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NOVEL *THETA* CLASS GLUTATHIONE *S*-TRANSFERASES Yrs-Yrs' AND Yrs'-Yrs' IN RAT LIVER CYTOSOL: THEIR POTENT ACTIVITY TOWARD 5-SULFOXYMETHYLCHRYSENE, A REACTIVE METABOLITE OF THE CARCINOGEN 5-HYDROXYMETHYLCHRYSENE

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Summary. Two novel *theta* class glutathione *S*-transferases (GSTs), designated Yrs-Yrs' and Yrs'-Yrs', were isolated from rat liver cytosol and purified to homogeneity. Polyclonal antibody raised against the previously reported *theta* class GST Yrs-Yrs (Hiratsuka, A. *et al.*, *J. Biol. Chem.* **265**, 11973 (1990)) cross-reacted with GSTs Yrs-Yrs' and Yrs'-Yrs'. These three *theta* class GSTs had different pI values and were separated by chromatofocusing. The enzyme subunit Yrs' was separated from Yrs by reverse partition HPLC, but identical to Yrs in the first 37 *N*-terminal amino acid sequence. Like GST Yrs-Yrs, both GSTs Yrs-Yrs' and Yrs'-Yrs' were not retained on an *S*-hexyl-GSH affinity column and had little activity toward 1-chloro-2,4-dinitrobenzene. However, they showed potent activities toward the reactive sulfate ester of the carcinogen, 5-hydroxymethylchrysene. GSTs Yrs-Yrs and Yrs'-Yrs' showed much higher GSH peroxidase activities toward arachidonate hydroperoxide than did rat liver *alpha* class GST Ya-Ya.

The occurrence of the newest *theta* class glutathione (GSH) *S*-transferase (GST) Yrs-Yrs in rat liver cytosol was first demonstrated by Hiratsuka, *et al.* (1) during the course of their investigation on bioinactivation of reactive sulfate esters metabolically formed from carcinogenic polycyclic arylmethanols (2-6), including 5-sulfoxymethylchrysene (SMCR) from 5-hydroxymethylchrysene (7). After that, Meyer, *et al.* (8) demonstrated GSTs 5-5 and 12-12 in rat liver and GST T1-1 in human liver to show strong similarity in *N*-terminal amino acid sequence to GST Yrs-Yrs.

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GST Yrs-Yrs, a homodimeric Y protein catalyzing the GSH-conjugation of reactive sulfate esters of carcinogenic polycyclic arylmethanols, is very unique in view of the facts that it has little activity toward 1-chloro-2,4-dinitrobenzene (CDNB) (1) used as a typical substrate for detection of all other classes of GSTs in various species (9) and also that it is not retained on an *S*-hexyl-GSH-affinity column (1). Very weak identity of the subunit Yrs in amino acid sequence to subunits of *alpha*, *mu*, and *pi* class GSTs (1) was in good accordance with the fact that polyclonal antibody raised against purified GST Yrs-Yrs showed no cross-reactivity with any other class of rat liver GST (1).

In the previous paper, Hiratsuka, *et al.* (1) demonstrated the existence of at least another GST in rat liver cytosol, which was capable of GSH-conjugation of SMCR to afford the stable metabolite, *S*-(chrysene-5-methylenyl)GSH (CRM-SG) and eluted as a broad peak at pH slightly lower than GST Yrs-Yrs from a chromatofocusing column. These GSTs including Yrs-Yrs represented more than 95% of the SMCR-GSH-conjugating activity in rat liver cytosol (1). The present communication deals with 1) isolation and purification of two novel *theta* class GSTs showing strong immunohomology and highly structural identity to GST Yrs-Yrs from Sprague-Dawley (SD) rat liver cytosol, 2) characterization of the two isoenzymes as GSTs Yrs-Yrs' and Yrs'-Yrs', and 3) potent GSH peroxidase (Px) activity of GST Yrs-Yrs toward mono-hydroperoxides of endogenous polyunsaturated fatty acids.

MATERIALS AND METHODS

Materials. Sodium salts of 1-menaphthyl sulfuric acid (10) and SMCR (7), and free forms of 13-hydroperoxy-9,11-octadecadienoic acid (linoleic acid 13-hydroperoxide), 13-hydroperoxy-6,9,11-octadecatrienoic acid (linolenic acid 13-hydroperoxide), and 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid (arachidonic acid 15-hydroperoxide) (11), and 13-hydroxy-9,11-octadecadienoic acid, 13-hydroxy-6,9,11-octadecatrienoic acid, and 15-hydroxy-5,8,11,13-eicosatetraenoic acid (12) were synthesized as previously described. Mr marker proteins were obtained from Sigma Chemical Co., St Louis, Mo. Other reagents used were of reagent grade.

Enzyme assay. GSH-conjugating activities of GSTs toward SMCR (1) and other substrates (13) and their GSH Px activities toward cumene hydroperoxide were determined as previously described (14).

GSH Px activities of GSTs toward fatty acid mono-hydroperoxides were determined by high performance liquid chromatography (HPLC) of enzymatically formed hydroxyfatty acids. The enzymatic reaction mixture consisted of 0.5-2.0 μg of a purified GST, 25 nmol of a fatty acid mono-hydroperoxide dissolved in 10 μl of ethanol, 2 μmol of GSH, and 2.5 μmol of EDTA in a final volume of 0.5 ml of 0.1 M Tris/HCl buffer, pH 7.4. After pre-incubation for 5 min at 37°C, the reaction was initiated by the addition of the ethanolic solution of the hydroperoxide. After a 2.5-min incubation, the reaction was terminated by immersing the reaction vessel in an ice-water bath, followed by immediate extraction of the chilled mixture with 2 ml of cold ether containing 0.5 mM mandelic acid, an internal standard for HPLC. From the organic phase separated, the solvent was evaporated at 30°C under a gentle stream of nitrogen, and the residue was dissolved in cold ether containing an excess of diazomethane. After 30 min at 2°C, the ethereal solution was concentrated under nitrogen as mentioned above and subjected to HPLC. Samples were chromatographed isocratically on a Finepak SIL-NH_2 column (4 x 250 mm, Japan spectroscopic, Ltd., Tokyo) with *n*-hexane/acetonitrile/*iso*-propanol (100:1:1, v/v/v) as a mobile phase at a flow rate of 3 ml/min. In chromatograms obtained by monitoring the absorbance at 233 nm, methyl esters of enzymatically formed hydroxyfatty acids appeared together with methyl esters of the corresponding hydroperoxides used as substrates and mandelic acid at retention times as follows: 5.5, 5.8, and 5.6 min for the hydroxyfatty acids from linoleic acid 13-

hydroperoxide (8.0 min), linolenic acid 13-hydroperoxide (9.3 min), and arachidonic acid 15-hydroperoxide (8.1 min), respectively, and 12 min for the internal standard.

Enzyme purification. A pooled flow-through fraction (330 mg protein) from an *S*-hexyl GSH Sepharose 6B column was obtained and condensed by ultrafiltration via chromatography on a DE-52 column of a cytosolic fraction (4,544 mg protein) from a male SD rat (270-290 g) liver homogenate as previously described (1). Chromatofocusing of the condensed pooled flow-through fraction (300 mg protein) was carried out as follows: a chromatofocusing gel (PBE 94) column (1.6 x 61 cm) pre-equilibrated with 25 mM ethanolamine-HCl buffer (pH 8.5) containing 1 mM EDTA and 2 mM mercaptoethanol was used, and the column was eluted with 1,500 ml of 1:20-diluted Polybuffer 96 adjusted to pH 6.5 with HCl. GSTs Ya-Ya, Ya-Yc, and Yb1-Yb2 were isolated from male SD rat liver cytosol and purified to homogeneity by the method of Mannervik and Jennson (15).

Electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli (16). Proteins were stained with Coomassie Brilliant Blue R-250.

Reverse partition HPLC of GSTs. HPLC analysis of GSTs Yrs-Yrs, Yrs-Yrs', and Yrs'-Yrs' was carried out on a μBondasphere C_{18}-300 Å column (5 μm in particle size, 3.9 x 300 mm, Nihon Waters Ltd., Tokyo) with an Atto model Constametric-II liquid chromatograph.

Western blot. Immuno-blotting was performed according to the method of Towbin, *et al.* (17). Antiserum raised against purified GST Yrs-Yrs was obtained and used for immuno-staining of GSTs as described previously (1).

Amino acid sequencing. Automatic Edman degradation of purified GST Yrs'-Yrs' was carried out with an Applied Biosystems model 470 A gas phase sequence analyzer and a Spectra Physics SP8700XR phenylthiohydantoin analyzer.

Protein determination. Protein concentrations were determined by the method of Lowry, *et al.* (18) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Purification and Characterization of GSTs Yrs-Yrs' and Yrs'-Yrs'

A pooled fraction containing GSTs active toward SMCR was obtained by direct application of a rat liver cytosolic fraction to a DE-52 column, followed by complete removal of *alpha* and *mu* class GSTs on an *S*-hexyl-GSH affinity column as previously reported (1). The SMCR-GSH-conjugating activity of the pooled fraction was separated into three peaks I, II, and III eluted at pH 7.9, 7.8, and 7.7, respectively, by chromatofocusing carried out under a condition of slower decrease in pH gradient than previously reported (1) (Fig. 1). Further purifications of these GSTs to homogeneity from the enzyme activity peaks I, II, and III were carried out by successive chromatography on a blue Sepharose and a TSK gel G3000SW gel filtration columns as previously reported (1). Purification folds (% yield) were 3.7 (43%), 10.4 (6.9%) and 8.7 (20%) for GSTs from the pooled fractions I, II, and III, respectively, so far as determined by SMCR as a substrate.

SDS-PAGE and Western blot analyses indicated all of the three purified GSTs to have subunits appearing at a molecular mass of 26 kDa and to be immuno-stained to the same extent by polyclonal antibody raised against purified *theta* class GST Yrs-Yrs (Fig. 2). The three purified GST proteins were eluted at 50 kDa from a TSK gel G3000SW gel filtration column calibrated with Mr marker proteins as previously described (1), indicating all of them to be dimeric proteins.

The purified GST from the above pooled fraction for the peak I was identified as Yrs-Yrs by co-elution with the previously isolated authentic specimen from an octadecylsilica column

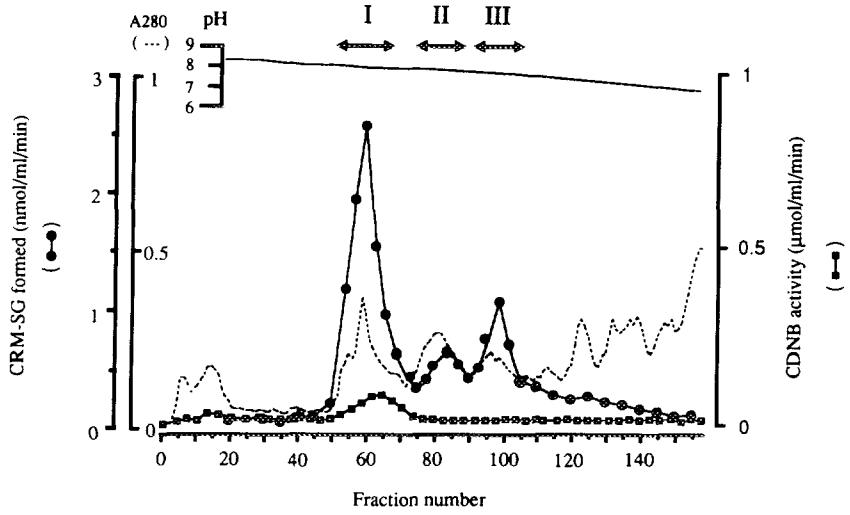

Fig. 1. Separation of rat liver cytosolic GSTs catalyzing GSH conjugation of SMCR by chromatofocusing. Enzymes catalyzing the GSH conjugation of SMCR were quantitatively collected from rat liver cytosol in a single fraction after successive chromatography on a DE-52 and an *S*-hexyl-GSH Sepharose 6B columns as previously reported (1). The enzyme in the single flow-through fraction from the *S*-hexyl-GSH affinity column was concentrated by ultrafiltration and separated by chromatofocusing at pH 8.5-6.5 (solid line) as described in Methods. Enzyme activities were determined by SMCR (●) and CDNB (■) as substrates and protein concentrations (broken line) by absorptiometry at 280 nm. Horizontal arrows numbered with I, II, and III represent fractions pooled for subsequent purification of the enzymes.

by HPLC. The subunit of homodimeric GST Yrs-Yrs was eluted as a single peak at a retention time of 27 min from the hydrophobic HPLC column (Fig. 3 A). In contrast to Yrs, the subunit (Yrs') of GST from the pooled fraction for the peak III was eluted as a single

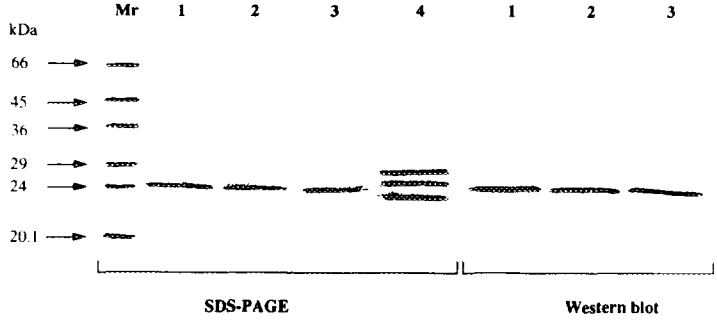

Fig. 2. SDS-PAGE and Western blot analyses of GSTs Yrs-Yrs, Yrs-Yrs', and Yrs'-Yrs' purified from rat liver cytosol. SDS-PAGE was performed in a 15% acrylamide gel plate. Lane 1, purified GST Yrs-Yrs (5 μg); lane 2, GST Yrs-Yrs' (5 μg), lane 3, purified GST Yrs'-Yrs' (5 μg), and lane 4, a mixture of purified GSTs Ya-Yc (10 μg) and Yb1-Yb2 (5 μg) purified from rat liver cytosol. For Western blot analysis, the protein samples were resolved by SDS-PAGE, transfered to a nitorocellulose membrane, and stained by rabbit anti-serum raised against purified GST Yrs-Yrs as previously reported (1). Numerals on the left side of the gel plate represent Mr values obtained by marker proteins in lane Mr, 66,000, serum albumin; 45,000, egg albumin; 36,000, rabbit muscle glyceraldehyde 3-phosphate dehydrogenase; 29,000, carbonic anhydrase; 24,000, bovine pancreas trypsinogen; and 20,100, soybean trypsin inhibitor.

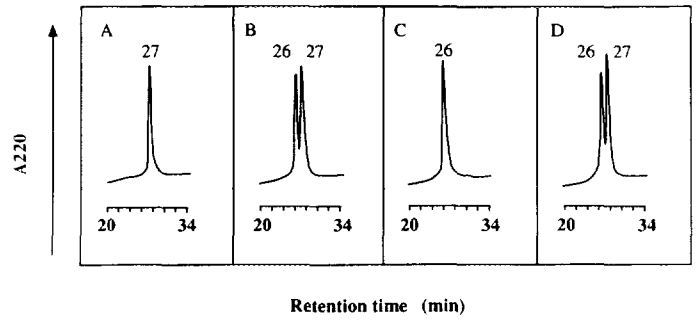

Fig. 3. Separation of GST subunits Yrs and Yrs' by reverse partition HPLC. A μBondasphere C_{18}-300Å column (3.9 x 300 mm) was eluted at a flow rate of 1 ml/min with a 30-50% (v/v) linear gradient of acetonitrile in water, both containing 0.1% (v/v) trifluoroacetic acid. Chromatograms were monitored by absorbance at 220 nm. A, purified GST Yrs-Yrs (10 μg); B, purified GST Yrs-Yrs' (20 μg); C, purified GST Yrs'-Yrs' (10 μg); and D, a mixture of A and C (10 μg each). Numerals above subunit protein peaks represent retention times (min).

peak at a slightly earlier retention time of 26 min under the same HPLC conditions as used for GST Yrs-Yrs, indicating the GST to be a homodimer Yrs'-Yrs' (Fig. 3 C). However, HPLC of the GST purified from the pooled fraction for the peak II demonstrated the enzyme to be a heterodimer of Yrs and Yrs', which was eluted as a doublet peak with equal peak areas at retention times (27 and 26 min) identical to those of Yrs and Yrs' (Fig. 3 B). A mixture of an equal molar ratio of GSTs Yrs-Yrs and Yrs'-Yrs' showed the same chromatographic pattern as obtained with GST Yrs-Yrs' (Fig. 3 D).

The first 40 chemically determined *N*-terminal amino acid sequence of GST Yrs'-Yrs' (GLELYLDLLSQPSRAVYIFAKKNGIPFQLRTVDLLKGXHL) was completely identical, except for the unidentified amino acid residue (X) at a position of 38, to that of GST Yrs-Yrs whose first 25 *N*-terminal and total amino acid sequences had been chemically determined by Hiratsuka, *et al.* (1) and deduced from a cDNA for Yrs by Ogura, *et al.* (19), respectively.

Substrate Specificities of GSTs Yrs-Yrs, Yrs-Yrs', and Yrs'-Yrs'

Specific SMCR-GSH-conjugating activities of the novel *theta* class GSTs Yrs-Yrs' and Yrs'-Yrs' were almost the same as that of GST Yrs-Yrs (Table I). Therefore, areas of the enzyme activity peaks I, II, and III shown in Fig. 1 are directly correlated to the relative ratios of GSTs Yrs-Yrs, Yrs-Yrs', and Yrs'-Yrs', 10:1:3, existing in rat liver cytosol. Based on the specific enzyme activity toward SMCR, these three GSTs corresponded to about 0.1% of total rat liver cytosolic protein.

Menaphthyl sulfate ester, an analogue to SMCR, was a better substrate than the latter for these *theta* class GSTs showing no appreciable difference in conjugating activity toward both sulfates (Table I). The GSTs showed very similar substrate specificities to other electrophiles used as substrates. They had little activity toward 1-chloro-2,4-dinitrobenzene (CDNB) compared to rat liver *alpha* class GST Ya-Ya and had no detectable activity toward

Table I. Substrate specificities of GSTs Yrs-Yrs, Yrs-Yrs' and Yrs'-Yrs' in comparison with *alpha* class GST Ya-Ya

Substrate	Activities of GSTs (μmol/mg protein/min)			
	Yrs-Yrs	Yrs-Yrs'	Yrs'-Yrs'	Ya-Ya
5-Sulfoxymethylchrysene (SMCR)	0.2	0.2	0.2	n.d.[1]
1-Menaphthyl sulfate	0.4	0.4	0.4	n.d.[2]
1-Chloro-2,4-dinitrobenzene (CDND)	< 0.1	< 0.1	< 0.1	33.0
1,2-Dichloro-4-nitrobenzene (DCNB)	n.d.[3]	n.d.[3]	n.d.[3]	0.05
1,2-Epoxy-3-(4'-nitrophenoxy)propane (EPNP)	n.d.[2]	n.d.[2]	n.d.[2]	0.13
Ethacrynic acid	0.4	1.6	2.5	0.16
4-Nitrobenzyl chloride	n.d.[4]	n.d.[4]	n.d.[4]	- [5]
4-Nitrophenyl acetate	n.d.[2]	n.d.[2]	n.d.[2]	- [5]
Cumene hydroperoxide	2.0	2.0	2.0	2.6
Arachidonic acid 15-hydroperoxide	13.6	- [5]	7.6	1.6
Linoleic acid 13-hydroperoxide	9.7	- [5]	0.9	2.0
Linolenic acid 13-hydroperoxide	4.6	- [5]	0.9	2.7

n.d. (not detectable): 1) less than 0.05 nmol/mg protein/min, 2) less than 10 nmol/mg protein/min, 3) less than 2 nmol/mg protein/min, 4) less than 5 nmol/mg protein/min, and 5) not determined.

2,4-dichloro-1-nitrobenzene (DCNB), 1,2-epoxy-3-(4'-nitrophenoxy)propane (EPNP), ethacrynic acid, 4-nitrobenzyl chloride, and 4-nitrophenyl acetate.

The observed substrate specificities of the rat *theta* class GSTs having Yrs or/and Yrs' are very similar to that reported on human Theta class GST T2-2* (20), but differ from those reported on other rat *theta* class GSTs 5-5 and 12-12, both of which are active toward the epoxide, EPNP, and 4-nitrobenzyl chloride (8). However, the very poor activity toward CDNB and considerably high activity as a GSH Px toward cumene hydroperoxide are most likely to be a common property of the reported *theta* class GSTs in the rat (1,8) and human (8,20).

GSH Px Activities of GSTs Yrs-Yrs and Yrs'-Yrs' toward Polyunsaturated Fatty Acid Hydroperoxides

The major enzyme Yrs-Yrs of the above Yrs family of GSTs was found to act as a GSH Px markedly on mono-hydroperoxides of arachidonic, linoleic, and linolenic acids (Table I). The GSH Px activity of GST Yrs-Yrs toward these hydroperoxides was much higher than that of purified rat liver *alpha* class GST Ya-Ya, the latter of which had been reported to have the highest GSH Px activity toward hydroperoxides of arachidonate (21) and linoleate (22) among all of known rat liver GSTs. Despite their equal activity toward cumene

hydroperoxide, GSTs Yrs-Yrs and Yrs'-Yrs' showed a marked difference in GSH Px activity toward the fatty acid hydroperoxides. GST Yrs'-Yrs' showed a much higher activity only toward the arachidonate hydroperoxide than did GST Ya-Ya. The linolate and linolenate hydroperoxides were poorer substrates for GST Yrs'-Yrs' than for the *alpha* class GST.

The above fact strongly suggests that the *theta* class GSTs, especially Yrs-Yrs, may play an important role in retarding the oxidative stress induced by hydroperoxides of polyunsaturated fatty acids derived by phospholipase A_2 from phospholipid hydroperoxides formed in hepatic intracellular membranes. A study on the structural difference between Yrs and Yrs' is now in progress in our laboratory.

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