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IDENTIFICATION OF THE FATTY ACID BINDING SITE ON GLUTATHIONE S-TRANSFERASE P

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Summary: Glutathione S-transferase P (GST-P) bound a series of endogenous fatty acids (C_{12}-C_{18}). To clarify the function and the binding site of the fatty acids, interaction between fatty acids and GST-P was investigated by using 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K. The fluorescence-conjugated fatty acid noncompetitively inhibited GST activity. After GST-P was covalently labeled with the fatty acid, the enzyme was digested with Lysyl Endopeptidase. From the peptide mapping, a single fluorescence-labeled peptide was obtained. By the sequence analysis, the peptide binding fatty acid was determined as the residues of 141-188 from the amino terminus.

Glutathione S-transferase (GSTs) [EC 2.5.1.8] are important enzymes for detoxification because of their ability to conjugate a variety of electrophilic compounds with glutathione (1, 2). Recently, species-independent classification of the isoenzymes has been proposed (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.

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Abbreviations: GST, glutathione S-transferase; GSH, glutathione; CDNB, 1-chloro-2,4-dinitrobenzene; IPTG, isopropyl-β-D(-)-thiogalactopyranoside; PBS, phosphate buffer saline; GC-MS, gas-liquid chromatography and mass-spectrometry; EDTA, ethylenediaminetetraacetic acid; SDS-PAGE, sodium dodecyl-sulfate polyacrylamide-gel electrophoresis.

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Each subunit consists of 209 amino acids and they differ in only 30 amino acids (85.6 % homology) (5).

In addition to the conjugating activity, transferases bind nonsubstrate ligands such as hemin, bilirubin and fatty acid (6-8). During the course of our studies on GST-P (9, 10), we have found that recombinant GST-P also binds a variety of hydrophobic compounds *in vitro*. We have expressed the GST-P in E. coli, purified and extracted the total lipid to identify endogenous hydrophobic ligands bound to the enzyme. The lipid analysis showed that GST-P was associated with a series of fatty acids. Identification of the fatty acid binding site is important to study the effect of hydrophobic ligand on GST activity from the structure-function relationship. In this report, we show the effect of fatty acid on GST activity, and reveal the binding site by affinity labeling using a fatty acid conjugated with Woodward's reagent K.

EXPERIMENTAL PROCEDURES

Materials

The following materials were obtained from commercial sources: Lysyl Endopeptidase, NdeI and BamHI from Takara (Kyoto, Japan); 12-(9-anthroyloxy) stearic acid from Lambda Probes & Diagnostics (Graz, Austria); Woodward's reagent K from Aldrich (Milwaukee, USA); reduced glutathione (GSH) from Yamanouchi (Osaka, Japan); isopropyl-β-D(-)-thiogalactopyranoside (IPTG) from Wako (Tokyo, Japan), 1-chloro-2,4-dinitrobenzene (CDNB) from Tokyo Kasei (Tokyo, Japan). All other chemicals were of analytical grade.

Expression and purification of Glutathione S-transferase P

GST-P was expressed in E. coli harboring GST-P expression plasmid (10). In brief, the sequence containing ATG initiation codon of GST-P cDNA was converted to NdeI recognition sequence by PCR mediated mutagenesis and inserted in NdeI-BamHI site of expression vector pET3a. E. coli, BL21(DE3)LysS, transformed with this plasmid was cultured and GST-P was induced by addition of IPTG. After E. coli was collected, washed and disrupted by French pressure cell disrupter at 1000 psi, the homogenate was centrifuged at 105,000 x g for 1 hr. The supernatant was used as the cytosolic fraction. GST-P was purified as previously described (11). GST activity was measured by the method of Habig et al. (12). Concentration of protein was spectrophotometrically determined by using $E^{280}_{1\%}$=10.5. The molar extinction coefficient was estimated by amino acid composition analysis.

Gas-liquid chromatography and mass-spectrometry (GS-MS)

Purified GST-P was dialyzed against distilled water for fatty acid analysis. Lipids were extracted from the enzyme by the method of Folch et al. (13). The extracted lipids were dried

under nitrogen, and free fatty acids were esterified with diazomethane at 22°C for 30 min. The resulting fatty acid methyl esters were analyzed by GC-MS using a JEOL JMS-DX 300 equipped with a 1 % OV1 column (2 m). Conditions were as follows; injector temperature, 250°C; column temperature, 150-250°C at a rate of 4 °C/min; ion source, 250°C, ionizing voltage, 70 eV. Pentadecanoic acid was used as an internal standard.

Synthesis of fatty acid conjugated with Woodward's reagent K

12-(9-anthroyloxy) stearic acid (0.2 mmol) was reacted with Woodward's reagent K (0.6 mmol) at 20°C for 45 min in 20 ml of acetonitrile containing triethylamine (2.0 mmol). Following evaporation *in vacuo*, the residue was dissolved in 5 ml of deionized water and filtrated. The filtrate was fractionated on Sephadex G-25 column (1 x 25 cm) equilibrated with H_2O at a flow rate of 40 ml/hr. 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K was further purified by HPLC (C5 reverse phase column, 4 x 150 mm, Wako) with a linear gradient of acetonitrile (0-90%).

Affinity labeling of fatty acid to GST-P

GST-P (3 μmol) was dissolved in 4 ml of PBS to which 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K (WF) (2.8 μmol) was added. The mixture was stirred under nitrogen at 22°C in the dark for 1 hr. After EDTA (1 mM) was added to the reaction mixture, pH was adjusted to 9.4 with imidazole (ca. 11 mmol). The reaction was further continued for 8 hrs, and the resulting fluorescence-labeled GST-P was purified by SDS-PAGE.

Peptide mapping of GST-P digested by Lysyl Endopeptidase

GST-P covalently labeled with 12-(9-anthroyloxy) stearic acid was dissolved in a Tris/HCl buffer (10 mM, pH 9.0) and digested with Lysyl Endopeptidase (enzyme : substrate = 1 : 200 (mol/mol)) at 30°C for 12 hrs. Peptides were separated on reverse phase HPLC (ODS column, 4 x 250 mm, Toso) by a Shimazu 130 liquid chromatography system. Absorbance of peptides was detected at 210 nm, and fluorescence intensity was measured at 460 nm as excited at 380 nm. Amino acid sequence analysis was carried out by a ABI 477A peptide sequencer.

RESULTS

Expression and purification of GST-P

The final enzyme preparation gave a single band with molecular weight of 25 kDa upon SDS-PAGE (data not shown). The molecular weight of the enzyme was about 50 kDa by Sephadex G-100 column chromatography under non-denatured conditions. These results indicated that GST-P existed as a homodimer form. The specific activity of the final preparation was 12 μmol/min/mg protein. This value was comparable with that of GST-P from rat placenta (14).

Identification of fatty acid associated with GST-P

Analysis of the endogenous fatty acids associated with GST-P revealed that the enzyme noncovalently bound long-chain (C_{12} -

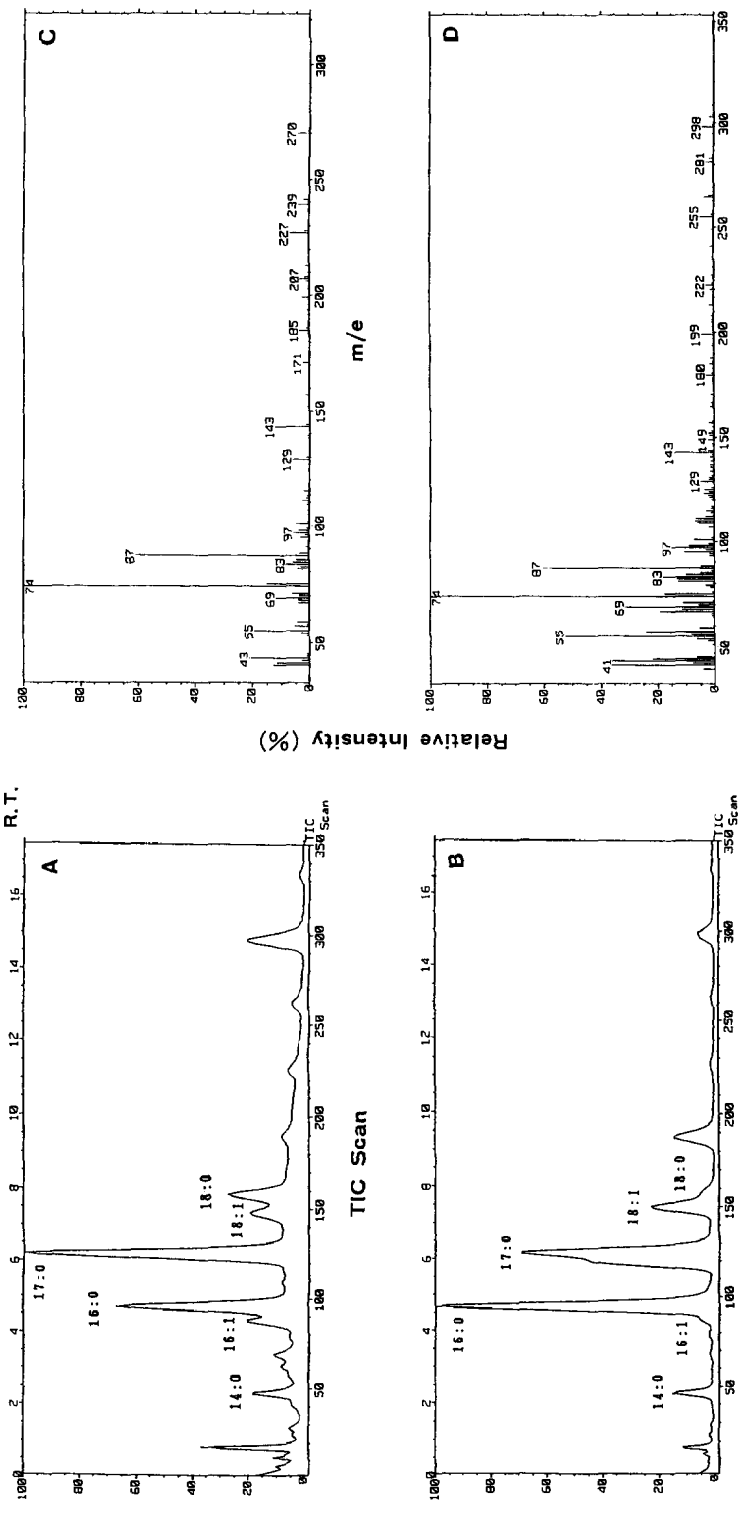

Fig. 1. GC-MS of fatty acids extracted from E. coli and purified GST-P. Gas-liquid chromatograms of fatty acid extracted from purified GST-P (5 mg) (A) and E. coli (10^8 cells) (B). Mass-spectrums on the peaks labeled as 16:0 (C) and 18:0 (D).

C_{18}) saturated and unsaturated fatty acids (Fig. 1). Palmitic acid and stearic acid were the major species, and composition of these fatty acids were similar to that synthesized by E. coli. Fatty acid analysis was also carried out on GST-P delipidated by chloroform and methanol. Any covalently associated fatty acid molecule was not detected on the delipidated enzyme after hydrolysis by 6 N HCl at 110°C for 24 hrs (data not shown).

Effect of 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K on GST activity

The effect of 12-(9-anthroyloxy) stearic acid conjugated with Woodward's reagent K (WF) on GST activity was evaluated by adding WF to the enzyme solution. From the double reciprocal plots of catalytic velocity and the substrates, GSH and CDNB, it was determined that WF inhibited GST activity in a noncompetitive manner (Fig. 2). The inhibition constant (Ki) was approximately 8 μM for these substrates.

Identification on fatty acid binding site

A single fluorescence-labeled peptide was identified in the peptide map of GST-P digested by Lysyl Endopeptidase (Fig. 3).

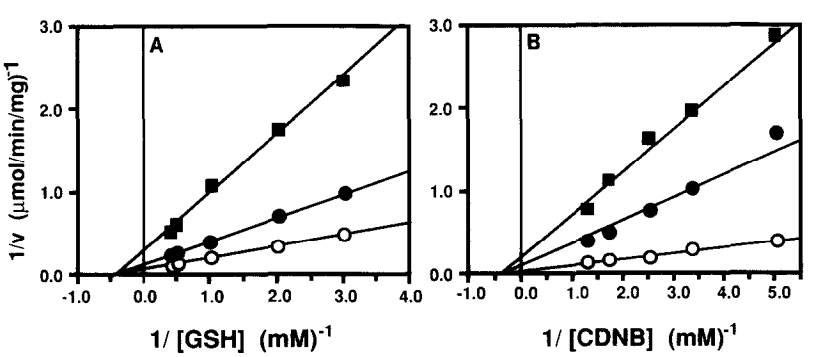

Fig. 2. Kinetics of GST-P in the presence of Woodward's reagent K conjugated 12-(9-anthroyloxy) stearic acid (WF). The plots are 1/(specific enzymatic activity) against 1/[GSH] (A) and 1/[CDNB] (B). The concentration of WF were 0 (○), 2.5 (●) and 5 μM (■).

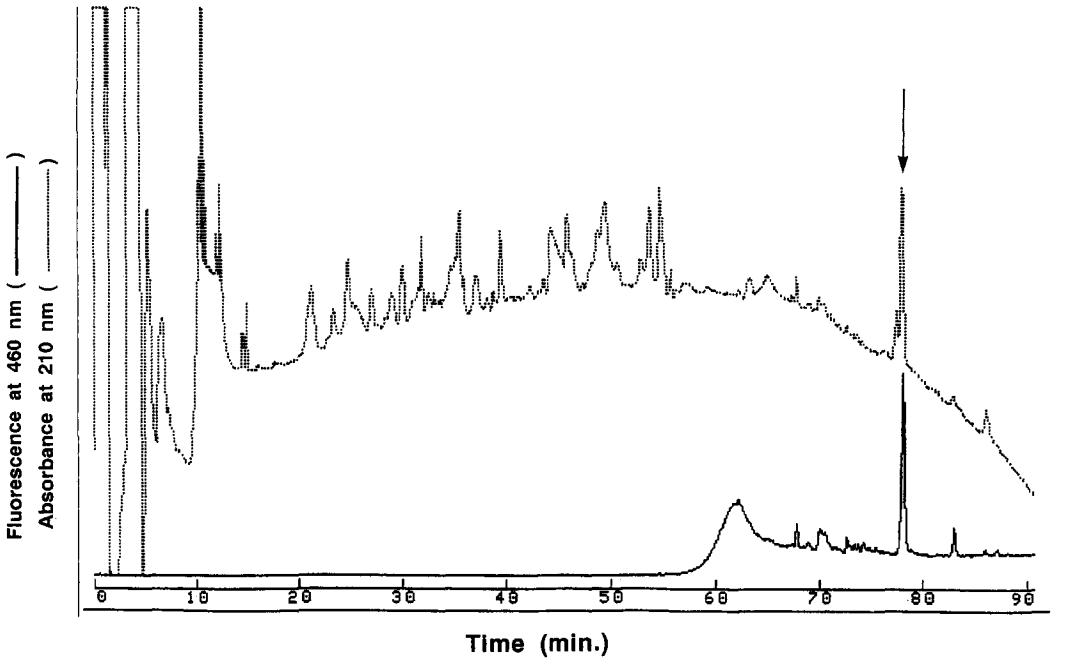

Fig. 3. Peptide map of GST-P labeled with 12-(9-anthroyloxy) stearic acid. Peptides were separated by linearly increased acetonitrile from 0 to 80 %. The peptide indicated by an arrow was collected for amino acid composition and sequence analysis.

As the peptide was chemically bound with the fluorescent hydrophobic fatty acid, it was eluted at higher concentration of acetonitrile clearly separated from other peptides. Collecting the peptide, the amino acid sequence was determined. Sequence analysis showed that the fluorescence-labeled peptide was 141-188 residues from the amino terminus. As the cysteine 169 was not identified by the peptide sequence analysis, it was concluded that the thiol group of this cysteine residue reacted chemically with the carboxyl group of the fatty acid. Amino acid composition of this peptide supported the result (data not shown).

DISCUSSION

GSTs bound various endogenous and exogenous hydrophobic compounds as substrates or nonsubstrates (6-8). GST-B (ligandin), was originally discovered as a binding protein capable of forming

complexes with various hydrophobic compounds as well as with xenobiotics (15). GST 1-1 and GST 2-2 possess a single binding site per dimer for heme, bilirubin and bile acid (16). In this study, we showed that GST-P bound endogenous long-chain fatty acid (Fig. 1). The chain lengths ranged from 12 to 18 carbons, and the composition was similar to that being synthesized by E. Coli. This fact indicated that GST-P did not selectively bind any specific fatty acid. The structure of the fatty acid binding site could also accept other hydrophobic compounds, such as bilirubin (17).

Analyses on an interaction between fatty acids and proteins are often accompanied by difficulties because of insolubility of hydrophobic ligands. When a fatty acid is conjugated with Woodward's reagent K, solubility is increased as in the case of bilirubin (18). The conjugate (WF) bound specifically to GST-P, which inhibited the enzymatic activity in a noncompetitive manner (Fig. 2). A putative hydrophobic ligand, anilinonaphthalenesulfonic acid (ANS), also inhibited the enzymatic activity (data not shown). In addition, both ANS and WF bound the same binding site of GST-P. These results suggest that GST-P has a nonsubstrate ligand binding site which regulates GST activity.

The fatty acid conjugated with Woodward's reagent K is useful characteristic to determine its binding site : the carboxyl group of fatty acid once conjugated with Woodward's reagent can covalently bind a side chain of basic amino acid residue at alkaline pH. The carboxyl group of 12-(9-anthroyloxy) stearic acid reacted with the thiol group of Cys169 (Fig. 3). As Cys169 was buried in the hydrophobic protein core, the carboxyl group should be localized in the protein core (10). These results demonstrate the topological feature of fatty acid within the binding site : the carboxyl group of fatty acid may be localized in the hydrophobic protein core as in the case of fatty-acid-binding protein (19).

The recent crystallographic study on class π GST from the porcine lung demonstrated that class π GST consisted of two domain structures, domain I and II (20). This structural analysis indicated that domain I (N-terminal side) had the substrate binding site, and domain II (C-terminal side) was abundant of the hydrophobic amino acid residues. Structural analyses by site-directed mutagenesis on class π GSTs supported the observation of crystallography on the location of the active site (14, 21). We showed that the fatty acid binding site was localized in domain II by affinity labeling in this study. When fatty acids bound the domain II, enzymatic activity was inhibited (Fig. 2). Taken these facts together, domain I and II represent the catalytic and the regulatory domain, respectively. This hypothesis is consistent with the result of enzyme kinetic study. Further study on the structure-function relationship with regard to fatty acid binding is under way by using nuclear magnetic resonance.

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REFERENCES

1. Chasseau, L. F. (1979) Adv. Cancer Res. 29, 175-274.
2. Jakoby, W. B., and Habig, W. H. (1980) in Enzymatic Basis of Detoxication (Jakoby, W. B., ed.), Vol. 2, pp. 63-94, Academic Press, New York.
3. Mannervik, B., Alin, P., Guthenberg, C., Jensson, H., Tahir, M. K., Warholm, M., and Jornvall, H. (1985) Proc. Natl. Acad. Sci. USA, 82, 7202-7206.
4. Mannervik, B., Awasthi, Y. C., Board, P. G., Hayes, J. D., Di-Ilio, C., Ketter, B., Listowsky, I., Morgenstern, R., Muramatsu, M., Pearson, W. R., Pickett, C. B., Sato, K., Widersten, M., and Wolf, C. R. (1992) Biochem. J. 282, 305-306.
5. Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T., and Muramatsu, M. (1985) Nucleic Acids Res. 13, 6049-6957.
6. Mannervik, B. (1985) Adv. Enzymol. 57, 357-417.
7. Mannervik, B., and Guthenberg, C. (1981) Methods Enzymol. 77, 231-235.
8. Li, Ming, and Ishibashi, T. (1990) J. Biochem. 108, 462-465.
9. Nishihira, J., Ishibashi, T., Sakai, M., Nishi, S., and Kumazaki, T. (1992) Biochem. Biophys. Res. Commun. 167, 331-338.

10. Nishihira, J., Ishibashi, T., Sakai, M., Nishi, S., Kumazaki, T., Hatanaka, Y., Tsuda, S., and Hikichi, K. (1992) Biochem. Biophys. Res. Commun. (in press).
11. Tu, C. P. D, Weiss, M. J., Li, N. Q., and Reddy, C. C. (1983) J. Biol. Chem. 258, 4659-4662.
12. Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) J. Biol. Chem. 249, 7130-7139.
13. Folch, J., Lees, M., and Sloane-Stanley, G. H. (1957) J. Biol. Chem. 226, 497-509.
14. Tamai, K., Shen, H., Tsuchida, S., Hatayama, I., Satoh, K., Yasui, A., Oikawa, A., and Sato, K. (1991) Biochem. Biophys. Res. Commun. 179, 790-797.
15. Litwack, G., Ketterer, B., and Arias, I. M. (1971) Nature 234, 466-467.
16. Ketley, J. N., Habig, W. H., and Jakoby, W. B. (1975) J. Biol. Chem. 250, 8670-8673.
17. Satoh, K., Hatayama, I., Tsuchida, S., and Sato, K. (1991) Arch. Biochem. Biophys. 285, 312-316.
18. Boyer, T. D. (1986) J. Biol Chem. 261, 5363-5367.
19. Sacchettini, J. C., Gordon, J. I., and Banaszak, L. J. (1989) J. Mol Biol. 208, 327-339.
20. Reinemer, P., Dirr, H. W., Ladenstein, R., Schaffer, J., Gally, O., and Huber, R. (1991) EMBO J. 10, 1997-2005.
21. Kong, K., Inoue, H., and Takahashi, K. (1991) Biochem. Biophys. Res. Commun. 181, 748-755.