Polymorphism of the glutathione transferase subunit 3 in Sprague–Dawley rats involves a reactive cysteine residue

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Comparison of Hirosaki hairless rat (HHR) and Sprague–Dawley (SD) rat liver glutathione transferase (GST) subunits by HPLC revealed differences in subunit 3; a new peak was detected in HHR GSTs and this was tentatively named X. By chromatofocusing, the HHR GST form composed of peak X and SD rat GST 3-3 were eluted at pH 8.8 and 9.1 respectively. The former was more sensitive to the SH reagent *N*-ethylmaleimide (NEM) than the latter. GSSG treatment of peak X resulted in a shift of retention time (peak Y) by HPLC analysis. However, such conversion was not observed for the SD rat GST 3-3 following GSSG or dithiothreitol (DTT) treatment. Peak Y exhibited *m/z* values of 26091.9 and 26125.4 by matrix-assisted laser-desorption ionization–time-of-flight MS, higher than those of peak X by 304–307, equivalent to the molecular-mass value of GSH. On treatment with DTT, peak Y was converted into peak X, with release of a substance with HPLC-characteristics of GSH. This substance was confirmed to be GSH by liquid chromatography/MS. These results thus indicated peak Y to be a glutathionylated form of peak X. Quantification revealed the release of

4 nmol of GSH from 0.12 mg of the peak Y protein, corresponding to 4.8 nmol (M_r 25000). The nucleotide sequence of HHR GST subunit 3 cDNA proved identical to that reported for pGTA/C44, possessing asparagine and cysteine as the 198th and 199th amino acid residues, respectively, corresponding to lysine and serine in subunit 3 of the SD rat. Thus peak X appeared to be the product of HHR GST subunit 3 cDNA. Treatment with *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide, a coloured analogue of NEM, followed by trypsin-treatment and sequencing of labelled peptides, identified the reactive cysteine residue of HHR GST subunit 3 to be located at position 199. Unlike SD rat GST 3-3, HHR GST 3-3 was not activated by treatment with xanthine and xanthine oxidase. These results suggest polymorphism of the rat GST subunit 3 gene with individual gene product variation in sensitivity to oxidative stress.

Key words: glutathionylation, matrix-assisted laser-desorption–ionization time-of-flight mass spectrometry, oxidative stress.

INTRODUCTION

Glutathione transferases (GSTs; EC 2.5.1.18) are a family of multifunctional dimeric enzymes that catalyse the conjugation of GSH with many electrophiles, including carcinogens [1]. The many molecular forms identified so far have been grouped into five classes, Alpha, Mu, Pi, Theta and Sigma, in a species-independent classification [2,3]. The major forms in rat liver are GST 1-1, 1-2 and 2-2 in the Alpha class, and GST 3-3, 3-4 and 4-4 in the Mu class. The forms in the Alpha class possess high glutathione peroxidase activity towards lipid hydroperoxides [4]. Conjugation reactions of the Mu-class forms are activated by active oxygen species [5], whereas the Pi class is inactivated by SH reagents and H_2O_2 ; modification of cysteine residues being responsible [6,7]. The GST forms have been suggested to play important roles in the prevention of tissue damage by oxidative stress [3,8].

Although cysteine residues of GSTs are not directly involved in enzyme activity [6,9], those of other enzymes and proteins play important roles in processes, such as catalysis [10], protein folding [11] and DNA binding [12]. Modification of cysteine residues with GSH has been reported for many enzymes, such as carbonic anhydrase [13] and fatty acid synthetase [14], and the amounts of glutathionylated forms may be increased in response

to oxidative stress [15]. This usually decreases enzymic activities, but has been suggested to protect cysteine residues from irreversible oxidation [16].

Human GST forms such as GST M1-1 and T1-1 are not expressed in approximately half of the population, and hereditary differences in their expression are due to deletion of the encoding genes [17,18]. Loss of these genes has been suggested as a possible marker for greater susceptibility to development of lung cancer, as well as other cancers [19,20]. However, whether such polymorphisms also exist in the rat has remained unclear. Although three cDNA clones have been reported for rat GST subunit 3 [21–23], it remains to be clarified whether these reflect either polymorphism of the subunit or highly related but different subunits. Furthermore, whether the forms encoded by the individual cDNAs differ in activity and other properties remains to be determined.

Hirosaki hairless rats (HHRs) are a mutant strain established from the Sprague–Dawley (SD) rat strain in the Institute for Animal Experiments, Hirosaki University School of Medicine, Hirosaki, Japan [24]. Breeding experiments revealed the trait to be autosomal and recessive and the responsible gene was designated as *hhr*.

In the present study, an HPLC comparison between HHR and SD rat liver GST subunits revealed variation in the GST

Abbreviations used: HHR, Hirosaki hairless rat; SD, Sprague–Dawley; GST, glutathione transferase; NEM, *N*-ethylmaleimide; CDNB, 1-chloro-2,4-dinitrobenzene; DDPM, *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide; DPCC, diphenylcarbonyl chloride; TFA, trifluoroacetic acid; MALDI-TOF, matrix-assisted laser desorption-ionization–time-of-flight; DTT, dithiothreitol; RT-PCR, reverse transcriptase PCR.

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subunit 3, and these two GST 3-3 forms demonstrated variation in sensitivity to an SH reagent. The GST subunit 3 in HHR was further found to differ from its counterpart in the SD rat by possessing Asn¹⁹⁸ and Cys¹⁹⁹ in place of lysine and serine respectively. These results suggest the presence of polymorphism in the rat GST subunit 3 gene.

GST 3-3 is now commonly referred to as the rGST M1-1 in accordance with the nomenclature introduced for the human enzyme.

MATERIALS AND METHODS

Materials

Epoxy-activated Sepharose 6B, Polybuffer exchanger (PBE) 118, and Pharmalyte 8-10.5 were obtained from Pharmacia Biotech (Uppsala, Sweden). GSH, diphenylcarbamyl chloride (DPCC)-treated trypsin, and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma. *N*-ethylmaleimide (NEM) was obtained from Nacalai Tesque (Kyoto, Japan). *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide (DDPM) was purchased from Aldrich (Milwaukee, WI, U.S.A.) and acetonitrile was obtained from Kanto Chemicals (Tokyo, Japan). All other chemicals were of analytical grade.

Purification of GSTs from rat livers

Livers from male SD rats (250–300 g body weight) or HHRs (250–300 g body weight) were homogenized with 9 vol. of 10 mM Tris/HCl (pH 7.8)/0.2 M NaCl. The supernatants obtained by centrifugation at 105 000 *g* for 45 min were subjected to *S*-hexyl-GSH-Sepharose affinity chromatography and the GST forms were eluted with 5 mM *S*-hexyl-GSH, as described by Guthenberg and Mannervik [25]. GST 3-3s from SD rat and HHR were further purified by chromatofocusing (pH 10.5–7.0), as described previously [26].

Assay for GST activity

GST activity was assayed with CDNB and other substrates according to the methods of Habig et al. [27] or Keen and Jakoby [28]. One unit of GST activity is the amount of enzyme that catalyses the conjugation of 1 μ mol of substrate/min at 25 °C. In some experiments to determine the K_m and V_{max} values, the CDNB and GSH concentrations were varied from 0.02 to 1 mM.

Protein content

Protein content was measured by the methods of Lowry et al. [29] or Bradford [30], using BSA as a standard.

Electrophoresis and immunoblotting

SDS/PAGE was performed according to the method of Laemmli [31]. Protein was stained with Coomassie Brilliant Blue R-250. Immunoblots were performed according to the method of Towbin et al. [32] using an anti-(GST 3-3) antibody raised in a rabbit, as described previously [33].

Separation of GST subunits and GSH by HPLC

Individual GST subunits were separated by HPLC, essentially as described by Ostlund-Farrants et al. [34]. HPLC was carried out using a TSK-Gel Octadecyl-4PW column (4.6 mm $\times$ 150 mm; Tosoh, Tokyo, Japan). Elution was performed with the following gradient system of solvent A (water) and solvent B [80 % (v/v)

acetonitrile/0.1 % trifluoroacetic acid (TFA)] at a flow rate of 1.4 ml/min: 0–5 min, 100 % solvent A; 5–15 min, 0–40 % solvent B; 15–80 min, 40–70 % solvent B. All runs were performed at 35 °C, and column effluent was monitored at 220 nm [35]. GSH was also resolved by HPLC on a TSK-Gel ODS-80TS column (4.6 mm $\times$ 250 mm; Tosoh). Elution was performed with the following gradient system of solvent A (water in 0.1 % TFA) and solvent B (80 % acetonitrile/0.1 % TFA) at a flow rate of 0.85 ml/min: 0–5 min, 50 % solvent A; 5–25 min, 50–60 % solvent B. All runs were performed at 35 °C, and column effluent was monitored at 220 nm.

N-terminal amino acid sequence analysis

Edman degradations were performed with a gas-phase protein sequencer (model 492; Applied Biosystems, Foster City, CA, U.S.A.). Amino acid phenylthiohydantoin derivatives were identified and quantified as reported previously [36].

Treatment with NEM, H₂O₂, and xanthine and xanthine oxidase

Each GST (1 unit/0.1 ml of 0.1 M Tris/HCl, pH 7.8) was treated with 0.02–0.4 mM NEM for 10 min at 25 °C. An appropriate amount (0.5 unit/ml) of each GST was also incubated at 25 °C with 0.4 mM xanthine and xanthine oxidase (15 m-units/ml) in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA [5]. At the indicated incubation times, aliquots were taken and assayed for GST activity. In some experiments, GSTs were treated with 1 mM H₂O₂ in 0.2 M Tris/HCl (pH 8.0) for 30 min at 25 °C [37].

Treatment with GSSG or dithiothreitol (DTT)

GST composed of peak X, purified from HHR livers by chromatofocusing (120 μ g protein/0.1 ml of 0.1 M Tris/HCl, pH 7.8), was mixed with 0.1 ml of 0.1 M Tris/HCl (pH 7.8) without or with 50 mM GSSG and then incubated at 25 °C for 30 min [38]. For experiments using DTT, DTT was added instead of GSSG at a concentration of 50 mM.

MS

Liquid chromatography/MS of HPLC eluates and GSH was performed using a JMX-AX505H double-focusing mass spectrometer fitted with a fast atom bombardment ('FAB') gun (Model FAB 05A; Jeol, Tokyo, Japan) and operated with a 3 keV xenon beam. Scanning was performed from *m/z* 50 to 500, and also at 308, a *m/z* value for GSH at 3 keV acceleration voltage, with a scanning rate of 5 s/decade [39]. Matrix-assisted laser-desorption ionization–time-of-flight (MALDI–TOF) spectra of GSTs were obtained with a Voyager-DESTR mass spectrometer (PerSeptive Biosystems Inc., Framingham, MA, U.S.A.). The matrix used was 3,5-dimethoxy-4-hydroxy-cinamic acid (cinapinic acid) dissolved at a concentration of 10 mg/ml of 0.1 % TFA in acetonitrile/water (7:3, v/v). Each sample was analysed twice, by setting linear and positive modes, the accelerating voltage at 25 kV, the grid voltage at 92.8 %, and the extraction delay at 350 ns [40]. Spectra were calibrated externally using the [*M*+H]⁺ ions from horse apomyoglobin monomers and dimers (average *m/z* values of 16952.6 and 33904 respectively) and bovine trypsinogen monomer (average *m/z* value of 23982.1).

DDPM treatment and trypsin digestion

In order to identify the reactive cysteine residue of HHR GST subunit 3, the enzyme (300 μ g protein) was treated with 1 mM

DDPM, as reported previously [6]. After digestion with DPCC-treated trypsin, peptides were separated by HPLC and DDPM-labelled peptides were detected by absorbance at 320 nm. GST 3-3 purified from SD rat livers was also subjected to DDPM treatment and trypsin digestion.

cDNA cloning of GST 3-3 by reverse transcriptase (RT)-PCR

Total RNA was isolated from HHR and SD rat livers by guanidium thiocyanate/phenol/chloroform extraction [41]. Oligo-dT-primed liver cDNAs were synthesized with a cDNA synthesis kit (Takara, Siga, Japan) and PCR was performed [42] with primers, 5'-CATGCCTATGATACTGGGATA-3' and 5'-CTACTTGTTACTCCATTGGGC-3', designed on the basis of the reported cDNA sequence of the rat GST subunit 3 [21]. PCR products were sequenced using an automated DNA sequencer (ABI 377; Applied Biosystems).

RESULTS

Different elution patterns of GST subunits between HHR and SD rat livers

HHR and SD rat liver GST subunits bound to *S*-hexyl-GSH-Sepharose were analysed by HPLC. Individual peaks were identified as GST subunits as shown in Figure 1 by SDS/PAGE, and immunoblotting with anti-GST antibodies (results not shown). The elution pattern of SD rat liver GSTs (Figure 1A) was consistent with previous reports [34,35]. However, in the case of HHR liver GSTs (Figure 1B), instead of subunit 3, a peak named X was detected between GST subunits 3 and 4. Other subunits did not differ either in retention time or in other properties examined between HHR and SD rat liver GSTs. To investigate the properties of peak X, it was examined by SDS/PAGE in addition to SD rat GST subunit 3. As shown in

Figure 1 Separation of rat liver GST subunits by HPLC

Aliquots (100–200 µg of protein) of GSTs partially purified by *S*-hexyl-GSH affinity chromatography from SD rat livers (A) and HHR livers (B) were subjected to HPLC. Elution was performed as described in the Materials and methods section. Numbers by peaks denote the respective GST subunits. X was assigned to the HHR peak appearing in place of SD subunit 3.

Figure 2 SDS/PAGE of rat liver GST subunits resolved by HPLC

SDS/PAGE was performed using a 12.5% polyacrylamide gel and proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, molecular-mass markers; lane 2, 30 µg of GSTs partially purified by *S*-hexyl-GSH affinity chromatography from HHR liver, including GST subunits 1, 2, 3 and 4; lane 3, 5 µg of HHR peak X separated by HPLC; lane 4, 5 µg of the SD rat subunit 3. The positions of molecular-mass markers are indicated (in kDa) to the left.

Figure 3 Separation of SD rat and HHR liver GSTs bound to *S*-hexyl-GSH-Sepharose by chromatofocusing (pH 10.5–7.0)

SD rat (A) and HHR (B) liver GSTs bound to *S*-hexyl-GSH-Sepharose were applied on to a column (1.0 cm × 28 cm) of chromatofocusing gel (PBE 118) equilibrated with 25 mM triethylamine/HCl, pH 10.5. Elution was carried out with 400 ml of Pharmalyte (pH 8–10.5), which was diluted 80-fold with deaerated water and adjusted to pH 7.0 with 1 M HCl. The flow rate was set at 15 ml/h and fractions of 2.6 ml were collected. To show the elution profile of Mu-class GSTs, only data for fraction numbers 80–200 are presented. ●, GST activity towards CDNB; ○, pH. Numbers by peaks denote respective GST subunits, while X represents the subunit shown in Figure 1.

Figure 4 Inactivation of HHR GST composed of peak X (●) and SD rat GST 3-3 (○) by NEM

GSTs (1.0 unit/0.1 ml of 0.1 M Tris/HCl, pH 7.8) were mixed with 0.1 ml of 0.1 M Tris/HCl (pH 7.8) containing 0.04–0.8 mM NEM, and then incubated at 25 °C for 10 min. Aliquots were assayed for GST activity with CDNB.

Figure 2, the two preparations exhibited similar electrophoretic mobilities, and were stained with anti-(GST 3-3) antibody upon immunoblotting (results not shown). Furthermore, the N-terminal amino acid sequence of peak X perfectly matched that of the subunit 3 [21] for the first 20 residues examined (results not shown).

HHR and SD rat liver GSTs bound to the affinity column were also resolved under non-denaturing conditions by chromatofocusing (pH 10.5–7.0) to compare the properties of active forms. Figure 3 illustrates chromatofocusing elution profiles of Mu-class GSTs. Active fractions were analysed by HPLC to identify the constituent subunits. In the SD rat (Figure 3A), GSTs 3-3, 3-4 and 4-4 were separated as individual peaks, eluting at pH 9.1, 9.0 and 8.6, respectively, consistent with values reported by other investigators [43,44]. Although separation was incomplete in the case of HHR, peak X was eluted at pH 8.8 (fraction 112 in Figure 3B) and that for subunit 4 and peak X at pH 8.7 (fraction 114). Both HHR and SD rat GST 4-4s eluted at the same pH value. Elution profiles of Alpha class GST forms, GST 1-1, 1-2 and 2-2, were the same for the SD rat and HHR (results not shown).

Effects of NEM or GSSG treatment on HHR peak X

The effect of an SH reagent, NEM, on enzymic activity was compared between HHR GST peak X and SD rat GST 3-3. As shown in Figure 4 preincubation of the HHR GST form with 0.02–0.4 mM NEM for 10 min resulted in a dose-dependent loss of activity, more than 90% being inactivated at a concentration of 0.4 mM, whereas SD rat GST 3-3 was less affected (15–45% inactivation) under the same conditions. Furthermore, the HHR GST form was treated with 25 mM GSSG at 25 °C for 30 min and then analysed by HPLC. This resulted in a shift of retention time (peak Y in Figure 5). However, this conversion was not observed with SD rat GST 3-3 following GSSG treatment. To confirm glutathionylation of peak Y, molecular masses of peak X, peak Y and subunit 3 of SD rat were examined by MALDI-TOF-MS. Peak X exhibited two dominant signals with similar counts, 25788.3 and 25818.3 (Figure 6A). Peak Y also showed two signals, 26091.9 and 26125.4 (Figure 6B).

Figure 5 Conversion of HHR peak X to peak Y by GSSG treatment

GST composed of peak X (fraction 112 in Figure 3B) (A and B) or SD rat GST 3-3 (fraction 88 in Figure 3A) (C and D), separated by chromatofocusing [120 µg of protein/0.1 ml of 0.1 M Tris/HCl (pH 7.8)] were mixed with 0.1 ml of 0.1 M Tris/HCl, pH 7.8, without (A and C) or with 50 mM GSSG (B and D) and then incubated at 25 °C for 30 min. These samples were analysed by HPLC for alteration of GST subunits. The HHR peak X was partly converted to a new peak (peak Y in B) while SD rat GST 3-3 was not altered (D).

However, subunit 3 showed a single signal of 25800.4 (Figure 6C). The molecular mass of peak Y was higher than that of peak X by approx. 304–307, equivalent to the molecular mass of GSH (i.e. 307), suggesting that peak Y was a glutathionylated form of peak X. Peak Y was also treated with 25 mM DTT at 25 °C for 30 min. The result was complete conversion into peak X, with

Figure 6 MALDI-TOF mass analysis of HHR peak X, HHR peak Y and SD rat GST subunit 3

Aliquots of protein (0.25 µg) of HHR peaks X (A) and Y (B) and SD rat subunit 3 (C) resolved by HPLC were analysed by MALDI-TOF-MS, as described in the Materials and methods section. Numbers by peaks denote m/z values.

Figure 7 Conversion of HHR peak Y to peak X by DTT treatment and concomitant release of GSH

The HHR peak Y, separated by HPLC (120 μ g of protein/0.1 ml of 0.1 M Tris/HCl, pH 7.8) was mixed with 0.1 ml of 0.1 M Tris/HCl (pH 7.8) without (A and C) or with 50 mM DTT (B and D) and then incubated at 25 $^{\circ}$ C for 30 min. These samples were analysed by HPLC for alteration of the GST subunit (A and B) and also release of GSH (C and D). To test for conversion of peak Y to peak X, subunit 4 was added as an internal standard (A and B). HPLC was performed as described in the Materials and methods section.

Figure 8 Liquid chromatography/MS analysis of the low-molecular-mass substance released from peak Y by DTT treatment

The HHR peak Y (120 μ g of protein), was treated with 25 mM DTT, as described in Figure 7. Liquid chromatography/MS analysis was performed as described in the Materials and methods section. (A) A mass chromatogram of the substance monitored at 308, an m/z value for GSH ($[M+H]^+$ ion). (B) A mass spectrum of the substance, showing a protonated molecular ion.

release of a substance with GSH features on HPLC analysis (Figure 7), and the expected molecular mass on mass chromatography (Figure 8A) and MS (Figure 8B). Quantification of

Figure 9 HPLC patterns of trypsin digests of DDPM-treated HHR GST 3-3 and SD rat GST 3-3

Aliquots of 300 μ g protein of HHR GST 3-3 (A) and SD rat GST 3-3 (B) in 50 mM sodium phosphate (pH 7.0) were treated with 1 mM DDPM for 1 min and the reaction was stopped with 1 mM DTT. After digestion with 5 μ g of DPCC-treated trypsin at 30 $^{\circ}$ C for 16 h, peptides were separated by HPLC on a TSK-Gel ODS-80TS column (4.6 mm $\times$ 250 mm; Tosoh). Elution was performed with a linear gradient from water/0.1% TFA to acetonitrile/0.1% TFA. The flow rate was 0.85 ml/min and peptides were detected by absorbance at 320 nm. Amino acid sequencing of the P1 and P2 peaks was performed as described in the Materials and methods section and the sequences were compared with those deduced from cDNA sequences. Amino acid sequences of the P1 and P2 peaks are expressed in single letter code, where X denotes an unknown residue.

GSH by HPLC revealed that 4 nmol of GSH were released from 120 μ g of the peak Y protein, corresponding to 4.8 nmol (M_r 25000). These data also supported the binding of one mole of GSH per mole of peak X subunit.

Identification of the reactive cysteine residue in HHR subunit 3

In order to identify the reactive cysteine residue of HHR peak X, DDPM, a coloured analogue of NEM, was employed. The HHR GST form treated with DDPM was digested with trypsin and the peptides were separated by HPLC. Two major peptides, P1 and P2, were detected at 320 nm (Figure 9A). Amino acid sequencing revealed that these peptides had the following sequences: P1, Lys-His-His-Leu-Xaa-Gly-Thr-Glu-Glu; P2, Ile-Ser-Ala-Tyr-Met-Asn-Xaa-Ser (where Xaa corresponds to an unknown amino acid). P1 was also observed with a similarly treated SD rat GST 3-3 preparation (Figure 9B). The sequences of P1 and P2 could be found from Lys⁸² to Glu⁹² and from Ile¹⁹³ to Ser²⁰⁰, respectively, of the amino acid sequence of rat GST subunit 3 [21]. Thus the unidentified residues of P1 and P2 were concluded to be the cysteine residues at positions 86 and 199, respectively, modified with DDPM. Since the cysteine residue at position 86 was shared by SD rat GST 3-3, the other cysteine residue was concluded to be the residue responsible for the inactivation of the HHR GST form by NEM.

cDNAs encoding the HHR and SD rat subunit 3 were generated by RT-PCR using the primers described in the Materials and methods section. Nucleotide sequencing of HHR subunit 3 cDNA revealed identity with the sequence for rat subunit 3 cDNA (pGTA/C44) reported by Ding et al. [22]. The deduced amino acid sequence suggested asparagine and cysteine residues at positions 198 and 199 respectively. Corresponding amino acid residues were lysine and serine for the subunit 3 of SD rat, in accordance with the sequence (pGTR200) reported by Lai et al. [21]. The other amino acid residues did not differ between HHR subunit 3 and SD rat subunit 3. These results suggested that peak X was the product of HHR subunit 3 cDNA.

Table 1 Substrate specificity of HHR and SD rat GST 3-3s

The results represent the mean $\pm$ S.E.M. for three independent experiments, each assayed in triplicate.

Substrate	Specific activity (units/mg protein)	
	HHR GST 3-3	SD rat GST 3-3
CDNB	35.1 $\pm$ 3.6	33.4 $\pm$ 3.8
1,2-Dichloro-4-nitrobenzene	2.87 $\pm$ 0.32	2.44 $\pm$ 0.25
Bromosulphophthalein	0.32 $\pm$ 0.03	0.43 $\pm$ 0.05
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)-propane	0.11 $\pm$ 0.02	0.07 $\pm$ 0.02
<i>trans</i> -4-Phenyl-3-buten-2-one	0.07 $\pm$ 0.01	0.03 $\pm$ 0.01
Ethacrynic acid	0.05 $\pm$ 0.01	0.10 $\pm$ 0.02

Figure 10 Effects of xanthine and xanthine oxidase treatment on activities of HHR and SD rat GST 3-3s

Purified HHR GST 3-3 (●) and SD rat 3-3 (○) (0.5 unit/ml of each) were incubated with 0.4 mM xanthine and xanthine oxidase (15 m-units/ml) in 50 mM potassium phosphate (pH 7.8) containing 0.1 mM EDTA, at 25 °C. At the indicated incubation times, aliquots were taken and assayed for GST activity.

Although SD rat subunit 3 possesses three cysteine residues per subunit, the unique cysteine residue at position 199 of HHR subunit 3 was suggested to be involved in its glutathionylation.

Enzymic properties of HHR and SD rat GST 3-3s

Specific activity values of HHR GST 3-3 towards various substrates were compared with those of SD rat GST 3-3. As shown in Table 1, the values were not significantly different between the two enzymes. The K_m values of HHR GST 3-3 for GSH and CDBN were calculated to be 46 μ M and 14 μ M, respectively, both being half of the values for SD rat GST 3-3. Treatment of SD rat GST 3-3 with xanthine oxidase and xanthine resulted in an approx. 2-fold increase in the specific activity value, confirming our previous result [5]. However, in the case of HHR GST 3-3, such activation was not observed with the same treatment (Figure 10). Activities of both enzymes were unaltered by treatment with 1 mM H_2O_2 (results not shown).

DISCUSSION

In the present study, the HHR GST subunit 3 was separated from that of SD rat by HPLC. Nucleotide sequencing of cDNAs indicated differences at two amino acid residues in HHR and SD rat GST subunit 3, with Asn¹⁹⁸ and Cys¹⁹⁹ in HHR, but Lys¹⁹⁸

and Ser¹⁹⁹ in SD rat subunit 3. These sequences are in agreement with those reported for pGTA/C44 [22] and pGTR200 [21], respectively, both obtained from SD rat livers. Reactivity of the Cys¹⁹⁹ residue, unique to HHR subunit 3, with SH reagents was demonstrated by binding experiments with a coloured SH reagent, DDPM, followed by trypsin treatment and peptide sequencing analysis.

Three cDNA clones encoding the rat GST subunit 3 have been reported and these differ from each other in one or two amino acid residues [21–23]. The present study demonstrated that the lysine/serine form encoded by pGTR200 [21] is expressed in SD rat liver, while the asparagine/cysteine form, produced by pGTA/C44 [22], is expressed in HHR. Inheritance of *hhr* is autosomal and recessive. Rats heterologous and homologous for the mutant gene both exhibited only the asparagine/cysteine form (results not shown), indicating that the encoding gene is not linked to the *hhr* gene. Furthermore, segregated expression of the asparagine/cysteine and lysine/serine forms in HHR and SD rats, respectively, suggested that their genes are polymorphic.

Peak X exhibited two signals with similar counts using MALDI-TOF-MS, with molecular-mass differences of approx. 30. Nucleotide sequencing of HHR GST subunit 3 cDNA did not suggest the presence of a variant with different primary structure, and thus, post-translational modification could be concluded.

We examined whether the two GST 3-3 forms, the lysine/serine form and the asparagine/cysteine form, differed in enzyme activity, because these regions, adjacent to the C-terminus, are thought to be responsible for binding of hydrophobic substrates [45]. However, we did not observe any marked variation in specific activity values with various substrates. A previous site-directed mutagenesis study revealed that the three cysteine residues of the lysine/serine form were not involved in enzyme activity [9]. The presence of reactive cysteine residues has been reported in the Pi class GST forms [6,46]. Both GST-P (7-7) and GST 3-3 are increased in rat pre-neoplastic hepatic lesions [47], but whereas the Pi class are inactivated by H_2O_2 through disulphide bond formation between two cysteine residues [7], this is not the case for either the asparagine/cysteine or lysine/serine forms of GST 3-3. This suggests that formation of a disulphide bond does not occur, possibly because only the residue at position 199 out of the four cysteine residues is reactive. The fact that the asparagine/cysteine form was not activated by treatment with xanthine and xanthine oxidase, while the lysine/serine form was, as reported previously [5], indicates that the two GST 3-3 forms differ in their sensitivity to oxidative stress.

Modification of cysteine residues with GSH has been reported for many enzymes such as carbonic anhydrase [13] and fatty acid synthetase [14]. Such glutathionylation usually decreases enzyme activities but enhances them in some cases, for example with fructose 1,6-bisphosphatase [48,49]. The specific activity value of glutathionylated GST 3-3 was found to be approx. one-third of that for the unglutathionylated form (results not shown). The amounts of glutathionylated carbonic anhydrase and other proteins are demonstrated to be increased in response to oxidative stress [15] and glutathionylation is suggested to protect cysteine residues from irreversible oxidation [16]. Modified cysteine residues can subsequently be deglutathionylated to restore enzyme activities by glutaredoxin and thioltransferase [50].

In conclusion, the present study indicates that the GST subunit 3 in HHR differs from SD rat subunit 3 in possessing a reactive cysteine residue at position 199 so that mixed disulphides are formed with GSH. The results point to polymorphism of the rat GST subunit 3 gene associated with variation in sensitivity to oxidative stress.

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