.9	39.6	0.65 ± 0.04	72.6 ± 1.8	111.7
Y108A	0.20 ± 0.02	11.2 ± 0.9	56.0	1.68 ± 0.01	41.1 ± 3.1	24.5
Y108F	0.16 ± 0.04	5.8 ± 0.1	36.0	0.75 ± 0.07	69.7 ± 8.00	92.9
Y108W	0.19 ± 0.04	7.5 ± 1.1	39.0	0.38 ± 0.05	124.0 ± 9.0	326.0

Values are means ± SD, generally based on $n \geq 3$.

Table 4. Enzymatic kinetic parameters for GSH-[Ethacrynic acid] conjugation

Enzyme	GSH			Ethacrynic acid		
	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)	K_m (mM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}s^{-1}$)
Wild type	0.15 ± 0.04	0.31 ± 0.08	2.0	0.18 ± 0.07	1.12 ± 0.08	6.3
Y108A	0.14 ± 0.05	0.29 ± 0.01	2.1	0.59 ± 0.03	0.90 ± 0.02	1.5
Y108F	0.12 ± 0.07	0.09 ± 0.06	0.8	0.85 ± 0.05	0.22 ± 0.04	0.3
Y108W	0.17 ± 0.03	0.19 ± 0.01	1.1	0.18 ± 0.07	1.82 ± 1.43	10.1

Values are means ± SD, generally based on $n \geq 3$.

Table 5. Inhibitory effect of *S*-hexylGSH and ethacrynic acid on GSH-[1-Chloro-2,4-dinitrobenzene] conjugation

Enzyme	*S*-HexylGSH		Ethacrynic acid	
	I_{50} (μM)	%	I_{50} (μM)	%
Wild type	20.2 ± 0.5	100	13.5 ± 0.3	100
Y108A	17.3 ± 0.1	86	26.9 ± 0.6	199
Y108F	20.5 ± 2.1	101	25.9 ± 0.1	192
Y108W	25.1 ± 0.1	124	23.0 ± 0.2	170

Values are Means = S.D., generally based on $n \geq 3$.

alanine and phenylalanine resulted in approximately 3-5 fold increases of K_m^{ETA} values, whereas the K_m^{GSH} values were similar to that of the wild type. On the other hand, the substitution of Tyr 108 with tryptophan scarcely affected the kinetic parameters for GSH-ETA conjugation. These results demonstrate that Tyr 108 in hGST P1-1 contributes to electrophilic substrate binding rather than to catalysis and GSH binding.

Inhibition studies. The inhibition parameters (I_{50}) of *S*-hexylGSH and ETA for GSH-CDNB conjugating activity were determined under the standard assay conditions. The substitutions of Tyr 108 with alanine, phenylalanine and tryptophan resulted in approximately 70-100% increases in the I_{50} values of ETA that competes with CDNB as an electrophilic substrate (Table 5). On the other hand, the I_{50} values of *S*-hexylGSH that compete with GSH for Tyr 108 mutants were similar to that of the wild type.

Discussion

The hGST P1-1 has attracted attention as reliable preneoplastic or neoplastic marker enzymes and it has been implicated in the development of resistance of tumors towards various anti-cancer drugs in resistant tumor cells.[35,36] Thus, hGST P1-1 has been extensively studied because of the clinical interest in it as a marker during chemical carcinogenesis and its potential role in the mechanism of cellular multidrug resistance against a number of antineoplastic agents.

The chemical modification and X-ray crystallographic studies have suggested that Tyr 108 in hGST P1-1 is located at or close to the substrate-binding site of the enzyme and it is in a structurally conserved position within the mu, pi and theta classes, and appears to be one of the few polar residues contributing to the H-site.[22-29] Indeed, it was found that the substitutions of Tyr 108 significantly affected K_m^{CDNB} and K_m^{ETA}, whereas scarcely affected K_m^{GSH} (Table 3 and 4). K_m^{CDNB} and K_m^{ETA} values of Y108A were approximately 2-3 fold larger than those of the wild type, but the K_m^{CDNB} of Y108W was a half-fold smaller than that of the wild type. Moreover, the substitutions of Tyr 108 also significantly affected I_{50} of ETA, an electrophilic substrate-like compound (Table 5). The I_{50} values of Y108A, Y108F and Y108W were approximately 2-fold larger than that of the wild type. These results suggest that tyrosine 108 in hGST P1-1 contributes to the binding of the electrophilic substrate. As shown by Table 3, K_m^{CDNB} values decreased as the size of side chain of the mutated amino acid increased (Ala < Phe < Trp), whereas K_m^{GSH} values were similar to that of the wild type. These results suggest that a major determinant in the binding of CDNB is the aromatic ring of Tyr108, not its hydroxyl group.

The substitution of Tyr 108 with phenylalanine resulted in approximately 90% decrease of the specific activity toward ETA (Table 2). This substitution significantly affected k_{cat}, K_m^{CDNB} and I_{50} of ETA, whereas scarcely affected K_m^{GSH} (Table 4 and 5). The three-dimensional structure of GST P1-1 in complex with ETA indicated that this substrate is bound to the H-site in a nonproductive manner in the binary complex.[37] On the contrary, crystallographic analysis of GST P1-1 in complex with GS-ETA showed a completely different orientation of ETA with its carbonyl oxygen in incipient hydrogen bonding geometry of Tyr 108. Taken together, the substitutions of this residue might affect the conformation of the active site of the enzyme as well as the interaction with electrophilic substrate.

In conclusion, this site-directed mutagenesis study indicates that Tyr 108 in hGST P1-1 is this residue responsible for electrophilic substrate binding and a major determinant in the binding of CDNB is the aromatic ring of Tyr 108, not its hydroxyl group.

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