Functional Studies of Cysteine Residues in Human Glutathione *S*-Transferase P1-1 by Site-Directed Mutagenesis

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To gain further insight into the relationship between structure and function of glutathione *S*-transferase (GST), the four cysteine mutants, C14S, C47S, C101S and C169S, of human GST P1-1 were expressed in *Escherichia coli* and purified to electrophoretic homogeneity by affinity chromatography on immobilized glutathione (GSH). The catalytic activities of the four mutant enzymes were characterized with five different substrates as well as by their binding to four different inhibitors. Cys14 seems to participate in the catalytic reaction of GST by stabilizing the conformation of the active-site loop, not in the GSH binding directly. The substitution of Cys47 with serine significantly reduces the affinity of GSH binding, although it does not prevent GSH binding. On the other hand, the substitution of Cys101 with serine appears to change the binding affinity of electrophilic substrate by inducing a conformational change of the α-helix D. Cys169 seems to be important for maintaining the stable conformation of the enzyme. In addition, all four cysteine residues are not needed for the steroid isomerase activity of human glutathione *S*-transferase P1-1.

Keywords: Cysteine residues, enzymatic properties, glutathione *S*-transferase, inhibition characteristics, substrate specificity, thermostability.

Introduction

Glutathione *S*-transferase (GST, EC 2.5.1.18) is a family of multifunctional proteins, catalyzing the formation of conjugates between reduced glutathione (GSH) and a wide variety of electrophilic compounds including alkyl- and aryl halides, epoxides, esters and alkenes.[1] Certain GSTs can also detoxify lipid and DNA hydroperoxide by their intrinsic peroxidase activity. 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.

GSTs are distributed in a wide range of organisms from mammals to *E. coli*.[2] Mammalian cytosolic GSTs, which exist as either homo- or hetero-dimers, are grouped into at least five distinct classes, alpha, mu, pi, sigma, and theta according to substrate specificity and primary structure.[3,4] Although each isozyme generally exhibits a broad substrate selectivity, most have unique catalytic attributes that are important in defining the role of a particular isoenzyme in the metabolism of endogenous and xenobiotic electrophiles.[5] Among various GSTs, the human pi-class (hGST P1-1) 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.[6] Moreover, the hGST P1-1 has been implicated in the development of resistance of tumors towards various anti-cancer drugs in resistant tumor cells.[7] Thus, the design of highly potent hGST P1-1 selective inhibitors may be useful in increasing the therapeutic index of commonly used anti-cancer agents.

Considerable effort has been expended to document the substrate preferences of numerous isozymes from the various gene classes and species. The crystal structures of representative members of each of the five classes have been determined and have been used to implicate several amino acid residues in catalysis and binding of GSH.[8-12] The electrophilic substrate-binding site has also been localized; however, the structural basis of differential electrophilic substrate recognition by various isozymes is still not well understood. Moreover, there is little information available concerning the precise enzyme-substrate interactions that may be responsible for the catalytic properties of GST and the identification or specific role of individual residues remains largely unknown.

Therefore, in this study, the four mutants of cysteine residues (at position 14, 47, 101, and 169) in human GST P1-1 have been extensively investigated regarding the effect on the substrate specificity and the response to inhibition. This study provides 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 chemotheraphy 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)propane, ethacrynic acid, glutathione-agarose, *S*-hexylGSH and *S*-methylGSH were obtained from Sigma (St. Louis, USA). Δ^5- androstene-3,17-dione was pur-

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chased from Steraloids Inc. (Wilton, N. H. USA). *S*-(2,4-dinitrophenyl)glutathione was synthesized by the method of Schramm *et al*..[13] 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[14] in *E. coli* as described in the previous paper.[15] Oligonucleotides 5'-TCGAGGCCGCTCCGCGGCCCTGC-3' (Cys14 → Ser), 5'-CAAAGCCTCCTCCCTATACGGGC 3' (Cys47 → Ser), 5'-GGACCTCCGCTCCAAATACATCT-3' (Cys101 → Ser) and 5'-ACGCATCCAGGGAGCCAGGGGCT-3' (Cys169 → Ser) were synthesized for site-directed mutagenesis experiments. Mutagenesis was performed according to the procedure of Kunkel using a Muta-Gene kit (Bio-Rad).[16] 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,[15] with the exception of purification by affinity chromatography on GSH-agarose. 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 15 mL column of GSH-agarose equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1 mM 2-mercaptoethanol (buffer A). The column was extensively washed with the same buffer. The enzyme was eluted with a 50 mM Tris-HCl buffer (pH 9.6) containing 10 mM GSH and dialyzed against buffer A. The dialyzed enzyme was used for the next experiment. Unless otherwise indicated, all purification procedures were carried out either at 4 °C or on ice. The purified enzyme was 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[17] 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-catalyzed conjugation of GSH with 1-chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB) or 1,2-epoxy-3-(*p*-nitrophenoxy)propane (EPNP) as described by Habig and Jakoby.[18] 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 adding 20 μL of 50 mM CDNB, DCNB or EPNP to 960 μL of 100 mM potassium phosphate (pH 7.5) containing, in order of addition, 20 μL of 50 mM GSH and 20 μL of the enzyme. Nonenzymatic reaction rates served as controls, and were subtracted from enzymatic rates. CDNB, 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 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 so 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 $mM^{-1}cm^{-1}$), (b) 1 mM DCNB, 5 mM GSH, 345 nm ($\Delta\varepsilon$ = 8.5 $mM^{-1}cm^{-1}$) and (c) 0.5 mM EPNP, 5 mM GSH, 360 nm ($\Delta\varepsilon$ = 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^5-androstene-3,17-dione (0.1 mM) to Δ^4-androstene-3,17-dione, and was determined spectrophotometrically from the change in absorbance at 248 nm ($\Delta\varepsilon$ = 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 ($\Delta\varepsilon$ = 6.6 $mM^{-1}cm^{-1}$) as substrate at 30 °C as described.[19]

Kinetic studies. Kinetic studies with GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were carried out at 30 °C as described by Ivanetich and Goold.[20] The enzyme was pre-incubated for 2 min at 30 °C with a desired concentration of CDNB. The concentration of enzyme used for initial rate studies was varied in the range of 1-50 μg/mL. The concentration of GSH and CDNB were varied in the range of 0.075-1 and 0.1-1 mM, respectively. Kinetic parameters were obtained from hyperbolic saturation curves by least squares fit of the initial velocity data to the equation of rapid equilibrium random sequential Bi Bi mechanism.

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 a 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 (pH 6.5) with 1 mM GSH and 1 mM CDNB at 30 °C. The enzyme was incubated in buffer A at various temperatures for 10 min and then cooled in ice.

Results

Purification of mutant enzymes. The mutants of hGST P1-1 expressed in *E. coli* under the control of *tac* promoter were isolated and purified by affinity chromatography on immobilized GSH. The mutants C101S and C169S were isolated in a yield of approximately 2 mg per liter of cultures as in the case of the wild type. However, in the cases of C14S

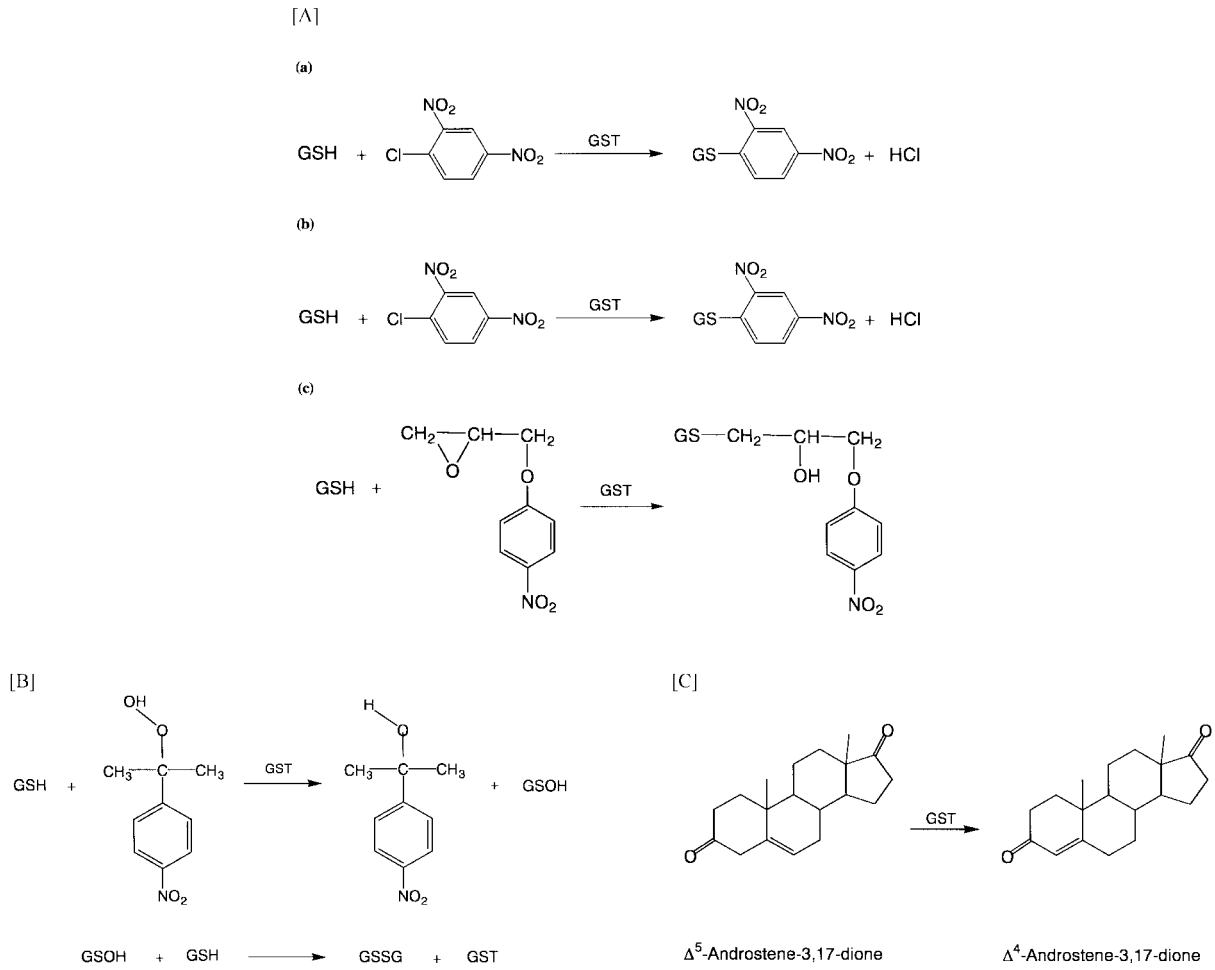

Figure 1. The scheme of the GST-catalyzed reactions. [A] GSH-conjugation activities towards (a) 1-chloro-2,4-dinitrobenzene, (b) 1,2-dichloro-4-nitrobenzene, (c) 1,2-epoxy-3-(*p*-nitrophenoxy)propane. [B] GSH-peroxidase activity towards cumene hydroperoxidase. [C] Steroid isomerase activity towards Δ^5-Androstene- 3,17-dione.

and C47S, the amounts of the isolated enzymes were 0.2-0.5 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, C101S and C169S were more than 70%, but those for the other mutants were less than 10%. Thus, the binding abilities of C101S and C169S to GSH-agarose were not so much different from that of the wild type, but C14S and C47S had low affinities for GSH-agarose. The purified mutants gave a single band on SDS-PAGE with an apparent *M*r of 25 kDa equivalent to that of the wild type and the antibody against the purified wild-type hGST P1-1 prepared from *E. coli* recognized the mutant GSTs specifically (data not shown).

Substrate specificity. The initial step in mercapturic acid formation is conjugation of the foreign compound with GSH, a reaction catalyzed by GSTs for many substrates (Figure 1-A).[3] The specific activities of the mutants for GSH conjugation reaction are shown in Table 1. The substitutions of the Cys14 and Cys47 with serine resulted in approximately 40-60% decrease in the specific activities towards CDNB, DCNB and EPNP. On the other hand, the substitutions of Cys101 and Cys169 with serine had a negligible effect on the GSH conjugation activity, except that the substitution of Cys101 with serine resulted in 34% decrease in the specific activity toward EPNP.

Organic hydroperoxides are substrates for GSTs. A study involving seven cytosolic homodimeric rat transferases demonstrated that linoleate hydroperoxide and arachidonate hydroperoxide in most cases gave activities comparable to the model substrate cumene hydroperoxide.[21] The GST-catalyzed reaction with cumene hydroperoxide represents the "nonselenium" glutathione peroxidase activity and is believed to occur in two steps involving an unstable glutathione sulfenic acid intermediate (Figure 1-B). Reduced GSH is regenerated from the produced glutathione disulfide (GSSG) by the action of glutathione reductase.[3] The GSH peroxidase activities of the mutants are shown in Table 2. The substitutions of Cys47 with serine resulted in approximately 30% decrease in the specific activity toward cumene hydroperoxide. On the other hand, the substitutions of Cys14, Cys101 and Cys169 with serine had a negligible effect on the activity.

Several GSTs can catalyze the *cis-trans* isomerization of maleylacetone to fumarylactone and maleylacetoacetic acid

Table 1. Specific activity of the wild type and mutants for GSH-conjugation reaction towards 1,2-dichloro-4-nitrobenzene and 1,2-epoxy-3-(*p*-nitrophenoxy)propane

Enzymes	1-Chloro-2,4-dinitrobenzene[a]		1,2-dichloro-4-nitrobenzene		1,2-Epoxy-3-(*p*-nitrophenoxy) propane	
	Specific activity (μmol/min/mg)	Relative activity (%)	Specific activity ($\times 10^{-2}$ μmol/min/mg)	Relative activity (%)	Specific activity ($\times 10^{-2}$ μmol/min/mg)	Relative activity (%)
Wild type	76.6 ± 1.5	100	11.8 ± 1.1	100	36.0 ± 3.8	100
C14S	42.9 ± 1.8	56	5.9 ± 0.6	50	19.5 ± 1.8	54
C47S	31.7 ± 1.9	41	4.5 ± 1.0	38	20.9 ± 1.4	58
C101S	93.4 ± 2.2	122	12.7 ± 0.8	108	23.8 ± 1.2	66
C169S	76.1 ± 1.8	99	11.4 ± 1.3	97	38.5 ± 1.6	107

Values are Means ± S.D., generally based on n $\geq$ 5. [a]Kong *et al*., 1991.

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

Enzymes	Cumene hydroperoxide	
	Specific activity ($\times 10^{-2}$ μmol/min/mg)	Relative activity (%)
Wild type	3.72 ± 0.33	100
C14S	3.93 ± 0.21	106
C47S	2.60 ± 0.15	70
C101S	4.00 ± 0.22	108
C169S	3.77 ± 0.19	101

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

Table 3. Specific activity of the wild type and mutants for steroid isomerase activity towards Δ^5-androstene- 3,17-dione

Enzymes	Δ^5-Androstene-3,17-dione	
	Specific activity ($\times 10^{-2}$ μmol/min/mg)	Relative activity (%)
Wild type	78.3 ± 0.5	100
C14S	69.7 ± 0.6	89
C47S	72.0 ± 1.0	92
C101S	70.5 ± 0.9	90
C169S	76.0 ± 1.4	97

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

to fumarylacetoacetic acid. An even smaller number of GST isozymes possess ketosteroid isomerase activity and catalyze the conversion of Δ^5-3-ketosteroids to Δ^4-3-ketosteroids (Figure 1-C). The steroid isomerase activities of the mutants are shown in Table 3. The substitutions of four cysteine residues with serine had a negligible effect on the steroid isomerase activity toward Δ^5-androstene-3,17-dione. This result indicates that the cysteine residues in hGST P1-1 are not essential for the steroid isomerase activity.

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.[1] Table 4 summarizes the kinetic parameters of the mutants for GSH-CDNB conjugation. The substitution of Cys14 with serine resulted in a 25% decrease of k_{cat} and 1.7-fold increase of K_m^{GSH}, whereas the K_m^{CDNB} was similar to that of the wild type. The substitution of Cys47 with serine resulted in a 5-fold increase in K_m^{GSH}, similar k_{cat} to that of the wild type and a 29-fold decrease in k_{cat}/K_m^{GSH}, whereas the K_m^{CDNB} was only 1.4-fold larger than that of the wild type. The substitution of Cys101 with serine resulted in 15% increase in k_{cat} and approximately 2-fold decrease in K_m^{CDNB}, whereas K_m^{GSH} was similar to that of the wild type. On the other hand, the replacement of Cys169 with serine scarcely affected the kinetic parameters.

Inhibition studies. The inhibition parameters (I_{50}) of various kinds of inhibitors, *S*-hexylGSH, *S*-methylGSH, *S*-(2,4-dinitrophenyl)glutathione and ethacrynic acid for GSH-CDNB conjugating activity were determined under the standard assay conditions. The substitution of Cys47 resulted in approximately 2-fold increase in the I_{50} values of *S*-hexylGSH and *S*-methylGSH that compete with GSH (Figure 2). The substitution of Cys14 resulted in 2.5-fold increase in the I_{50} value of *S*-hexylGSH. On the other hand, the I_{50} values of *S*-hexylGSH and *S*-methylGSH for Cys101 and Cys169 were similar to those of the wild type.

The I_{50} values of *S*-(2,4-dinitrophenyl)glutathione, a conjugation product of GSH with CDNB, for C14S, C101S and

Table 4. Enzymetic kinetic parameters for GSH-[1-chloro-2,4-dinitrobenzene] conjugation

Enzymes	k_{cat} (s^{-1})	GSH		1-chloro-2,4-dinitrobenzene	
		K_m^{GSH} (mM)	k_{cat}/K_m^{GSH} ($M^{-1}s^{-1}$)	K_m^{CDNB} (mM)	k_{cat}/K_m^{CDNB} ($M^{-1}s^{-1}$)
Wild type	61.7 ± 3.2	0.15 ± 0.01	4.1×10^5	0.82 ± 0.04	7.5×10^4
C14S	46.1 ± 2.3	0.25 ± 0.01	1.8×10^5	0.72 ± 0.10	6.4×10^4
C47S	58.6 ± 1.7	0.76 ± 0.07	1.4×10^4	1.38 ± 0.13	4.2×10^4
C101S	70.7 ± 4.1	0.17 ± 0.04	4.2×10^5	0.45 ± 0.02	1.6×10^5
C169S	58.9 ± 2.7	0.18 ± 0.02	3.3×10^5	0.80 ± 0.04	7.4×10^4

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

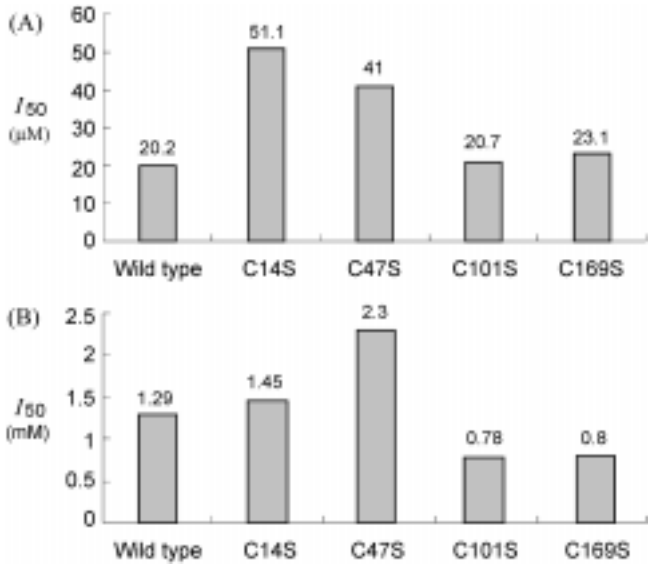

Figure 2. Inhibition effect of the wild type and mutants on GSH-[1-chloro-2,4- dinitrobenzene] conjugation with *S*-hexylGSH (A) and *S*-methylGSH (B).

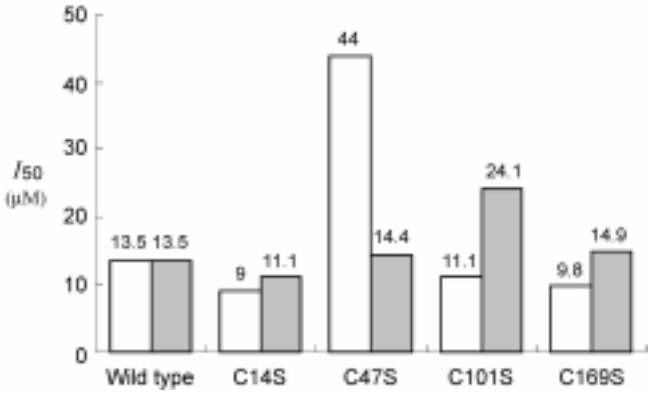

Figure 3. Inhibition effect of the wild type and mutants on GSH-[1-chloro-2,4-dinitrobenzene] conjugation with *S*-(2,4-dinitrophenyl)glutathione (□) and ethacrynic acid (■).

C169S were a little lower than the wild-type value, as shown in Figure 3. On the other hand, the I_{50} value for C47S was significantly higher about 3-fold than the value for wild-type. The substitution of Cys101 resulted in 1.8-fold increase in the I_{50} value of ETA that competes with CDNB as an electrophilic substrate (Figure 3). On the other hand, the I_{50} values for Cys14, Cys47, and Cys169 were similar to that of the wild type.

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 4). The wild-type, C14S, and C47S were fairly stable to such an incubation at temperature up to 50 °C, but were inactivated irreversibly above 50 °C. The C169S mutant was less stable than the wild type. The midpoints of inactivation were 55-57 °C for the wild type, C14S, C47S, and C101S, whereas it was 52 °C for C169S mutant.

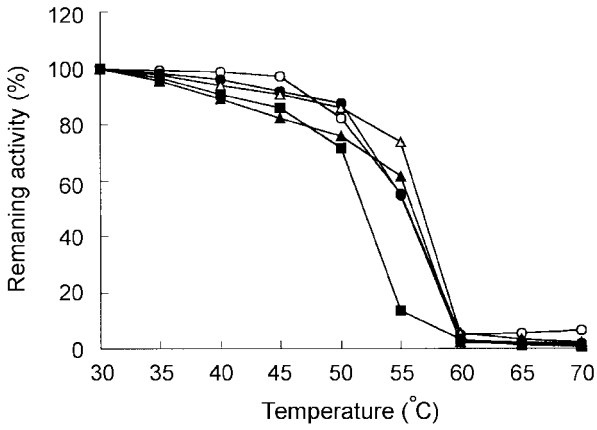

Figure 4. Thermostability of the wild type and mutants. O, wild type; ● , C14S; △ , C47S; ▲ , C101S; ■ , C169S.

Discussion

Human GST 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.[6] By chemical modification studies, cysteine residues have been suggested to be present at or near the active site of GSTs and to play essential roles in catalysis.[22-24] The site-directed mutagenesis, however, suggested that cysteine residues in GST are not essential for catalytic activity.[15,25-27] A recent affinity-labeling study suggested that cysteine residues are located in the hydrophobic substrate-binding site of GST.[28] The three-dimensional structures of GST also suggested the possibility that cysteine residues play an essential role in substrate binding affinity or catalysis.[29,30] In the present study, to elucidate the precise enzyme-substrate interactions responsible for the catalytic properties of hGST P1-1, we introduced site-directed mutations into four cysteine residues putatively involved in catalysis, substrate binding and/or non-substrate ligands binding and examined the enzymatic properties of the mutated enzymes.

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 substrate. The essential active-site residue has been revealed to be an evolutionarily conserved Tyr7 in hGST P1-1 by our previous studies.[31,32] According to the three-dimensional structure of GST, Cys14 is located in the active-site loop (residues 8 to 14) that is of significance for the reactivity of Tyr7.[33] Thus, Cys14 is expected to participate in catalytic functions of the enzyme. The substitution of Cys14 with serine decreased the catalytic activity. This substitution resulted in about 50% decreases in GSH conjugation activities towards CDNB, DCNB and EPNP (Table 1). The analysis of the kinetic parameters of Cys14 for GSH-CDNB conjugation indicated that the replacement resulted in 25% decrease in k_{cat} and a little increase in K_m^{GSH} (Table 4). The I_{50} of *S*-hexylGSH of C14S was 2.5-fold larger than that of the wild type (Figure 2).

Moreover, the affinity of C14S for GSH-agarose was lower than that of the wild type. These results indicate that Cys14 contributes not only to the catalytic activity but also to some extent to the binding of GSH. According to the three-dimensional structure of hGST P1-1, no direct interaction is expected between Cys14 and the enzyme-bound GSH.[34] Furthermore, Tyr7 is located in a hydrophobic pocket formed by the side chains of Phe8, Val10, Gly12, Cys14, and Pro53, and is only partially exposed to solvent.[33] An analysis of the electrostatic environment at the active site of GST P1-1 also indicates that the peptide dipoles at Gly12, Arg13, and Cys14 generate an extremely high positive electrostatic potential at the position of the hydroxyl group of Tyr7.[35] Taken together, Cys14 seems to participate in the catalytic reaction of GST by stabilizing the conformation of the active-site loop, but no in the GSH binding directly.

Among the four-cysteine residues in hGST P1-1, Cys47 was believed to be an essential catalytic residue because of its high reactivity and because its chemical modification affects the enzyme activity.[30] Indeed, it was found that the single amino acid substitution of Cys47 with serine decreased the affinity for GSH-agarose and the specific activities on GSH-conjugation reaction (Table 1). The analysis of the kinetic parameters of C47S for GSH-CDNB conjugation indicated that the substitution significantly affected K_m^{GSH}, but scarcely k_{cat} and K_m^{CDNB} (Table 4). The K_m^{GSH} of C47S was 5-fold larger than that of the wild type. Moreover, the I_{50} values of *S*-hexylGSH, *S*-methylGSH, and DNPG were 2~3-fold larger than that of the wild type (Figures 2 and 3). These results suggest that the substitution of Cys47 with serine generally decreases the binding affinity of the enzyme to GSH. The three-dimensional structure of GST P1-1 shows that Cys47, a residue located on α-helix 2 which forms a wall of the G-site, is removed by about 12 Å from the active site.[34] The three-dimensional structure of the Cys47-carboxymethylated GST P1-1 also shows that the side chain of Cys47 is buried by the side chains of Leu43, Thr46, Lys53, and Tyr63 in the native complexes and is essentially inaccessible to solvent. Therefore, the substitution of Cys47 with serine seems to affect the residues implied in the GSH binding by causing severe contacts with the surrounding side chains, but it does not seem to affect the residues close to the glutathione sulfur site like Tyr7 that might be involved in catalysis.

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.[34] Most of the residues (Arg13, Lys44, Gln51, Gln64, and Asp98) necessary for binding of GSH occupies a site on domain I (G-site). However, little is known about the location of the binding site of electrophilic substrates (H-site). The substitution of Cys101 with serine significantly affected K_m^{CDNB}, whereas scarcely affected k_{cat} and K_m^{GSH} (Table 4). The K_m^{CDNB} of C101S was only half of that of the wild type. Moreover, its I_{50} of ETA, an electrophilic substrate-like compound, was 1.8-fold larger than that of the wild type (Figure 3). These results suggest that the substitution of Cys101 with serine generally changes the binding affinity of electrophilic substrates to the enzyme. Reinemer *et al.*[10] suggests that one of the possible sites for H-site is a hydrophobic pocket composed of residues found near the middle of α-helix D (residues 81 to 107). By the three-dimensional structures of hGST P1-1 in complex with GSH and various inhibitors, however, Cys101 does not to interact with electrophilic substrates directly, although this residue is located in α-helix D.[36-38] Taken together, the substitution of Cys101 is thought to change the binding affinity of electrophilic substrates by inducing a conformational change of the α-helix D.

Cys169 is suggested to be located not in the G-site but apart from the catalytic site by the three-dimensional structure of hGST P1-1.[34] This residue was expected not to participate directly in the catalytic mechanism. The substitution of Cys169 with serine scarcely affected the enzyme activity and kinetic parameters (Tables 1-3). However, the substitution lowered the thermostability of the enzyme. Remaining activities after 10 min incubation at various temperatures (Figure 4) showed that the midpoint of the temperature-stability curve was 57 °C for the wild type and 52 °C for C169S. Therefore, Cys169 seems to be important for maintaining the stable conformation of the enzyme.

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