Rat lung glutathione *S*-transferases

Evidence for two distinct types of 22000-*M_r* subunits

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Two immunologically distinct types of 22000-*M_r* subunits are present in rat lung glutathione *S*-transferases. One of these subunits is probably similar to Ya subunits of rat liver glutathione *S*-transferases, whereas the other subunit Ya' is immunologically distinct. Glutathione *S*-transferase II (pI 7.2) of rat lung is a heterodimer (YaYa') of these subunits, and glutathione *S*-transferase VI (pI 4.8) of rat lung is a homodimer of Ya' subunits. On hybridization *in vitro* of the subunits of glutathione *S*-transferase II of rat lung three active dimers having pI values 9.4, 7.2 and 4.8 are obtained. Immunological properties and substrate specificities indicate that the hybridized enzymes having pI 7.2 and 4.8 correspond to glutathione *S*-transferases II and VI of rat lung respectively.

Multiple forms of glutathione (GSH) *S*-transferase are present in different rat tissues (Habig *et al.*, 1974a; Bass *et al.*, 1977; Scully & Mantle, 1981; Mannervik & Jensson, 1982). GSH *S*-transferases of rat liver arise from the subunits of three different sizes of *M_r*, 25000 (Yc), 24000–23500 (Yb) and 22000 (Ya). Rat liver GSH *S*-transferase B, which was previously thought to be identical with ligandin (Habig *et al.*, 1974b), has the subunit composition of YaYc (Boyer *et al.*, 1983), whereas GSH *S*-transferases A and C both have the composition of YbYb. Until recently the reasons for the observed differences in the physical and kinetic characteristics of GSH *S*-transferases A and C despite their seemingly identical subunit structure were not clear. However, recently it has been suggested that 23500-*M_r* (Yb) subunits consist of two distinct peptides Yb and Yb' having similar sizes (Mannervik & Jensson, 1982; Frey *et al.*, 1983; Beale *et al.*, 1983; Hayes, 1983). Heterogeneity in the population of Ya subunits of rat liver GSH *S*-transferases has also been suggested by Boyer *et al.* (1983). However, definitive evidence for the heterogeneity in the population of Ya subunits of rat liver GSH *S*-transferases is still lacking.

In the present paper we provide evidence for the existence of two distinct types of 22000-*M_r* subunits in rat lung GSH *S*-transferases. By the use of techniques of hybridization similar to those adopted by Kitahara & Sato (1981) and Hayes *et al.* (1981), these two subunits were separated as dimers and studied for their substrate specificities and immunological characteristics. Hybridization *in vitro* of these two subunits in the three possible combinations was achieved. Studies reported in the present paper explain the reasons for the differences in the characteristics of two of the GSH *S*-transferases of rat lung, which have a significant difference in their pI values (7.2 and 4.8) despite the fact that both are dimers of 22000-*M_r* subunits.

Materials and methods

Materials

GSH, 1-chloro-2,4-dinitrobenzene, NADPH, glutathione reductase (yeast, type III), bovine serum albumin, Sephadex G-100, guanidinium chloride and epoxy-activated Sepharose 6-B were purchased from Sigma Chemical Co., St Louis, MO, U.S.A. 1,2-Dichloro-4-nitrobenzene, *p*-nitrobenzyl chloride and ethacrynic acid were obtained from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Acrylamide, bisacrylamide, Tris, *NNN'*-tetramethylethylenediamine, 2-mercaptoethanol and SDS were purchased from Bio-Rad, Richmond, CA, U.S.A.

Abbreviations used: GSH, reduced glutathione; SDS, sodium dodecyl sulphate.

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GSH *S*-transferase activities with 1,2-dichloro-4-nitrobenzene, 1-chloro-2,4-dinitrobenzene, *p*-nitrobenzyl chloride and ethacrynic acid were determined by the methods of Booth *et al.* (1961) and Habig *et al.* (1974a). One unit of enzyme activity utilized 1 μ mol of substrate/min at 25°C. Protein content was measured by the method of Bradford (1976), with bovine serum albumin as a standard. Urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis by the method of Laemmli (1970) was performed in an LKB vertical-slab-gel apparatus at 40mA/gel current.

Purification of GSH S-transferases from rat lung

Male Sprague-Dawley rats were killed by cervical dislocation and lungs were taken out and washed with ice-cold distilled water. All subsequent steps were performed at 4°C. The tissue was minced and homogenized in 10mM-potassium phosphate buffer, pH 7.0, containing 1.4mM-2-mercaptoethanol (buffer A) in a Sorvall Omnimixer at 4000 rev./min for 5 min. The homogenate (10%, w/v) was centrifuged at 27000g for 30 min, and the supernatant was collected. Solid $(\text{NH}_4)_2\text{SO}_4$ was added gradually to the supernatant to bring about 80% saturation. The suspension was stirred overnight and centrifuged at 27000g for 30 min. The pellet was resuspended and dialysed overnight against buffer A (40 vol., four changes) and centrifuged at 27000g for 30 min. The supernatant was applied to a column (1 cm $\times$ 10 cm) of GSH-linked epoxy-activated Sepharose 6B, which had been pre-equilibrated with 22mM-potassium phosphate buffer, pH 7.0, at a flow rate of 10 ml/h. The enzyme was eluted with 50mM-Tris/HCl buffer, pH 9.6, containing 10mM-GSH. The eluted enzyme (53% yield) was dialysed against buffer A and subjected to isoelectric focusing in an LKB-8100 column with Ampholines in the pH range 3.5–10, and GSH *S*-transferase activity was monitored in the 0.8 ml fractions with 1-chloro-2,4-dinitrobenzene as a substrate. On isoelectric focusing purified rat lung GSH *S*-transferases separated into six forms, designated as I (pI 8.8), II (pI 7.2), III (pI 6.8), IV (pI 6.0), V (pI 5.3) and VI (pI 4.8).

The preliminary studies on the subunit structure indicated that enzyme forms II (pI 7.2) and VI (pI 4.8) were dimers of 22000- M_r subunit. Since the pI of these two forms were significantly different despite their apparently similar subunit composition, present studies were centred around forms II (pI 7.2) and VI (pI 4.8). To isolate the forms II and VI, the fractions from isoelectric focusing of purified rat lung GSH *S*-transferases corresponding to peaks at pH 7.2 and 4.8 were pooled separately and subjected to isoelectric focusing. Both the enzymes focused at their respective pI

values (enzyme II at pI 7.2 and VI at pI 4.8), and these preparations were used for further characterizations. Antibodies against rat lung GSH *S*-transferase VI (pI 4.8) were raised in rabbits by the procedure described previously by us (Awasthi *et al.*, 1980). Attempts to raise antibodies against the form II (pI 7.2) were not successful.

GSH *S*-transferases were also isolated from the livers of male Sprague-Dawley rats. Purified total GSH *S*-transferases of rat liver were subjected to isoelectric focusing, and GSH *S*-transferases B (pI 9.4), C (pI 8.4) and A (pI 8.8) were separated. The identity of these forms was established by studying their subunit structures. On urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis GSH *S*-transferase B showed the presence of 25000- M_r and 22000- M_r subunits whereas GSH *S*-transferases A and C showed presence of only 23500- M_r subunits. Antibodies were raised in rabbits against GSH *S*-transferase B and the mixture of GSH *S*-transferases A and C.

Dissociation and reconstitution of subunits of GSH S-transferase II and VI

Approximately equimolar amounts of rat lung GSH *S*-transferases II and VI (about 0.2 units of activity towards 1-chloro-2,4-dinitrobenzene for each) were incubated with 10 ml of 50mM-potassium phosphate buffer, pH 7.5, containing 20mM-2-mercaptoethanol and 6M-guanidinium chloride (buffer A) at 25°C for 1 h. The incubation mixture was diluted 9-fold with 5mM-potassium phosphate buffer, pH 6.7, containing 0.1 mM-EDTA and 25% (v/v) glycerol (buffer B). The diluted mixture was incubated further for 1 h at 25°C. The reaction mixture was then dialysed at 4°C against the buffer B for 36 h (40 vol., three changes). At the end of the dialysis, the total enzyme activity of the reaction mixture was about 75% of that used to start the reaction. No visual precipitation of enzyme protein was observed at any stage of the reaction. The enzyme solution was then subjected to isoelectric focusing in an LKB-8100 column with Ampholines in the pH range 3.5–10 and the enzyme activity of the column fractions was monitored with 1-chloro-2,4-dinitrobenzene as substrate.

Denaturation and reconstitution of subunits of GSH S-transferase II (pI 7.2)

GSH *S*-transferase II (pI 7.2) was also denatured with guanidinium chloride and reconstituted as described above. About 0.4 unit of GSH *S*-transferase II was incubated with 10 ml of buffer A at 25°C. After 1 h of incubation, the reaction mixture was diluted 9-fold with the buffer B and left to stand at 25°C for 1 h. It was then dialysed for 36 h at 4°C against buffer B (40 vol, three changes).

The dialysed reaction mixture was subjected to isoelectric focusing.

Immunological studies

Immunotitration studies were performed by incubating a fixed amount of enzyme with different amounts of appropriate antisera. The reaction mixture (200 μ l) in 10 mM-potassium phosphate buffer, pH 7.0, was incubated overnight at 4°C. Subsequently, anti-rabbit immunoglobulin G (1:4 dilution, 50 μ l) was added and the reaction mixture was incubated for an additional 16 h at 4°C. It was then centrifuged at 10000g for 30 min, and supernatants were used for enzyme activity determination. Immunodiffusion was performed as described by Ouchterlony (1958).

Inhibition studies in immunotitrations

Immunotitration curves for the enzymes with antibodies were obtained as described above. To study the inhibitory effects of the hybridized enzymes, pI 4.8 and 9.4, these enzymes were heat-inactivated at 90°C for 3 min in 8 M-urea. The denatured proteins thus obtained had no enzyme activity. From the immunotitration curves the concentrations of the antibodies were selected that caused about 40% immunoprecipitation of the enzyme. In the reaction mixtures for immunotitrations the enzyme was incubated with respective denatured proteins and left to stand for 4 h at 4°C. Antisera equivalent to the amount that causes 40% precipitation of enzyme activity were then added and incubated at 4°C for 16 h. Anti-rabbit immunoglobulin G (1:4 dilution, 50 μ l) was then added and the reaction mixture was incubated for 16 h at 4°C. After centrifugation the enzyme activity was determined in the supernatant.

Results

GSH *S*-transferases of rat lung were purified by using (NH₄)₂SO₄ fractionation, affinity chromatography and Sephadex gel filtration. On isoelectric focusing the purified GSH *S*-transferases of rat lung separated into six forms, designated as GSH *S*-transferases I (pI 8.8), II (pI 7.2), III (pI 6.8), IV (pI 6.0), V (pI 5.3) and VI (pI 4.8). On urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis forms II (pI 7.2) and VI (pI 4.8) were found to be dimers of 22000-*M_r* subunit (Fig. 1). The subunit structures of forms III (pI 6.8), IV (pI 6.0) and V (pI 5.3) could not be ascertained, and form I (pI 8.8) was found to be a dimer of 25000-*M_r* subunit (results not shown). The interesting observation that the forms II (pI 7.2) and VI (pI 4.8) had widely varying pI values despite their apparently similar subunit composition made these forms the focus of our

Fig. 1 Urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis of rat lung GSH *S*-transferases II (pI 7.2) and VI (pI 4.8)

Lane A, Standards in order of increasing distance from origin: bovine serum albumin (*M_r* 67000); ovalbumin (*M_r* 43000); carbonic anhydrase (*M_r* 30000); soya-bean trypsin inhibitor (*M_r* 20100); β -lactoglobulin (*M_r* 18000). Lanes B, C and D contain GSH *S*-transferases II (pI 7.2), VI (pI 4.8) and a mixture of II (pI 7.2) and VI (pI 4.8) of rat lung respectively.

present studies. Peak fractions containing forms II (pI 7.2) and VI (pI 4.8) were pooled and separately subjected to isoelectric focusing, where each of these forms focused as a single peak at their respective pI (Figs. 2a and 2b). The subunit composition of these forms was confirmed by urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis, where each of these forms showed presence of only 22000-*M_r* subunits. It was argued that rat lung GSH *S*-transferases may have two distinct types of 22000-*M_r* subunits, and hybridization studies similar to those reported by Kitahara & Sato (1981) and Hayes *et al.* (1981) were performed with these two forms.

Hybridization of rat lung GSH *S*-transferase II and VI subunits

Hybridization *in vitro* of the subunits of GSH *S*-transferase II of rat lung (Fig. 3) yielded three enzymes having isoelectric points of 9.4, 7.2 and 4.8. The relative proportions of these peaks were 1:4:1.5 respectively. Also, when equimolar mixtures of GSH *S*-transferases II and VI were used for hybridization studies, three enzyme peaks were obtained. The isoelectric points of these enzymes (Fig. 4) were similar to those obtained by the

Fig. 2. Isoelectric focusing profiles of (a) rat lung GSH S-transferase II (pI7.2) and (b) rat lung GSH S-transferase VI (pI4.8)

Experimental details are given in the text. ○, pH gradient; ●, enzyme activity.

Fig. 3. Isoelectric-focusing profile of reconstituted enzymes obtained by the hybridization of subunits of rat lung GSH S-transferase II (pI7.2) alone

Experimental details are given in the text. Fractions of volume 0.8 ml each were collected and GSH S-transferase activity was determined with 1-chloro-2,4-dinitrobenzene as substrate. ○, pH gradient; ●, enzyme activity.

hybridization of GSH S-transferase II alone. In this case the relative proportions of the peaks at pH 9.4, 7.2 and 4.8 were found to be 1:2:1.4 respectively (Fig. 4). When each of the peaks of hybridized enzymes were pooled and subjected to isoelectric focusing separately, they focused at their respective pI values and no additional

Fig. 4. Isoelectric focusing profile of reconstituted enzymes obtained by hybridizing subunits of rat lung GSH S-transferases II (pI7.2) and VI (pI4.8)

See the Materials and methods section for experimental details. Fractions of volume 0.8 ml each were collected and enzyme activity was determined with 1-chloro-2,4-dinitrobenzene as substrate. ○, pH gradient; ●, enzyme activity.

peaks of the enzyme activity were seen. That the enzyme peaks at pH 9.4, 7.2 and 4.8 (Figs. 3 and 4) represented the active enzymes as dimers of 22000- M_r subunits was established by Sephadex G-100 gel filtration and urea/SDS/2-mercaptoethanol/polyacrylamide-slab-gel electrophoresis. When the reconstituted subunits of either GSH S-transferase II alone or the mixture of GSH S-transferases II and VI were subjected to gel filtration before isoelectric focusing, only one peak of enzyme activity was seen, corresponding to M_r about 45000. Gel electrophoresis under reduced denaturing conditions revealed the presence of only one polypeptide, of M_r 22000, in each of the peaks. Hybridization of the constituent subunits of GSH S-transferase VI alone was also attempted, where only one enzyme peak corresponding to the pI of the original enzyme (4.8) was obtained. These results can best be explained if GSH S-transferase II is a heterodimer, GSH S-transferase VI is a homodimer and one of the subunits of II is common to those of VI. In this case one would expect the formation of three enzymes on hybridization of either GSH S-transferase II subunits or the mixture of GSH S-transferases II and VI subunits. Two of the hybridized enzymes, with pI 7.2 and 4.8, exist *in vivo* in lung (GSH S-transferases II and VI), but the third enzyme, with pI 9.4, was not detected in rat lung during these studies. As discussed below in this section, the immunological properties and substrate specificities of the hybridized enzymes with pI 7.2 and 4.8 were similar to those of rat lung GSH S-transferases II and VI respectively.

Table 1. *Immunological properties of the peaks obtained after hybridization*

Antigens used to raise antibodies	Hybridized enzyme ...	Cross-reactivity		
		pI9.4	pI7.2	pI4.8
Rat lung GSH S-transferase VI		—	+	+
Rat liver GSH S-transferase B		+	+	—
Mixture of rat liver GSH S-transferases A and C		—	—	—

Fig. 5. *Immunodiffusion of hybridized enzymes with the antibodies raised against lung and liver GSH S-transferases* (a) Centre well contains antibodies raised against GSH S-transferase VI (pI4.8) of rat lung. Outer wells contains: A, the hybridized enzyme with pI7.2; B, the hybridized enzyme with pI9.4; C, the hybridized enzyme with pI4.8. (b) Centre well contains antibodies raised against GSH S-transferase B of rat liver. The contents of outer wells A, B and C are the same as in (a).

Immunological properties of the hybridized enzymes

Immunological properties of the hybridized enzyme peaks at pH9.4 and 4.8 (Table 1) clearly indicate that these two enzymes are immunologically distinct from each other. In the immunotitration studies the more basic form (pI9.4) obtained either from the hybridization of the subunits of GSH S-transferase II alone or the mixture of GSH S-transferases II and VI is precipitated by the antibodies raised against GSH S-transferase B (YcYa) of rat liver. The acidic form of the hybridized enzyme (pI4.8) is not precipitated by these antibodies. This indicates that the subunits of the basic enzyme (pI9.4) have immunological similarity with GSH S-transferase B and that the Ya subunit may be present in the basic enzyme. Furthermore, this suggests immunological dissimilarity between the acidic (pI4.8) and the basic enzymes (pI9.4). Neither the acidic nor the basic enzyme is precipitated by the antibodies raised against the mixture of GSH S-transferases A and C of liver. More revealing is the observation that the basic enzyme (pI9.4) is not precipitated by the antibodies raised against GSH S-transferases VI of rat lung, which precipitate the acidic enzyme

(pI4.8). The hybridized enzyme having pI7.2 has immunological properties similar to those of GSH S-transferase II and is precipitated by the antibodies raised against GSH S-transferase B of liver as well as those raised against GSH S-transferase VI of rat lung. Immunodiffusion studies (Fig. 5) confirm the findings of the immunotitration studies. These results strongly suggest that the constituent subunits of M_r 22000 present in the basic (pI9.4) and the acidic (pI4.8) enzyme forms are immunologically distinct and that the intermediate peak (pI7.2) shares the immunological characteristics of both the acidic and the basic enzymes.

Further evidence for the distinct immunological nature of the basic (pH9.4) and acidic (pI4.8) enzymes is provided by studies of competitive inhibition by the inactivated enzyme proteins of the acidic and the basic enzymes in the immunotitration studies. GSH S-transferase II (pI7.2) was immunotitrated separately with the antibodies raised against GSH S-transferase VI of rat lung. As shown in Fig. 6, the antibodies precipitate this enzyme. The basic and the acidic enzymes obtained by hybridization were inactivated by boiling in 8M-urea and then were used to study the competitive inhibition in the immunoprecipitation

studies. During the immunotitration of GSH *S*-transferase II (pI7.2) with the antibodies raised against GSH *S*-transferase VI, the precipitation was inhibited by the inactive protein of the acidic enzyme (pI4.8) and not by that of the basic (pI9.4) enzyme (Fig. 6 inset). This also indicates that the acidic and the basic enzymes obtained by the denaturation and reconstitution of the subunits present in GSH *S*-transferase II are immunologically distinct.

Substrate specificities of the hybridized enzymes

Differences in the characteristics of the basic (pI9.4) and the acidic (pI4.8) enzymes are also reflected in their substrate specificities. Both these enzymes have high activity towards 1-chloro-2,4-dinitrobenzene but have noticeable differences

in their activities towards *p*-nitrobenzyl chloride, 1,2-dichloro-4-nitrobenzene and ethacrynic acid (Table 2). Lower specific activities of the basic enzyme (pI9.4) towards *p*-nitrobenzyl chloride, and 1,2-dichloro-4-nitrobenzene indicate its kinetic similarities with the Ya subunit of rat liver GSH *S*-transferases (Boyer *et al.*, 1983). Relatively higher specific activities (7–10-fold) of the acidic enzyme towards these substrates strongly suggest that the constituent subunits of this enzyme have different kinetic characteristics from those of the Ya subunits of liver GSH *S*-transferases.

Discussion

The present study provides the evidence for two types of 22000-*M_r* subunit in the GSH *S*-transferase II (pI7.2) of rat lung. One of these subunits is common to those present in the GSH *S*-transferase VI (pI4.8) and is presumably acidic, whereas the other subunit, which appears to be basic, is different from the subunits of GSH *S*-transferase VI of lung. When the subunits of GSH *S*-transferase II were dissociated under denaturing conditions and re-associated by the removal of the denaturing agent, the two subunits reconstituted the active enzyme, yielding, as expected, three active dimers. The enzyme peaks at pH9.4 and 4.8 represent the homodimers of the basic and the acidic subunits respectively and the peak at pH7.2 represents the heterodimer of the acidic and the basic subunits. It is noteworthy that only two of these hybrids, those with pI7.2 and 4.8, are present *in vivo*. These correspond to rat lung GSH *S*-transferases II and VI respectively. The third hybrid, with pI9.4, has not been detected in rat lung. When the mixture of GSH *S*-transferases II and VI of rat lung were hybridized, the isoelectric-focusing profiles, substrate specificities and immunological properties of the reconstituted enzymes were the same as those obtained by the hybridization of GSH *S*-transferase II alone. Hybridization of subunits of GSH *S*-transferase VI alone yielded only one active enzyme, corresponding to the original enzyme.

Fig. 6. Immunotitration of rat lung GSH *S*-transferase II (pI7.2) with the antibodies raised against GSH *S*-transferase VI (pI4.8) of rat lung

Details are given in the text. In the inset, the reaction mixture was preincubated with hybridized denatured enzyme proteins as described in the text: ●, preincubated with denatured hybridized acidic enzyme (pI4.8); ○, preincubated with denatured hybridized basic enzyme (pI9.4) protein; □, preincubated without any denatured enzyme protein.

Table 2. Substrate specificities of three enzyme forms obtained by hybridizing the subunits of rat lung GSH *S*-transferases II (pI7.2) and VI (pI4.8)

Substrate	Hybridized enzyme ...	Specific activity (units/mg of protein)		
		pI9.4	pI7.2	pI4.8
1-Chloro-2,4-dinitrobenzene		2.9	2.8	2.4
1,2-dichloro-4-nitrobenzene		0.01	0.01	0.07
<i>p</i> -Nitrobenzyl chloride		0.15	0.30	1.8
Ethacrynic acid		0.25	0.28	2.1

Rat liver GSH S-transferase B has a 22000- M_r subunit (Ya). Antibodies raised against this enzyme cross-reacted with the hybridized basic enzyme having pI9.4 and 7.2 and not with the acidic enzyme (pI4.8). On the other hand, the antibodies raised against GSH S-transferase VI (pI4.8) of the rat lung cross-reacted with the hybridized enzyme peaks with pI4.8 and 7.2 and not with that with pI9.4. Immunological studies suggest that one of the 22000- M_r subunits of GSH S-transferase II or the hybridized enzyme peak at pH7.2 is similar to the 22000- M_r subunit present in GSH S-transferase B of liver. The other subunit of this dimer is same as those present in the GSH S-transferase VI of rat lung. Rat lung GSH S-transferase II (pI7.2) therefore is a heterodimer having subunit composition YaYa'. Dissociation and reconstitution of active enzyme yields the expected combinations of YaYa (pI9.4), YaYa' (pI7.2) and Ya'Ya' (pI4.8). The suggestion that one of the subunits of the lung enzyme II (pI7.2) is the same as GSH S-transferase B of liver is substantiated by the immunological properties and the substrate specificities of the hybridized enzyme peak at 9.4.

The present studies provide a clue for understanding the basis of the origin of multiple forms of GSH S-transferases in rat tissues and for the first time provide a proof for the heterogeneity in 22000- M_r subunits. Five subunits, Ya, Ya', Yb, Yb' and Yc, could account for up to 15 forms of GSH S-transferases in rat tissues in different dimeric combinations of the constituent subunits.

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