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CHARACTERIZATION OF CYSTEINE RESIDUES OF GLUTATHIONE S-TRANSFERASE P : EVIDENCE FOR STERIC HINDRANCE OF SUBSTRATE BINDING BY A BULKY ADDUCT TO CYSTEINE 47

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Summary: Glutathione S-transferase P (GST-P) lost the enzymatic activity by 7-fluoro-4-sulfamoyl-2, 1, 3-benzodiazole (ABD-F), a thiol-group chemical modifier, but did not by methylmethanethiolsulfonate. Both ABD-F and methylmethanethiolsulfonate reacted with Cys47 and Cys101. These two cysteine residues were site-directedly mutated with serine residues. Only the Cys101Ser lost the enzymatic activity by the treatment of ABD-F. On carbon 13 NMR experiments, a NMR signal of S-[^{13}C]CH_3 adduct to Cys47 did not show any change by the addition of S-hexylglutathione. These facts revealed that Cys47 did not locate at the active site, and a bulky adduct to Cys47 hindered the binding of substrates to the active site.

Glutathione S-transferases (GSTs) [EC 2.5.1.18] catalyze conjugation of a series of electrophilic xenobiotics with glutathione (1, 2). Species-independent classification of the iso-

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Abbreviations: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl-β-D(-)-thiogalactopyranoside; NMR, nuclear magnetic resonance; PCR, polymerase chain reaction; NEM, N-ethylmaleimide; ABD-F, 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole; DTNB, 5, 5'-dithio-bis (2-nitrobenzoic acid); SDS-PAGE, sodium dodecyl-sulfate polyacrylamide-gel electrophoresis.

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zymes has been proposed according to amino terminal amino acid sequence homology, substrate specificity, and common antigenicity (3, 4). This classification divides the isozymes into three classes, α, μ, and π. Both human placental GST (GST-π) and rat placental GST (GST-P) belong to class π enzyme. Their subunits consist of 209 amino acids residues and differ in only 30 amino acids (85.6 % homology) (5). Each subunit contains four cysteines at positions of 14, 47, 101, and 169 from the amino terminus.

Thiol-group modifiers inhibited Class π GST activity (6, 7). These suggested that a cysteine residue was involved in the catalytic function. But, a series of recent papers on the cysteine residues by site-directed mutagenesis clarified that the residue was not essential to the catalytic function (8, 9). However, the inhibition mechanism by thiol-group modifiers has not been understood.

To clarify the inhibition mechanism of GST activity by thiol-group modifiers, cysteine residues were chemically labeled with two different thiol-modifiers, 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F) and methylmethanethiolsulfonate. Reactive cysteine residues were replaced with an isostructural serine by site-directed mutagenesis, and structural analysis near the active site was carried out by chemical modification and nuclear magnetic resonance.

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from commercial sources: S-hexylglutathione, S-hexylglutathione-linked Sepharose from Sigma (St. Louis, USA); reduced glutathione (GSH) from Yamanouchi (Tokyo, Japan); Isopropyl-β-D(-)-thiogalactopyranoside (IPTG), and N-ethylmaleimide (NEM) from Wako Pure Chemical (Tokyo, Japan) ;1-chloro-2,4-dinitrobenzene (CDNB) from Tokyo Kasei (Tokyo, Japan); 7-fluoro-4-sulfamoyl-2,1,3-benzodiazole (ABD-F) from Dojindo Chemical (Tokyo, Japan); methylmethanethiolsulfonate from Aldrich (Milwaukee, USA); $[^{13}C]CH_3$Bromomethane from Nippon Sanso (Tokyo, Japan). Lysyl Endopeptidase, NdeI and BamHI from Takara

(kyoto, Japan). All other chemicals were of analytical grade.

Expression of Glutathione S-transferase P in E. coli

The primers used were listed in Table I. The sequence containing ATG initiation codon of GST-P cDNA was converted to NdeI recognition sequence by PCR mediated mutagenesis and inserted into NdeI-BamHI site for expression vector pET3a (pETGST-WTNde) (10). To construct the mutated GST-P plasmids, "Splicing by overlapped extension method" (SOE) was used with pETGST-WTNde template as previously described (10, 11). The E. coli, BL21(DE3)lysS, transformed by these plasmids were cultured and GST-P expression was induced by addition of IPTG. GST-P was isolated in a homogeneous state from E. coli possessing GST-P clone as described (12). GST activity was measured by the method of Habig et al.(13). Concentrations of GST-P (Mr 50,130) was spectrophotometrically determined using $E^{280}_{1\%}$=10.5. Molar concentration was estimated by amino acid composition analysis. The composition after hydrolysis (6 M HCl containing 0.05% phenol, 110°C, 24 hr) was determined on a Hitachi 835 amino acid analyzer.

Labeling of cysteine residue by ABD-F

GST-P was dissolved in borate buffer in the presence of ABD-F, and reacted at 37 °C for 30 min. After the reaction, the labeled GST-P was dialyzed against Tris/HCl (10 mM, pH 9.0) and digested with Lysyl Endopeptidase (enzyme : substrate = 1 : 200 (mol/mol)) at 30 °C for 12 hr. The peptide was separated on reverse phase high-performance liquid chromatography (ODS column, 4 x 250 mm, Toso) by a Shimazu 130 liquid chromatography system. Absorbance was detected at 210 nm and 280 nm, and fluorescence intensity was detected at 510 nm as excited at 380 nm. Amino acid sequence analysis was carried out on a ABI 477A peptide sequencer.

Organic synthesis of[^{13}C]methylmethanethiolsulfonate

Two mmol of potassium methanethiolsulfonate was suspended in 5 ml of anhydrous ethanol. 0.72 mmol of [^{13}C]bromomethane was slowly added to the solution and reacted at 22°C for 12 hrs. After the reaction, the mixture was cooled in ice. Excess bromoethane (1.28 mmol) was added and temperature was gradually returned to room temperature, and stayed for further 6 hrs. The reaction mixture was cooled down to 0°C, and the reactant was filtered with 2 ml of anhydrous ethanol three times. The filtrate was combined and dried in vacuo. The final preparation was carried out by distillation which produced clear yellow oil.

Carbon 13 nuclear magnetic resonance

To prepare the S-[^{13}C]CH_3 labeled enzyme, GST-P or its mutants was reacted with [^{13}C]methylmethanethiolsulfonate in 10 mM glycine-NaOH buffer (pH 7.8) for 1 hr at 22°C. The excess reagent was removed by Sephadex G-25 column chromatography equilibrated with the same buffer. GST-P or its mutant labeled with S-[^{13}C]CH_3 was dissolved in potassium phosphate buffer (50 mM, pH 6.5). Nuclear magnetic resonance (NMR) experiment was carried out by a JEOL GX-500 (500 MHz) spectrometer.

RESULTS

Purification of Enzyme

The final enzyme preparation was homogeneous upon SDS-PAGE and the molecular weight was calculated as about 25 kDa (data not shown). The molecular weight of the enzyme was about 50 kDa by

Table I. Oligonucleotide primers used for site-directed mutagenesis

Primer	Sequence	Note
Cys47Ser+	AAGTCCACTTCTCTGTATGGG	Forward
Cys47Ser-	CCCATACAGAGAAGTGGACTT	Inverse
Cys101Ser+	GACCTTCGATCTAAATATGGT	Forward
Cys101Ser-	ACCATATTTAGATCGAAGGTC	Inverse

Underline: position mutated.

Sephadex G-100 column chromatography under the non-denatured conditions. These data indicated that GST-P existed as a homodimeric form.

Labeling of cysteine residue with ABD-F

Enzymatic activity of wild type GST-P was totally lost by chemical modification with ABD-F (10 mM) (Table II). In the peptide map made by Lysyl Endopeptidase digestion, two major peptides labeled with the fluorescent agent were collected (Fig. 1). From peptide sequence analysis, peptide A was determined as a fragment from 45th to 54th from the amino terminus, and peptide B was that from 85th to 102th.

Peptide A : Ser-Thr-X-Leu-Tyr-Gly-Gln-Leu-Pro-Lys.

Peptide B : Glu-Ala-Ala-Leu-Val-Asp-Met-Val-Asn-Asp-Gly-Val-Glu-Asp-Leu-Arg-X-Lys.

X means an unidentified residue. Since fluorescence was detected in the fraction of X, the residue was determined as ABD-F labeled cysteine residue. In the presence of 0.2 % SDS, two other fluorescence-labeled peptide fragments were detected, corresponding to ABD-F adducts of Cys14 and Cys169 (data not shown). These facts indicated that cys47 and cys101 were selectively labeled

Table II. Chemical Modification by ABD-F

Enzyme	Remaining activity (%)
Wild type	100
Wild type + ABD-F	<0.1
Cys47Ser + ABD-F	97.5
Cys101Ser + ABD-F	<0.1
Cys47,101Ser + ABD-F	102.1

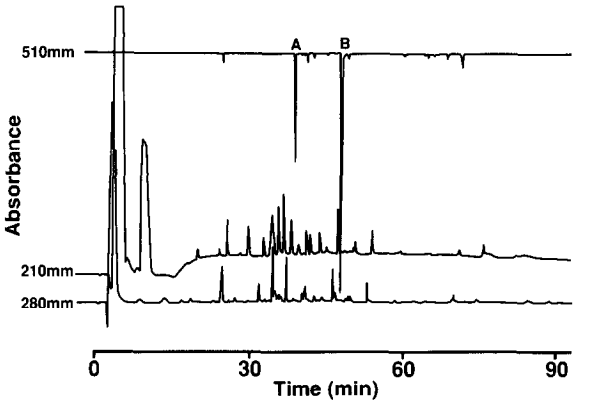

Fig. 1. Peptide analysis of ABD-F labeled GST-P. GST-P (100 nmol) was labeled with ABD-F (1 μg), and was digested with Lysyl Endopeptidase as described in Experimental Procedures. Peptides were separated in the condition that the concentration of acetonitoril was linearly increased from 0 to 80%.

with ABD-F in the native form of GST-P, and Cys14 and Cys169 were labeled in the denatured form.

Effect of replacement of cysteine with serine on GST activity

Little change of enzymatic activity was observed on three mutants, Cys47Ser, Cys101Ser, and Cys47,101Ser (Table III). These values of Km for GSH and CDNB were almost identical. For chemical modification of the mutants by ABD-F, only the Cys101Ser lost the enzymatic activity. The comparable results were obtained about chemical modification by NEM (data not shown). The results indicated that the ABD-F introduced to Cys47 caused GST-P to lose the activity.

On the other hand, either wild type GST-P or any mutant did not show any significant loss of GST activity when they were labeled with S-CH_3 (Table IV). These facts indicated that the

Table III. Kinetic parameters

Enzyme	Km (mM)		Vmax
	GSH	CDNB	(μmol/min/mg)
Wild type	0.15	1.15	14.5
Cys47Ser	0.17	1.15	12.5
Cys101Ser	0.16	1.16	13.7
Cys47,101Ser	0.15	1.18	12.1

Table IV. Chemical Modification by methylmethanethiolsulfonate

Enzyme	Remaining activity (%)
Wild type	100
Wild type + S-CH_3	89.0
Cys47Ser + S-CH_3	99.5
Cys101Ser + S-CH_3	86.1
Cys47,101Ser + S-CH_3	98.3

inhibition of GST activity depended on molecular sizes of an adduct introduced to Cys47.

Structural analysis near cysteine residues by carbon 13 NMR

To analyze the different effect of these thiol-modifiers to GST activity, carbon 13 NMR experiments was carried out. Two carbon 13 NMR signals were observed at 23.2 ppm and 25.3 ppm in the wild-type labeled with S-[^{13}C]CH_3 (Fig. 2). It showed that two cysteine residues out of the four were selectively labeled with S-[^{13}C]CH_3 as in the case with ABD-F. On Cys101Ser and Cys47Ser mutants, the respective carbon NMR signals were observed at 23.2 ppm and at 25.4 ppm. Since methylmethanethiolsulfonate reacted with Cys47 of the Cys101Ser and with Cys101 of the Cys47Ser, the resonance at 23.2 ppm was assigned to a signal of

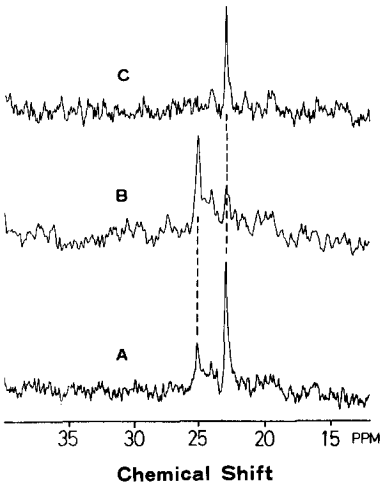

Fig. 2. Carbon NMR spectra of GST-P labeled with S-[^{13}C]CH_3. Ten nmol of GST-P or the mutants was dissolved in 0.5 ml potassium phosphate buffer, and NMR experiment was carried out as described in Experimental Procedures: Wild-type GST-P (A), Cys47Ser (B), and Cys101Ser (C).

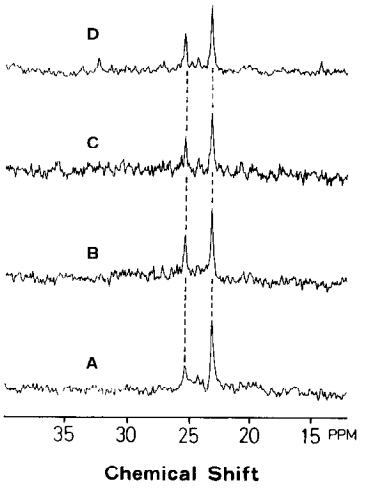

Fig. 3. Effect of S-hexylglutathione on NMR spectra of S-[^{13}C]-CH_3 GST-P. Ten nmol of the native GST-P was prepared as in Fig. 4, to which various amount of S-hexylglutathione was added, and NMR experiment was carried out. Molar ratio of S-hexylglutathione to S-CH_3 labeled GST-P: 0 : 1, (A); 20 : 1, (B); 50 ; 1, (C); 100 : 1, (D).

the Cys47 adduct and that at 25.4 ppm was to a signal of the Cys101 adduct.

We monitored the change of carbon 13 NMR spectra on S-[^{13}C]CH_3 labeled GST-P in the presence of various concentrations of S-hexylglutathione to clarify the structural relationship of Cys47 near the active site. Even if [S-hexylglutathione]/[S-CH_3 labeled GST-P] molar ratio was increased to 100, no appreciable change of NMR signals was observed (Fig. 3). The result showed that Cys47 did not exist in the active site.

DISCUSSION

A thiol-group of cysteine residue is chemically reactive by itself. In a several enzymes, a cysteine residue is unusually reactive and important for catalytic function (14, 15). By chemical modification of the cysteine residues in GST-π or GST-P, Cys47 had been considered to be essential for the catalytic activity (6, 7). Recently, a series of papers on the role of cysteine residues using site-directed mutagenesis have shown that

any of the cysteine residues is not essential for the catalytic function (8, 9). To evaluate the different outcome of chemical modifications from that of site-directed mutagenesis, we studied on the protein structure-function relationship at the active site.

Chemical modification have a risk to cause misunderstanding because of its untoward side reaction. The maleimide-derivatives were reported to react with both lysine and histidine residues as well as cysteine (16). In this regard, ABD-F is more favorable for labeling a cysteine residue because it reacts more selectively with thiol-groups under weak alkaline condition, and the adduct is fairly stable (17). In addition, the benzodiazole-derivate is well soluble in aqueous solution. By this selective labeling, we showed that GST-P had two highly reactive cysteine residues, Cys47 and Cys101. Numbers of reactive cystein residues measured by DTNB were 1.8 and 3.5 in the native GST-P and in the presence of 0.2 % SDS, respectively (data not shown). These facts indicated that two cysteine residues were located at the protein surface whereas other two residues were buried in the hydrophobic protein core. Papers on the reactive thiol-group of class π GST showed a single chemically modified cysteine residue, Cys47 (7, 18). The difference on calculated number of reactive cysteines from our present result may rely on the chemical reactivities or stabilities of these chemical adducts.

Replacements of cysteine residues including Cys14 and Cys169 (data not shown) with serine residues did not affect the enzymatic activity. The result showed that cysteine residues were not essential for the catalytic action as indicated by others (8, 9). But, chemical modification by ABD-F showed that the modification of Cys47 related with the loss of GST activity (Table II), even if the cysteine residue was not located in the active site. On

location of Cys47, carbon 13 NMR experiment revealed that Cys47 did not exist at the active site (Fig. 3). Actually, three-dimensional structure of class π GST from a pig lung showed that glutathionesulfonate did not contact with Cys45, corresponding to Cys47 of GST-P (19). Taken together with the fact that GST-P did not lose the enzymatic activity by a small adduct to Cys47 (Table IV), Cys47 is located near the active site, not within the site. Therefore, it is concluded that the bulky adducts to Cys47 caused inactivation of GST activity through the steric hindrance of the substrate binding.

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