Functional Studies of Tyrosine 108 Residue in the Active Site of Human Glutathione S-Transferase P1-1

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To gain further insight on the relationship between structure and functions of glutathione S-transferase (GST), the three tyrosine 108 mutants, Y108A, Y108F, and Y108W, of human GST P1-1 were expressed in Escherichia coli and purified to electrophoretic homogeneity by affinity chromatography on immobilized GSH. The substitution of Tyr 108 with alanine resulted in significant decrease of the GSH-conjugation activity and the GSH peroxidase activity, but approximately 63% increase of steroid isomerase activity toward ∆5-androstene 3,17-dione. On the other hand, the substitution of Tyr 108 with phenylalanine resulted in decreases of k_{cat} and k_{cat}/K_{EPNP} by 2 orders of magnitude, suggesting that Tyr 108 residue of hGSTP1-1 are considered to be important for the catalysis and the binding of the epoxide substrates. The substitution of Tyr 108 with tryptophan resulted in significant decreases of the specific activities toward EPNP, cumene hydroperoxide and ∆5-androstene 3,17-dione, but approximately 2-fold increase on the enzyme-catalyzed addition of GSH to DCNB. We conclude from these results that Tyr 108 in hGST P1-1 plays very different roles depending upon the nature of the electrophilic substrates.

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

Introduction

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a ubiquitous family of proteins whose primary functions are involved in the chemical and physical disposition of toxic substances. The chemical function of GSTs is to catalyze the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds including alkyl- and aryl halides, epoxides, esters, and alkenes, the first step in mercapturic acid biosynthesis. Certain GSTs can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity, while others catalyze the isomerization of certain steroids. Additionally they play an important role in the intracellular transport of numerous hydrophobic nonsubstrate ligands such as bile acids, billirubin, and a number of drugs. Although a number of investigations on GSTs have been performed, very little has been discovered concerning the relationship between their structures and functions.

GSTs are distributed in a wide range of organisms from mammals to Escherichia coli. 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. Human pi-class GST (hGST P1-1), a homodimeric protein of about 46 kDa has attracted attention as reliable preneoplastic or neoplastic marker enzymes because a number of human tumors have been shown to express raised levels of the hGST P1-1 enzyme. Moreover, the hGST P1-1 has been implicated in the development of resistance of tumors towards various anti-cancer drugs in resistant tumor cells. Thus, the design of highly potent hGST P1-1 selective inhibitors may have increasing the therapeutic index of commonly used anti-cancer agents.

Recent crystallographic studies indicate that the polypeptide is organized into two domains, a GSH binding domain (domain I) at the N terminus and a xenobiotic substrate binding domain (domain II) at the C terminus. The GSH-binding site (G-site) and the catalytic mechanism of these enzymes have been the targets of many investigations involving chemical modification, site-directed mutagenesis, and X-ray crystallographic analysis. Major roles of the active site in GST are the activation of the thiol group of reduced GSH for the nucleophilic reaction by deprotonation and the stabilization of the transition state for reactions with electrophilic substrates. The essential active-site residue has been revealed to be an evolutionarily conserved Tyr7 in hGST P1-1 by our previous studies. 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. 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. Only a few amino acid residues have been identified as key determinants of the H-site : Tyr 115 in rat

Abbreviations: CP, cumene hydroperoxide; DCNB, 1,2-dichloro-4-nitrobenzene; DNPG, S-(2,4-dinitrophenyl)glutathione; EPNP, 1,2-epoxy-3-(p-nitrophenoxy)propane; Escherichia coli, E. coli; GSH, glutathione; G-site, glutathione-binding site; GST, glutathione S-transferase; hGST, human GST; H-site, electrophilic substrate-binding site.
mu GST (isoenzymes 3-3 and 4-4). Met 208 in GST A1-1, Ile 104 in GST P1-1, and Val 10, Arg 11, and Val 104 in the murine class pi GST.

Previously, we reported the importance of Tyr 108 for the binding of the electrophilic substrate of hGST P1-1. As an extension of this study, we expressed the three tyrosine 108 mutants, Y108A, Y108F, and Y108W, of human GST P1-1 for mutagenesis, confirmation of mutation, construction of expression plasmid, expression and purification of the mutants, and characterization with respect to their enzymatic properties, toward four selected substrates, i.e., 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(p-nitrophenoxy)propene for GSH-conjugation activity, cumene hydroperoxide for GSH peroxidase activity, and Δ^2-androstene-3,17-dione for steroid isomerase activity. The results show that Tyr 108 plays very different roles depending upon the nature of the electrophilic cosubstrate. This study offers the information on the precise enzyme-substrate interactions responsible for the catalytic properties of hGST P1-1, and it will be of great value in designing new inhibitors that may prove useful in chemotherapy and new enzymes having different substrate specificity.

Materials and Methods

Materials. GSH and 1,2-dichloro-4-nitrobenzene were purchased from Kohjin Co. and Wako Pure Chem. Ind. (Osaka, Japan), respectively. Cumene hydroperoxide, 1,2-epoxy-3-(p-nitrophenoxy)propene and S-methylGSH were obtained from Sigma (St. Louis, USA). Δ^2-androstene-3,17-dione was purchased from Steraloids Inc. (Wilton, N. H. USA). S-(2,4-Dinitrophenyl)glutathione was synthesized by the method of Schramm et al. Glutathione Sepharose was purchased from Pharmacia Biotech (Uppsala, Sweden). All other reagents used were of the highest grade commercially available.

Preparation of mutant enzymes. Wild-type hGST P1-1 was obtained by expression of a cloned cDNA in E. coli as described in the previous paper. Oligonucleotides 5'-AAATACATCTCTCCTCCTGCCCACAACATGAG-3' (Tyr108Ala), 5'-AAATACATCTCCCTTCATCTCACCAGATGAG-3' (Tyr108Phe), and 5'-AAATACATCTCCCTTCATCTCACCAGATGAG-3' (Tyr108Trp) were synthesized for site-directed mutagenesis experiments. Mutagenesis was performed according to the procedure of Kunkele using a Mutant™.Super Express Km kit (Takara Shuzo Co.). Construction of single-stranded DNA template for mutagenesis, confirmation of mutation, construction of the expression plasmid, expression and purification of the mutant enzymes were performed as described in the previous paper. Unless otherwise indicated, all purification procedures were carried out either at 4 °C or on ice. The enzymes were stored at −70 °C until use.

Determination of protein concentration. Protein concentration of the wild type enzyme was determined by measuring the absorbance at 280 nm as described by Parker 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 and kinetic studies. The specific activities were determined by measuring the initial rates of the enzyme-catalysed conjugation of GSH with 1,2-dichloro-4-nitrobenzene (DCNB) or 1,2-epoxy-3-(p-nitrophenoxo)propane (EPNP) as described by Habig and Jakoby. 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 DCNB or EPNP 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. DCNB and EPNP were dissolved in ethanol. The concentration of ethanol in the reaction mixture (1 mL) was constant at 2% (v/v). GSH was dissolved in H2O 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 DCNB, 5 mM GSH, 345 nm (Δε = 8.5 mM−1 cm−1) and (b) 0.5 mM EPNP, 5 mM GSH, 360 nm (Δε = 0.5 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. Steroid isomerase activity was monitored by the catalyzed isomerization of Δ^2-androstene-3,17-dione (0.1 mM) to Δ^2-androstene-3,17-dione, and was determined spectrophotometrically from the change in absorbance at 248 nm (Δε = 16.3 mM−1 cm−1) in 100 mM potassium phosphate buffer, pH 6.5, at 30 °C in the presence of 3 mM GSH. GSH-dependent peroxidase activity was assayed in the presence of 5 mM GSH with 1.5 mM cumene hydroperoxide (Δε = 6.6 mM−1 cm−1) as substrate at 30 °C as described. Kinetic studies with GSH and electrophilic substrates were carried out at 30 °C as described by Chen et al.

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 1-chloro-2,4-dinitrobenzene (final concentration, 1 mM). The concentration of inhibitor giving 50% inhibition (I50) was determined from plot of residual activity against inhibitor concentration.

Heat inactivation assays. The enzyme was incubated at each temperature for 10 min at a protein concentration of 0.1 mg/mL in 20 mM potassium phosphate buffer (pH 7.0) containing 10 mM DTT and 10 mM EDTA to prevent the oxidative inactivation, and then cooled in ice. The remaining activity was assayed in 100 mM potassium phosphate buffer.
Table 1. Specific activity of the wild type and mutants for GSH-conjugation reaction towards 1,2-dichloro-4-nitrobenzene (DCNB) and 1,2-epoxy-3-(p-nitrophenoxy) propane (EPNP)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>DCNB (× 10⁻² µmol/min/mg)</th>
<th>Relative activity (%)</th>
<th>EPNP (× 10⁻² µmol/min/mg)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>11.8 ± 1.1</td>
<td>100</td>
<td>36.0 ± 3.8</td>
<td>100</td>
</tr>
<tr>
<td>Y108A</td>
<td>6.8 ± 0.2</td>
<td>58</td>
<td>13.3 ± 1.0</td>
<td>37</td>
</tr>
<tr>
<td>Y108F</td>
<td>11.8 ± 0.1</td>
<td>100</td>
<td>2.5 ± 0.1</td>
<td>7</td>
</tr>
<tr>
<td>Y108W</td>
<td>25.4 ± 1.7</td>
<td>215</td>
<td>12.4 ± 1.5</td>
<td>35</td>
</tr>
</tbody>
</table>

Values are Means ± S.D., generally based on n ≥ 5.

Enzymes for GSH conjugation reaction are shown in Table 1. The substitutions of Tyr 108 with alanine and tryptophan resulted in approximately 80-90% decreases of the specific activity toward cumene hydroperoxide. On the other hand, the substitution of Tyr 108 with phenylalanine had negligible effect on the activity.

Several GSTs can catalyze the cis-trans isomerization of maleylacetone to fumarylactone and maleylacetoacetic acid to fumarylacetoacetic acid. An even smaller number of GST isozymes possess ketosteroid isomerase activity and catalyze the conversion of Δ⁵-3-ketosteroids to Δ⁴-3-ketosteroids. The steroid isomerase activities of the mutant enzymes are shown in Table 3. The substitution of Tyr 108 with tryptophan resulted in a decrease of the specific activity to approximately 36% of that of wild type. On the other hand, Y108A and Y108F resulted in approximately 50-60% increase of specific activity toward Δ⁴-androstene 3,17-dione.

Table 2. Specific activity of the wild type and mutants for GSH peroxidase activity toward cumene hydroperoxide

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Cumene hydroperoxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes</td>
<td>Specific activity (× 10⁻¹ µmol/min/mg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>37.2 ± 3.3</td>
</tr>
<tr>
<td>Y108A</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>Y108F</td>
<td>28.8 ± 0.7</td>
</tr>
<tr>
<td>Y108W</td>
<td>6.1 ± 0.5</td>
</tr>
</tbody>
</table>

Values are Means ± S.D., generally based on n ≥ 5.

Table 3. Specific activity of the wild type and mutants for steroid isomerase activity toward Δ⁴-androstene 3,17-dione

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Δ⁴-Androstene 3,17-dione</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enzymes</td>
<td>Specific activity (× 10⁻¹ µmol/min/mg)</td>
</tr>
<tr>
<td>Wild type</td>
<td>7.8 ± 0.1</td>
</tr>
<tr>
<td>Y108A</td>
<td>12.7 ± 0.2</td>
</tr>
<tr>
<td>Y108F</td>
<td>11.7 ± 0.4</td>
</tr>
<tr>
<td>Y108W</td>
<td>2.8 ± 0.1</td>
</tr>
</tbody>
</table>

Values are Means ± S.D., generally based on n ≥ 5.
activity. The substitution of Tyr 108 with alanine resulted in a 1.4-fold increase of \( K_m \) for GSH and \( k_{cat} \) for DCNB, whereas the \( K_m \) for GSH value of the Y108F was a 3.6-fold lower than that of the wild type. The substitution of Tyr 108 with tryptophan resulted in approximately 2-3 fold decreases of \( K_m \) values, approximately 1.5-fold increases of \( k_{cat} \) values and approximately 4-6 fold increases of \( k_{cat}/K_m \).

Table 5 summarizes the kinetic parameters of the mutant enzymes for GSH-EPNP conjugation activity. The substitution of Tyr 108 significantly affected \( K_m \) for EPNP, whereas scarcely affected \( K_m \) for GSH. The substitution of Tyr 108 with phenylalanine resulted in a 7.4-fold increase of \( K_m \) for EPNP, a 6.5-fold decrease of \( k_{cat} \) and a 47-fold decrease of \( k_{cat}/K_m \).

On the other hand, the substitution of Tyr 108 with tryptophan resulted in a 1.9-fold decrease of \( K_m \) for EPNP, a 1.5-fold increase of \( k_{cat} \) and a 3-fold increase of \( k_{cat}/K_m \).

Table 6 summarizes the kinetic parameters of the mutant enzymes for GSH peroxidase activity. The substitution of Tyr 108 with alanine resulted in a 10-fold increase of \( K_m \) for GSH, whereas the Y108F and Y108W were similar to that of the wild type. On the other hand, the \( K_m \) values of the mutant enzymes were approximately 3-27 fold larger than that of the wild type. Particularly, the substitution of Tyr 108 with alanine resulted in a 27-fold increase of \( K_m \) for EPNP, a 48-fold decrease of \( k_{cat} \) and a 1280-fold decrease of \( k_{cat}/K_m \).

Table 7 summarizes the kinetic parameters of the mutant enzymes for steroid isomerase activity toward \( \Delta^1 \)-androstene-3,17-dione.
Functional Studies of Tyr108 Residue in hGST P1-1

Human GST P1-1 (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. Thus, studies on the relationship between structure and function of hGST P1-1 provide a rationale for the design of inhibitors and prodrugs to enhance therapeutic index. The hGST P1-1 subunit consists of the N-terminal 76 residues (domain I) and the C-terminal 127 residues (domain II) which are connected by a six residue linker. Most of the residues (Arg13, Lys44, Gln51, Gln64 and Asp98) necessary for binding of GSH occupied a site on domain I (G-site). On the other hand, little is known about the location of the binding site of electrophilic substrates (H-site). The chemical modification studies have suggested that Tyr residue in GST is located at or close to the substrate-binding site of the enzyme. Barycki and Colman suggested that Tyr 115 in class mu GST 4-4 contributes to xenobiotic substrate binding by chemical modification study using 4-(fluorosulfonyl)benzoic acid, a xenobiotic substrate analogue. Affinity labeling study using 4-(fluorosulfonyl)benzoic acid also provided another evidence that Tyr 106 of the pig lung class pi GST is located at or close to the substrate-binding site of the enzyme. X-ray crystallographic studies have also suggested that Tyr 108 residue in GST is located at or close to the substrate-binding site 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. In order to elucidate the precise enzyme-substrate interactions responsible for the catalytic properties of GST, the mutant enzymes of Tyr 108 residue in hGST P1-1 were expressed in E. coli, purified to electrophoretic homogeneity by affinity column chromatography and examined the enzymatic properties of the mutated enzymes. The substitutions of Tyr 108 with alanine, phenylalanine and tryptophan greatly affected the catalytic properties of hGST P1-1 toward a number of substrates, as discussed below.

Discussion

Heat inactivation of the wild type and mutant enzymes. After the enzymes were incubated in 20 mM potassium phosphate buffer (pH 7.0) at various temperatures for 10 min, the remaining activities were assayed in 100 mM potassium phosphate buffer (pH 6.5) at 30 °C (Figure 2). The wild type, Y108A and Y108W were fairly stable to such an incubation at temperature up to 50 °C, but were inactivated irreversibly above 50 °C. Y108W mutant was unstable than the wild type. The midpoints of inactivation were 57 °C for the wild type, whereas 53 °C for Y108W mutant.

Inhibition studies. The inhibition parameters (I_{50}) of various kinds of inhibitors, S-methylGSH, S-(2,4-dinitrophenyl)glutathione and hematin for GSH-CDNB conjugating activity were determined under the standard assay conditions (Figure 1). The substitutions of Tyr 108 with phenylalanine and tryptophan resulted in a 2-fold decrease of K_{mGSH}, whereas K_{mS-steroid} value resulted in a 7.6-fold increase. On the other hand, the K_{mS-GSH} values of the Y108A and Y108F were similar to that of the wild type. The substitution of Tyr 108 with tryptophan resulted in a 2-fold decrease of K_{mGSH}, whereas K_{mS-GSH} values of the Y108A and Y108F were a little lower than that of the wild type, as shown in Figure 1. On the other hand, the I_{50} value of S-methylGSH for Y108A was similar to that of the wild type. The I_{50} values of S-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with CDNB, for Y108A and Y108W were a little lower than that of the wild type, as shown in Figure 1. On the other hand, the I_{50} value for Y108F was significantly lower than the wild-type value about 2-fold. The I_{50} values of hematin, a nonsubstrate ligand, for mutant enzymes were significantly lower than that of the wild type about 2-3 fold.

Heat inactivation of the wild type and mutant enzymes. After the enzymes were incubated in 20 mM potassium phosphate buffer (pH 7.0) at various temperatures for 10 min, the remaining activities were assayed in 100 mM potassium phosphate buffer (pH 6.5) at 30 °C (Figure 2). The wild type, Y108A and Y108W were fairly stable to such an incubation at temperature up to 50 °C, but were inactivated irreversibly above 50 °C. Y108W mutant was unstable than the wild type. The midpoints of inactivation were 57 °C for the wild type, whereas 53 °C for Y108W mutant.

Discussion

Human GST P1-1 (hGST P1-1) has been extensively...
and steroid isomerase activity. The substitution of Tyr 108 with alanine resulted in approximately 40-60% decrease of the specific activities toward DCNB and EPNP and 98% decrease of the GSH peroxidase activity toward cumene hydroperoxide (Table 1 and 2). This substitution also resulted in large increases of $K_m$ values toward electrophilic substrates (Table 5 and 6) and a significant decrease of $I_{50}$ value for hematin (Fig. 1). On the other hand, the substitution of Tyr 108 with alanine resulted in approximately 63% increase of steroid isomerase activity toward $\Delta^2$-androstene 3,17-dione (Table 3). This substitution also resulted in about 7-fold increase of the $k_{cat}$ value for GSH compared to that of the wild-type enzyme (Table 7). This higher catalytic efficiency was explained with the removal of the hydroxyl function of Tyr 108 limits the product release (rate-limiting).

The substitution of Tyr 108 with phenylalanine had a dramatic effect on the enzyme-catalyzed addition of GSH to EPNP, reducing $k_{cat}$ and $k_{cat}/K_{m}$ by 2 orders of magnitude (Table 1 and 5). There were little differences in the activities of the Y108F mutant and the wild-type enzyme toward DCNB, cumene hydroperoxide and $\Delta^3$-androstene 3,17-dione (Table 1, 2 and 3). Thus, the severely impaired catalytic efficiency of the Y108F mutant was consistent with the removal of an interaction crucial for the chemical step of the reaction and not a deterious change in the conformation of the active site. Similar results about the effect of this residue on epoxide conjugation have been reported by Johnson et al., using site-directed mutagenesis and crystallographic analysis for isozyme 3-3 of rat GST.28 Ji et al. also suggested the role of the hydroxyl group of Tyr 108 as an electrophilic participant in the addition of GSH to epoxides.45 From these results, Tyr 108 residue of hGSTP1-1 are considered to be important for the catalysis and the binding of the epoxide substrates.

The substitution of Tyr 108 with tryptophan resulted in 65-84% decreases of the specific activities toward EPNP, cumene hydroperoxide and $\Delta^3$-androstene 3,17-dione (Table 1, 2 and 3). On the other hand, the substitution of Tyr 108 with phenylalanine resulted in approximately 2-fold increase on the enzyme-catalyzed addition of GSH to DCNB. This substitution resulted in 2-3 fold decreases of $K_m$ values on substrates and $I_{50}$ value on 5-methylGSH, and 4-6 fold increase $k_{cat}/K_m$ (Table 1, 4 and Fig. 1). Y108W mutant was unstable against heat than the wild-type enzyme (Fig. 2). These results suggest that the substitution of Tyr 108 with tryptophan changes the conformation of the active site to increase the affinity for substrates. Tyr 108 of hGST P1-1, the equivalent to Tyr 115 in class mu, was positioned in close contact to the xenobiotic substrate and was a possible candidate for involvement in H-site.

These rather striking differences in the response of the catalytic properties of the mutant enzymes are most likely to be correlated with structural differences between their active-sites, and the substitutions of Tyr 108 might affect the interaction between the thiol group and Tyr 7 through distorting the orientation of the GSH bound in the enzyme and/or the binding site of electrophilic substrates. We conclude from these results that Tyr 108 in hGST P1-1 can play different roles depending upon the nature of the electrophilic substrates.

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References

26. Wilce, M. C. J.; Board, P. G.; Feil, S. C.; Parker, M. W. *EMBO J.*
Functional Studies of Tyr108 Residue in hGST P1-1
