Rat spleen glutathione transferases

A new acidic form belonging to the Alpha class

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Cytosolic glutathione transferases (GSTs) were purified from the rat spleen by *S*-hexyl-GSH-Sepharose chromatography, and two major forms were identified as GSTs 2–2 and 7–7 (GST P). Besides these forms an acidic form (pI 5.8) was purified by chromatofocusing at pH 7–4 and it accounted for about 1 % of the total GST activity bound to *S*-hexyl-GSH-Sepharose. Two-dimensional gel electrophoresis revealed that it is a homodimer (subunit M_r 26000 with pI 5.8). Immunoblot analysis demonstrated that it was immunologically related to GSTs 2–2 and 1–1, and its *N*-terminal amino acid was apparently blocked, similarly to other forms of the class Alpha. This form had a low activity towards cumene hydroperoxide or 4-hydroxynon-2-enal, indicating that this form differed from GSTs 10–10 and 8–8 as well as from GSTs 1–1 and 2–2. These results suggest that it is a new form of GST belonging to the class Alpha.

INTRODUCTION

Glutathione transferases (GSTs) (EC 2.5.1.18) catalyse the conjugation of GSH with a large number of electrophilic compounds, thereby protecting cells against their potential toxicity [1–3]. Some of the molecular forms also show significant glutathione peroxidase [1–3] and prostaglandin isomerase activities [4,5].

All cytosolic forms of GST are dimeric proteins composed of identical or non-identical subunits [2,3]. So far at least ten subunits [6] and 15 molecular forms of this enzyme have been described in the rat [1–3,6]. The expression of GST forms appears to be tissue-specific [7–9], and the subunits are distinguished by their M_r values, isoelectric points and other properties [1–3]. They have been classified into three groups, the Alpha, Mu and Pi classes, on the basis of similarities in structural, immunological and kinetic properties [10]. In the Alpha class four subunits have been reported, namely subunits 1 (Ya), 2 (Yc), 8 (Yk) [11,12] and 10 (Y_{fetus}) [6,13]. GST 8–8 (GST K) from rat liver has a low affinity for *S*-hexyl-GSH-Sepharose [11,14] and has a high activity towards 4-hydroxynon-2-enal [12]. Recently we found in the rat testis a new subunit, Y_{n_2} (subunit 9 in the new nomenclature), together with a similar subunit Y_{n_1} (subunit 6), belonging to the Mu class [15]. In the present paper we characterize GST forms of rat spleen and describe a form that is a homodimer of a new acidic subunit belonging to the Alpha class. It has an M_r value similar to that of the subunit 9 (Y_{n_2}) but has a more acidic isoelectric point.

MATERIALS AND METHODS

Materials

Epoxy-activated Sepharose 6B, Polybuffer exchangers (PBE 118 and 94), Pharmalyte 10.5–8 and Polybuffer 74 were obtained from Pharmacia. Nitrocellulose membrane

and anti-(rabbit IgG) antibody conjugated to horseradish peroxidase were from Bio-Rad Laboratories. All other chemicals were of analytical grade.

Purification of GSTs from rat spleen

A 70 g batch of spleens from male Sprague–Dawley rats (200–250 g body wt.) was homogenized with 4 vol. of 10 mM-Tris/HCl buffer, pH 7.8, containing 0.2 M-NaCl. The supernatant obtained by centrifugation at 78000 *g* for 90 min was subjected to *S*-hexyl-GSH-Sepharose affinity chromatography as described by Guthenberg & Mannervik [16]. The GST forms eluted with 5 mM-*S*-hexyl-GSH were resolved by chromatofocusing at pH 7–4 as described previously [17]. GST 8–8 of rat spleen was purified from the fractions not bound to an *S*-hexyl-GSH-Sepharose column by GSH-Sepharose chromatography essentially as described by Hayes [11] followed by chromatofocusing at pH 7–4. Other rat GSTs were purified from hyperplastic nodule-bearing rat liver by *S*-hexyl-GSH-Sepharose affinity chromatography as described above.

GST assay

GST activity was assayed with 1-chloro-2,4-dinitrobenzene (CDNB) and other substrates as described by Habig *et al.* [18] and Keen & Jakoby [19]. Glutathione peroxidase activity was determined by the method of Paglia & Valentine [20] as modified by Lawrence & Burk [21]. Activity towards 4-hydroxynon-2-enal was assayed by the method of Jensson *et al.* [12]. Protein content was measured by the method of Lowry *et al.* [22] or that of Bradford [23], with bovine serum albumin as a standard.

Electrophoresis

SDS/PAGE was carried out as described by Laemmli [24]; immunoblot analysis was performed by the procedure by Towbin *et al.* [25], with anti-(GST 2–2), anti-

Abbreviations used: GST, glutathione transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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(GST 7-7) [anti-(GST P)], anti-(GST 3-4) and anti-(GST 9-9) [anti-(GST Y_nY_n)] antibodies. Two-dimensional gel electrophoresis was performed as reported previously [26,27].

Antibody preparations

Antibodies to rat GSTs 2-2, 7-7 and 9-9 were prepared in rabbits as described previously [14,26,27].

RESULTS

Rat spleen GST subunits bound to *S*-hexyl-GSH-Sepharose

Rat spleen GSTs bound to *S*-hexyl-GSH-Sepharose were resolved into five spots by two-dimensional gel electrophoresis (Fig. 1a). Four spots were identified as subunits 2, 3, 4 and 7 (Yp) by their isoelectric points and M_r values and by immunoblots with anti-(GST 7-7) (Fig. 1b), anti-(GST 2-2) (Fig. 1c) and anti-(GST 3-4) (Fig. 1d) antibodies. Subunit 2 was the major one among them, whereas subunits 3 and 4 were faint. A minor spot above subunit 2 had an M_r of 56000, twice that of subunit 2 (28000), and the same pI as it (Fig. 1a). Furthermore, this spot was also stained on immunoblotting with anti-(GST 2-2) antibody (Fig. 1c), suggesting that it is the dimer of subunit 2, partly not dissociated into monomers (spots marked 2 in Figs. 1a and 1c) under the conditions of two-dimensional gel electrophoresis. Subunit 1 was not detected in rat spleen. An acidic spot (M_r 26000 with pI 5.8) was stained by immunoblotting with anti-(GST 2-2) (Fig. 1c) and anti-(GST 1-1) (results not shown) antibodies and was tentatively named the Ys subunit to indicate its splenic origin.

Purification of a GST form containing the Ys subunit

To purify a GST form containing the Ys subunit, spleen GSTs that had been partially purified by *S*-hexyl-

Fig. 2. Separation of rat spleen GSTs by chromatofocusing at pH 7-4

GSTs partially purified by *S*-hexyl-GSH-Sepharose affinity chromatography were dialysed against 25 mM-imidazole/HCl buffer, pH 7.4, and then applied to a column (1.0 cm $\times$ 16 cm) of chromatofocusing gel PBE 94 equilibrated with the same buffer. Elution was carried out as described previously [17]. The flow rate was 20 ml/h and 2.7 ml fractions were collected. ●, GST activity towards CDNB; ----, pH.

GSH-Sepharose affinity chromatography were separated by chromatofocusing at pH 7-4 (Fig. 2). One peak (peak a in Fig. 2) was recovered in the breakthrough fraction and another (peak b) was eluted at pH 6.9. SDS/PAGE (Fig. 3a, lanes 3 and 4) revealed that these peaks contain GST subunits 2 and 7 respectively. The breakthrough fraction was further subjected to the second chromatofocusing at pH 11-8 and most activity was eluted at pH 8.9 (results not shown). These results support the view that the major form of the breakthrough fraction

Fig. 1. Two-dimensional gel electrophoresis of rat spleen GSTs bound to *S*-hexyl-GSH-Sepharose

In each experiment 70 μ g of the protein bound to *S*-hexyl-GSH-Sepharose was used. (a) Protein staining with Coomassie Brilliant Blue R-250. (b), (c) and (d) Immunoblots with anti-(GST 7-7) [anti-(GST P)], anti-(GST 2-2) and anti-(GST 3-4) antibodies respectively. Numbers on the electrophoretograms indicate the GST subunits. Yp and Ys indicate subunits of GST 7-7 (GST P) and the pI 5.8 form respectively.

Fig. 3. SDS/PAGE of rat spleen GSTs

Rat spleen GSTs separated by chromatofocusing at pH 7–4 were subjected to SDS/PAGE. (a) Protein staining. Lane 1, rat GSTs from hyperplastic hepatic nodules [subunits 1, 2, 3, 4 and 7 (Yp), 35 μ g]; lane 2, spleen GSTs bound to *S*-hexyl-GSH-Sepharose (10 μ g); lane 3, the breakthrough fractions of chromatofocusing at pH 7–4 (peak a in Fig. 2, 5 μ g); lane 4, GST 7–7 (GST P) (pI 6.9, peak b in Fig. 2, 5 μ g); lane 5, the pI 5.8 form (peak c, 2 μ g); lane 6, GST 8–8 purified from the spleen by GSH-Sepharose chromatography (1 μ g). Numbers at the left-hand side indicate the GST subunits. (b) and (c) Immunoblots with anti-(GST 2–2) and anti-(GST 9–9) [anti-(Yn₂Yn₂)] antibodies respectively. Lanes 1, spleen GSTs bound to *S*-hexyl-GSH-Sepharose; lanes 2, the pI 5.8 form; lane 3, GST 8–8. The same protein amounts were applied to the respective lanes as described for (a).

is GST 2–2. Although fractions 45–48 of chromatofocusing at pH 7–4 (peak c with pI 5.8 in Fig. 2) showed a low activity towards CDNB, a protein was eluted in these fractions, as shown in Fig. 3(a), lane 5. On SDS/PAGE this protein band showed an intermediate mobility (subunit M_r 26000) between those of the subunit 3 (26500) and subunit 1 (25000), and was larger than subunit 8 (M_r 24500, lane 6 in Fig. 3). Immunoblotting with anti-(GST 2–2) antibody revealed that this subunit as well as subunit 8 is immunologically related to GST 2–2 (Fig. 3b), suggesting that it belongs to the Alpha class. These results suggest that the subunit (M_r 26000) is identical with the subunit Ys shown in Fig. 1. On two-dimensional gel electrophoresis the peak c material gave a spot in a position identical with that of the Ys subunit (Fig. 4), indicating that it is a homodimer of the Ys subunit. The steps for the purification of this acidic form are summarized in Table 1. This form accounted for about 1% of the total GST activity bound to *S*-hexyl-

Fig. 4. Two-dimensional gel electrophoresis of the pI 5.8 form of GST from rat spleen

A 4 μ g portion of the pI 5.8 form of GST from rat spleen purified by chromatofocusing at pH 7–4 was subjected to two-dimensional gel electrophoresis and then stained with Coomassie Brilliant Blue as described for Fig. 1.

GSH-Sepharose. The M_r value and isoelectric point of the Ys subunit (M_r 26000 with pI 5.8) were similar to those of subunit 9 (Yn₂) (M_r 26000 with pI 6.0) [15]. However, the Ys subunit is not immunologically related to subunit 9, as shown in Fig. 3(c), indicating that they are not identical. Subunits 3 and 4 bound to *S*-hexyl-GSH-Sepharose were stained with anti-(GST 9–9) antibody (Fig. 3c, lane 1), consistent with our previous findings [15].

Substrate-specificity of the acidic form (pI 5.8) from rat spleen

As shown in Table 2, this form exhibited a low but significant activity towards CDNB, ethacrynic acid and cumene hydroperoxide, and very low activity towards 4-hydroxynon-2-enal, whereas GST 8–8 had a very high activity towards 4-hydroxynon-2-enal (Table 2). These results indicate that this acidic form (pI 5.8) differs from GST 8–8, another acidic form of the class Alpha GSTs. The pI 5.8 form did not show any significant activity towards substrates for the Mu class GSTs, such as *p*-nitrophenyl acetate, *p*-nitrophenethyl bromide and *trans*-4-phenylbut-3-en-2-one. A significant activity towards

Table 1. Purification of rat spleen GSTs

The purification steps are described in the text.

Step	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)
105 000 <i>g</i> supernatant	300	6360	195 (100%)	0.03
<i>S</i> -hexyl-GSH-Sepharose affinity chromatography	10.8	7.78	120 (61.5%)	15.4
Chromatofocusing pH 7–4				
Peak a (fractions 4–14)	30	2.01	36.9 (18.9%)	18.4
Peak b (fractions 28–39) (pI 6.9)	32	1.70	32.0 (16.4%)	18.9
Peak c (fractions 45–48) (pI 5.8)	11	0.35	1.1 (0.6%)	3.1

Table 2. Substrate-specificity of the pI 5.8 form of GST from rat spleen

The assays were performed as described by Habig *et al.* [18] and Keen & Jakoby [19]. For comparison the values for GST 2-2, GST 7-7 (GST P) and GST 8-8 (GST K) purified from rat spleen are also included. Abbreviation: N.D., no detectable activity.

Substrate	GST activity (units/mg of protein)			
	pI 5.8 form	GST 8-8 (GST K)	GST 2-2	GST 7-7 (GST P)
1-Chloro-2,4-dinitrobenzene (CDNB)	3.10	3.07	18.4	18.9
1,2-Dichloro-4-nitrobenzene	N.D.	N.D.	N.D.	N.D.
Ethacrynic acid	0.90	4.55	1.03	2.35
Cumene hydroperoxide	0.48	N.D.	6.17	0.12
4-Hydroxynon-2-enal	0.11	120	0.40	0.26
1,2-Epoxy-3-(<i>p</i> -nitrophenoxy)propane	0.30	N.D.	0.13	0.37
Bromosulphophthalein	N.D.	N.D.	N.D.	N.D.
<i>p</i> -Nitrophenethyl bromide	N.D.	N.D.	N.D.	N.D.
<i>p</i> -Nitrophenyl acetate	N.D.	N.D.	0.08	N.D.
<i>trans</i> -4-Phenylbut-3-en-2-one	N.D.	N.D.	0.34	N.D.

cumene hydroperoxide also supported the view that it belongs to the Alpha class. The substrate-specificities of GST 2-2 and GST 7-7 (GST P) purified from rat spleen were almost the same as those of GST 2-2 from the liver and the GST P form from hyperplastic hepatic nodules [3].

DISCUSSION

GSTs 2-2 and 7-7 are the two dominant forms in rat spleen GSTs (Figs. 1 and 2). The pI 5.8 form is only a minor form, about 0.6% of the total GST activity (Table 1), and has a low activity towards 4-hydroxynon-2-enal (Table 2). The subunit (Ys) of this form has an M_r similar to that of subunit 9 (Yn₂) of rat testis recently identified by us [15], but the former was more acidic (pI 5.8 on two-dimensional gel; Fig. 1a) than subunit 9 (pI 6.0) [15]. The non-identity of the Ys subunit and subunit 9 was confirmed by immunoblot analysis with anti-(GST 9-9) antibody (Fig. 3c). The pI 5.8 form is immunologically related to GST 2-2 (Fig. 3b) and GST 1-1. Although *N*-terminal amino acid sequence analysis was performed with a gas-phase protein sequencer, the *N*-terminal amino acid residue of this form, like that of subunit 1, was blocked. Alin *et al.* [28] have recently reported that liver GST 8-8 of the class Alpha is also *N*-terminally blocked. The subunit M_r of the pI 5.8 form is similar to that of subunit 10 (Y_{fetus}) [13], but the latter form has a very basic pI (9.8) [6,13] and a high activity towards cumene hydroperoxide. GST 8-8 purified from the spleen showed high activities towards 4-hydroxynon-2-enal and ethacrynic acid (Table 2), confirming the findings on liver GST 8-8 reported by Jensson *et al.* [12] and Meyer *et al.* [29]. The spleen pI 5.8 form differs from GST 8-8 in the following points: first, the pI 5.8 form showed low activities towards 4-hydroxynon-2-enal and ethacrynic acid (Table 2). Secondly, the M_r of subunit Ys of the pI 5.8 form (26 000) was larger than that of GST 8-8 (24 500; Fig. 4). Thirdly, the pI 5.8 form was bound to *S*-hexyl-GSH-Sepharose (Table 1), whereas GST 8-8 was not bound to that column, but instead was bound to GSH-Sepharose, as reported by Hayes [11]. These results

indicate that the Ys subunit differs from subunits 8 and 10. Alin *et al.* [28] have described the acidic form designated GST A(6), with high activities towards 4-hydroxynon-2-enal and *trans*-4-phenylbut-3-en-2-one. Although it is not clear yet whether GST A(6) belongs to the class Alpha or not, its substrate-specificity is different from that of the spleen pI 5.8 form. Urade *et al.* [30] have reported a prostaglandin D₂ synthetase enzyme in rat spleen that exhibited GST activity with CDNB. Immunoblot analysis with anti-(prostaglandin D₂ synthetase) antibody (generously given by Dr. Yoshihiro Urade, Kyoto Laboratory of Hayaishi Bioinformation Transfer Project, Research Development Corporation of Japan, Kyoto, Japan) did not show any cross-reactivity with the pI 5.8 form (results not shown), indicating that the pI 5.8 form is not identical with it. Thus this acidic form, a homodimer of Ys, seems to be a new form of the Alpha class GSTs. It showed only low activities towards several substrates, and the functions of this form in the spleen are unknown. The fact that the subunit of this form (Ys) was detected in the rat liver (results not shown) as well as in the spleen suggest that it might be involved in some functions of the reticuloendothelial system.

We are grateful to Professor Hermann Esterbauer, Institute of Biochemistry, Graz University, Graz, Austria, for providing 4-hydroxynon-2-enal and to Ms. Noriko Hebiguchi and Ms. Rumiko Nakamura for their technical assistance. This work was supported in part by a Grant in Aid for Cancer Research from the Ministry of Education, Science and Culture of Japan.

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Received 10 July 1989/11 September 1989; accepted 18 September 1989