Vol. 167, No. 1, 1990 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
February 28, 1990 Pages 331-338

SPECIFIC INACTIVATION OF GLUTATHIONE S-TRANSFERASES IN CLASS PI BY SH-MODIFIERS

Katsuto Tamai, Kimihiko Satoh, Shigeki Tsuchida, Ichiro Hatayama, Takako Maki, and Kiyomi Sato[1]

Second Department of Biochemistry, Hirosaki University School of Medicine, Hirosaki 036, Japan

Received January 10, 1990

Summary: Treatment of Class Pi glutathione S-transferases (GST) such as rat GST P (7-7), human GST π and mouse GST MII with 0.05 - 0.1 mM N-ethylmaleimide (NEM) in 0.1 M Tris-HCl (pH 7.8) resulted in almost complete inactivation of these forms, whereas no or less inactivation occurred for GSTs in Class Alpha and Mu under the same conditions. Inactivated GST P lost its S-hexyl-GSH-Sepharose column affinity. About 0.8 mol of [^{14}C]NEM was found to be covalently bound to 1 mol of GST P subunit when 80% of the activity was lost. Similar treatment with N-dimethyl-amino-3,5-dinitrophenyl maleimide, a colored analogue of NEM, followed by trypsin digestion, HPLC and amino acid sequence analysis revealed that one cysteine residue at the 47th position from the N-terminal of the GST P subunit was preferentially modified. Subunits of GST P and GST π are known to have 4 cysteine residues at the same corresponding positions. The present results suggest that the 47th cysteine residue may be located in the vicinity of the active site of Class Pi GSTs.
© 1990 Academic Press, Inc.

With the exception of one microsomal form the glutathione S-transferases (GST , EC 2.5.1.18) are a family of multifunctional dimeric proteins present in cytosolic fractions (1), the majority of which can be classified into three groups, Alpha, Mu and Pi in a species-independent classification (1).

---

[1] To whom correspondence should be addressed.

Abbreviations used are: CDNB, 1-chloro-2,4-dinitrobenzene; DDPM, N-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DTT, dithiothreitol; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; HPLC, high performance liquid chromatography; NEM, N-ethylmaleimide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

0006-291X/90 $1.50
Copyright © 1990 by Academic Press, Inc.
All rights of reproduction in any form reserved.

We earlier described the rat GST P (7-7)[2], a Class Pi form, as a reliable marker for rat hepatic preneoplastic lesions and have also proposed the related human GST π as a possible marker for neoplastic lesions in various organs (2). While the actual functions of GST P and GST π in (pre)neoplastic tissues remain unclear, these forms have recently been considered to participate in (multi)drug resistance mechanisms (2). In this paper, evidence is presented that GSTs in Class Pi have in common one cysteine residue, at the 47th from the amino(N)-terminus reactive to SH-modifiers such as NEM and that this residue may be located near the active site of the enzymes.

MATERIALS AND METHODS

Materials. NEM was obtained from Nakarai Chemical Ltd., Kyoto, GSH from Boehringer-Mannheim-Yamanouchi, Tokyo; epoxy-activated Sepharose 6B from Pharmacia, Uppsala; and N-ethyl[2,3-^{14}C]-maleimide from Amersham-Japan, Tokyo. All other chemicals were of analytical grade.

GST preparations and assay. Rat GST YaYa (1-1), YaYc (1-2) and Yb1Yb2 (3-4) were purified from rat normal liver (3) and GST P from rat liver bearing hyperplastic nodules (4). Human GST α and μ were from the liver and GST π from the placenta (5). Mouse GST MI, MII and MIII were from normal liver (6). GST activity was assayed using CDNB as the substrate (7). One unit of GST activity was defined as that catalyzing conjugation of 1 μmol of substrate per min at 25°C.

Treatment of GSTs with NEM. GSTs (0.1 unit each) were incubated with 0.02 - 0.2 mM NEM in 1 ml of 0.1 M Tris-HCl (pH 7.8) at 25°C. After 10 min preincubation, 100 μl aliquots of the mixture were removed and used for assay of GST activity.

S-hexyl-GSH affinity chromatography and SDS-PAGE of NEM-treated GSTs. An enzyme mixture containing GSTs YaYc (200 μg), Yb1Yb2 (100 μg)and GST P (100 μg) was incubated with 1 mM NEM in 3 ml of 0.1 M Tris-HCl (pH 7.8) at 25°C for 30 min, and then dialyzed against 10 mM Tris-HCl (pH 7.8). The mixture was subjected to S-hexyl-GSH Sepharose chromatography (column size 1 x 5 cm) as described previously (5). GSTs in breakthrough and bound fractions were respectively subjected to SDS-PAGE (8).

Labeling of GST-P with [^{14}C]NEM. GST P (2.0 nmol) was incubated with 0.1 μmol of [^{14}C]NEM (1670000 cpm/μmol) in 1.0 ml of 0.1 M Tris-HCl (pH 7.0) at 25°C. At indicated time intervals, aliquots (0.1 ml) were taken into 3 ml of 10% trichloroacetic acid followed by addition of 0.1 ml of bovine serum albumin (10mg/ml). Precipitated proteins were collected by centrifugation at 3000 rpm for 5 min, and dissolved in 0.7 ml 0.1 N NaOH. After repeat

---

2 In a new nomenclature, Ya, Yc, Yb1, Yb2 and Yp correspond to 1, 2, 3, 4, and 7, respectively (1).

of this procedure twice to remove free [^{14}C]NEM, aliquots (0.5 ml) of alkaline solution were mixed with 4 ml of scintillation cocktail (Aquasol-2, New England Nuclear, Boston) and GST P radioactivities were determined.

N-terminal amino acid sequencing. N-terminal amino acid sequencing based on the automated Edman degradation method was performed using a gas-phase sequencer (Model 470A, Applied Bio-Systems, Foster, Calif.). A solution (0.8 ml) of GST P (0.95mg/ml) in 50 mM phosphate (pH 7.0) was reduced at room temperature for 20 min by addition of 10 mM DTT (16 μl). After treatment with 10 mM DDPM (48 μl) for 1 min the reaction was stopped by addition of 10 mM DTT (48 μl). Unreacted reagents were removed on a PD-10 column (Pharmacia), which had been equilibrated with 10 mM Tris-HCl (pH 8.1). Fractions containing the DDPM-bound GST P were combined, concentrated to approx. 0.5 ml by a centrifree concentrator (Amicon-Grace, Japan), and digested with DPCC-treated trypsin (5 μg) at 30°C for 16 h. A 100 μl portion of the hydrolysate was applied to an HPLC apparatus, Model 638-30, Hitachi Koki Co., Ltd., Tokyo, equipped with an octadecylsilica column of μBondasphere C-18, 100 A (3.9 x 150 mm). Peptides were separated with a linear gradient from water/0.1% TFA to acetonitrile/0.1% TFA. The flow rate was 1 ml/min and peptides were detected at the two wave lengths of 210 and 280 nm.

RESULTS

Inactivation of GSTs in Class Pi by NEM. As shown in Fig. 1, preincubation of Class Pi GSTs (rat P (YpYp), mouse M-II and human π) with 0.05 - 0.2 mM NEM for 10 min resulted in time and dose-dependent loss of more than 90% of their activities, whereas GSTs in Class Alpha (rat YaYc, mouse M-I and human α) and Class

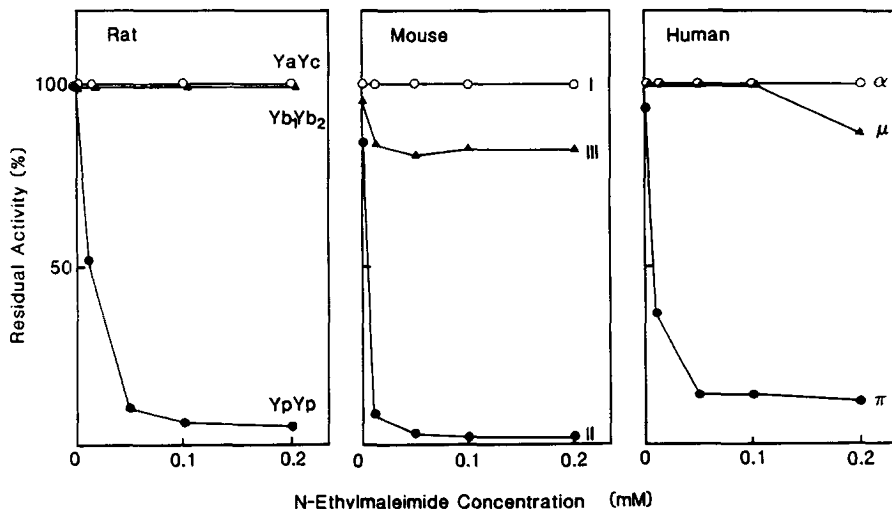

Fig. 1. Inactivation of rat GST P, mouse GST-MII and human GST π by NEM. Details of the procedure are described in the Methods section.

Mu (rat Yb1Yb2, mouse M-III and human μ) were far less affected (0 - 20% inactivation) under the same conditions (Fig. 1). Almost 90% of the Class Pi activity was lost on incubation with 0.1 mM NEM within 2 min, whereas 50% of the activity remained after 10 min with 0.01 mM NEM.

Loss of affinity of GST P for S-hexyl-GSH-Sepharose by NEM treatment. After treatment of a mixture of rat GSTs YaYc, Yb1Yb2 and YpYp with 1 mM NEM for 30 min, while most of the forms were still adsorbed on an S-hexyl-GSH-Sepharose column (Fig. 2A), SDS-PAGE (Fig. 2B) revealed YpYp in the breakthrough fractions, YaYc and Yb1Yb2 remained bound to the column until eluted with S-hexyl-GSH.

Stoichiometry of the modification of cysteine residues of GST P subunit with NEM. It has been reported that GST P and GST π have 4 cysteine residues in their respective subunits at the same

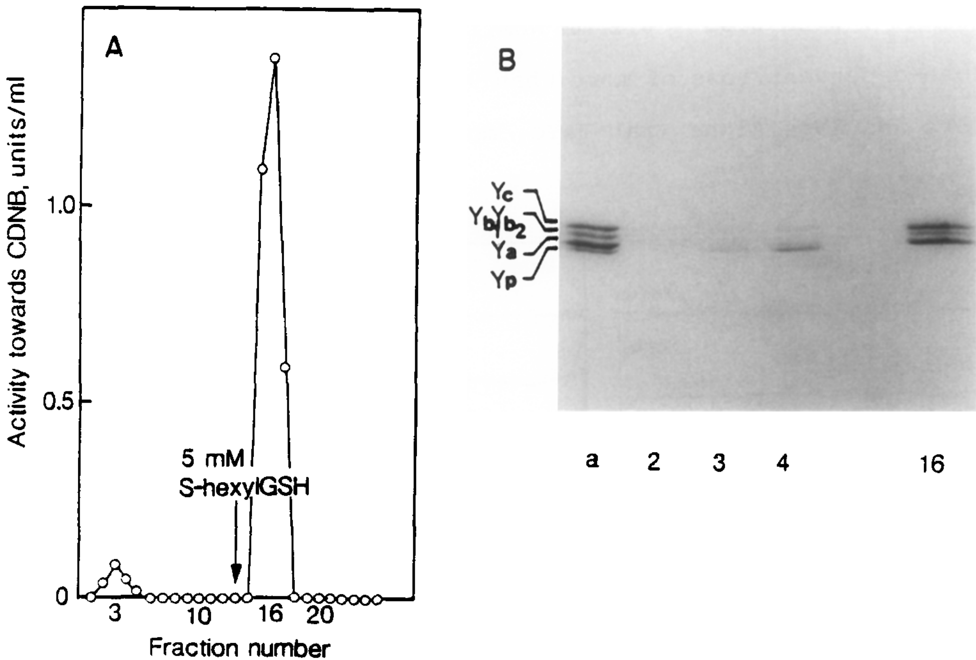

Fig. 2. S-hexy-GSH Sepharose chromatography of rat GSTs (A) and SDS-PAGE pattern of the GST subunits in breakthrough and bound fractions (B). Details of the procedure are described in the Methods section. In A, fraction volume was 3 ml. In B, lane numbers indicated are the same as fraction numbers in A. Lane a, a mixture of GSTs applied to the affinity column.

positions (9). Examination of the stoichiometry of GST P inactivation with NEM by quantitation of [^{14}C]NEM covalent binding to its subunit (Yp) revealed an increase in the amount of bound radioactivity during incubation (Fig. 3). When 80% of the activity was lost after incubation for 60 min, 0.77 nmol of [^{14}C]NEM was bound to 1.0 nmol of Yp. Under the same conditions, only 0.22 and 0.15 nmol of [^{14}C]NEM were bound to Ya and Ybl subunits, respectively. These data suggest that binding of one mole of NEM per one mole of Yp might result in complete loss of activity.

Identification of the reactive cysteine residue of GST P. In order to identify the reactive cysteine residue of GST P, DDPM, a colored analogue of NEM (10), was employed. GST P (0.76 mg) was treated with 0.1 mM DDPM at 20°C for 1 min, during which time more than 90% of GST P activity was lost (data not shown). After digestion of the modified enzyme with trypsin, peptides were separated by HPLC. Three major peptides, named I, II and III were observed at 280 nm (Fig. 4). On concentration, the three peptides became detectable as red precipitates. Amino acid sequencing and amino acid analysis revealed that these peptides

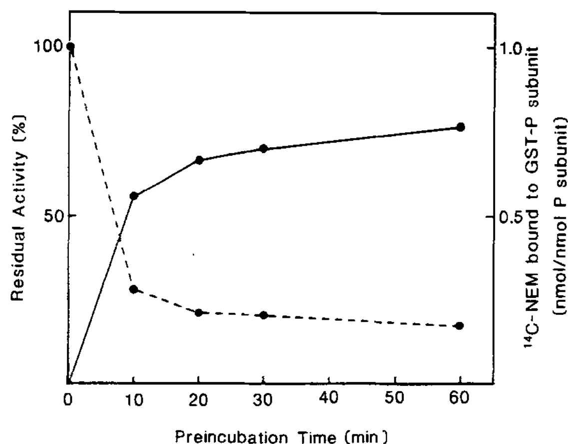

Fig. 3. Covalent binding of [^{14}C]NEM to GST P subunit. Details of the procedure are described in the Methods section. Dashed line, remaining GST activity; solid line, amount of [^{14}C]NEM bound to GST P subunit.

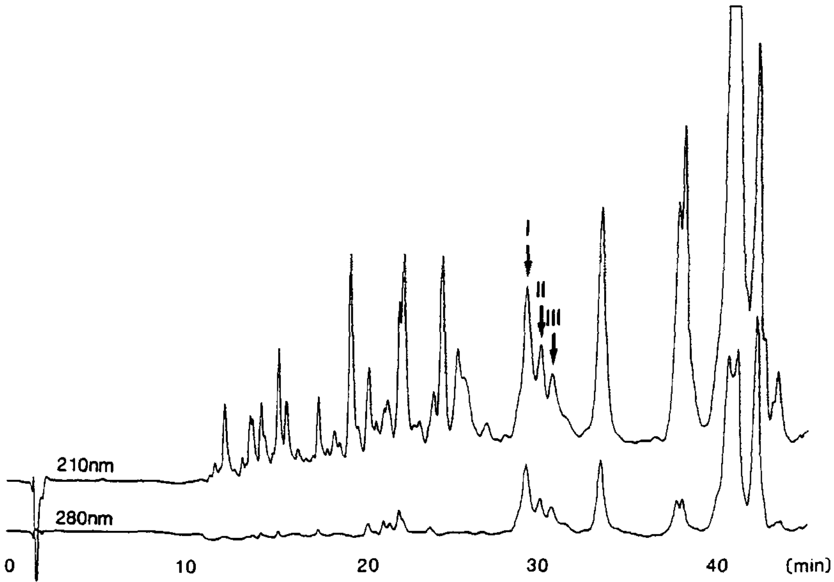

Fig. 4. HPLC pattern of trypsin digests of DDPM-treated GST P. Details of the procedure are described in the Methods section.

had the sequences; peptide I, SerThr(X)LeuTyrGlyGlnLeuProLys; peptide II, SerThr(X)LeuTyrGlyGlnLeu and peptide III, SerThr-(X)LeuTyrGlyGln, respectively. The sequence of the peptide I including those of peptides II and III can be found from Ser (45th) to Lys (54th) of the amino acid sequence deduced from base sequencing of GST P cDNA (11). Thus, the unidentified residue (X) was concluded as the 47th cysteine residue modified with DDPM.

DISCUSSION

Subunits of both GST P and GSTπ contain 4 cysteine residues at the same corresponding positions; i.e. at the 14th, 47th, 101th and 169th positions, while Ya contains two at the 17th and 111th, Yc only one at the 211th and Yb_1 and Yb_2 three at the 86th, 114th and 173th positions (see review (1)). Analysis of cDNA coding mouse GST MII suggested that this form also contains a cysteine residue in common at the 47th position (our unpublished data). Thus, the 47th cysteine residue appears unique to the

GSTs in Class Pi and the results described in this paper suggest that the residue is important for their catalytic activity.

Recently, Hoesch and Boyer (12) identified a common active site for rat GSTs YaYa and YcYc by photoaffinity labeling, comprising sequences between residues 212 and 218 for Ya and between 206 and 218 for Yc near the C-termini. Considering this report, a portion including the 47th cysteine residue in Class Pi might conformationally construct the active site with some portion of the C-terminal. However, it is also possible that the NEM modification of the 47th cysteine residue might cause conformational changes affecting catalytic activity even if distant from the active site.

Similar inactivation of GSTs in Class Pi by treatment with GSSG and hydrogen peroxide and complete reactivation by DTT (data not shown) suggest the involvement of disulfide formation and breakdown in control of enzyme activity. It remains, however, to be clarified whether the 47th cysteine residue is also responsible in these cases.

GST forms, and GST π in particular have been considered to participate in elevated (multi)drug resistance of some human neoplastic cells. Selective inactivation of GST π (P) by modification of a specific SH group may thus be useful for overcoming this resistance.

Acknowledgment This study was supported in part by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

1. Mannervik, B., and Danielson, U. H. (1988) CRC Critical Reviews in Biochemistry. 23, 283-337.
2. Sato, K. (1989) Adv. Cancer Res. 52, 205-255.
3. Kitahara, A., Satoh, K., and Sato, K. (1983) Biochem. Biophys. Res. Commun. 112, 20-28.
4. Satoh, K, Kitahara, A., Soma, Y., Inaba, Y, Hatayama, I., and Sato, K. (1985) Proc. Natl. Acad. Sci. USA. 82, 3964-3968.

5. Soma, Y., Satoh, K., and Sato, K. (1986) Biochim. Biophys. Acta. 869, 247-258.
6. Hatayama, I., Satoh, K., and Sato, K. (1986) Biochem. Biophys. Res. Commun. 140, 581-588.
7. Habig, W. H., Pabst, M. J., and Jakoby, W. B. (1974) J. Biol. Chem. 249, 7130-7139.
8. Laemmli, U. K. (1970) Nature. 227, 680-685.
9. Kano, T., Sakai, M., and Muramatsu, M. (1978) Cancer Res. 47, 5626-5630.
10. Witter, A., and Tuppy, H. (1960) Biochim. Biophys. Acta. 45, 429-442.
11. Sugioka, Y., Kano, T., Okuda, A., Sakai, M., Kitagawa, T., and Muramatsu, M. (1985) Nucleic Acids Res. 13, 6049-6057.
12. Hoesch, R. M., and Boyer, T. D. (1989) J. Biol. Chem. 264, 17712-17717.