Chem. Pharm. Bull. 36(11)4527—4533(1988)

Studies on the Subunits of Rabbit Hepatic Glutathione S-Transferases[1,2]

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(Received April 26, 1988)

We previously reported that the purified glutathione S-transferases from rabbit liver are homodimers or heterodimers of four different subunits, having molecular weight of 24500 (Y1), 25000 (Y2), 26500 (Y3) and 28000 (Y4). Their subunit compositions were also reported to be Y1Y1 (R1a), Y1Y3 (R3a), Y3Y3 (R3b), Y2Y2 (R2) and Y2Y4 (R1b, R1c, R1d).

In the present study, two additional isozymes, R3o and R3c, were obtained. They showed the same subunit compositions as R1a and R3b, respectively. Each subunit with the same molecular weight among different isozymes was demonstrated to have the same pI value by isoelectric focusing on polyacrylamide-gel plates containing 7.5 M urea (Y1, pI 8.49; Y2, pI 7.86; Y3, pI 8.41; Y4, pI 7.15). R3a (Y1Y3) in a reconstitution experiment using 4 M guanidine hydrochloride gave Y1Y1 homodimer (R3o), Y1Y3 heterodimer (R3a) and Y3Y3 homodimer (R3b). Similarly, the reconstituted R1c (Y2Y4) gave Y2Y2 homodimer (R2), Y2Y4 heterodimer (R1c) and an isozyme which probably has the subunit composition of Y4Y4. The failure of the *in vitro* formation of heterodimers such as Y1Y2, Y1Y4, Y2Y3 and Y3Y4 suggests that the possible combinations of subunits are limited.

Keywords——glutathione; glutathione S-transferase; isozyme; subunit; rabbit liver; reconstitution; isoelectric focusing

Introduction

The glutathione S-transferases (EC.2.5.1.18) are a family of enzymes which play an important role in drug biotransformation, xenobiotic metabolism and protection against peroxidatic damage.[3-6] The existence of multiple forms of glutathione S-transferase has been observed in rat,[7] human[8] and mouse[9] livers, *etc.* All of them have molecular weights of 40000—50000 and are dimeric proteins.

Historically, rat hepatic glutathione S-transferases have been the most extensively investigated and many isozymes (AA, A, B, C, D, E, ligandin, *etc.*) have been purified to homogeneity.[7,10-12] Sodium dodecyl sulfate (SDS)/polyacrylamide-gel electrophoresis of these isozymes has demonstrated the existence of three major classes of subunits: Ya (molecular weight 24000), Yb (molecular weight 25500) and Yc (molecular weight 27500).[13,14] The values of molecular weights of these subunits are cited from our previous data.[1] Hayes[15] reported that the Yb subunit is further divided into Yb1 and Yb2 subunits based on a reconstitution experiment with glutathione S-transferase C (Yb1Yb2). Mannervik and Jensson[16] also suggested that two subunits of similar size, Yb (Yb1) and Yb' (Yb2), could be distinguished in terms of the difference of substrate specificities among three isozymes having the subunit composition of YbYb. The analysis of subunit composition indicated that combinations of subunits were not random. YaYc (B) and Yb1Yb2 (C) heterodimers have been purified from rat liver. However, no YaYb or YbYc heterodimer has been identified.

We have previously reported that at least 10 species of glutathione S-transferase exist in

the diethyl aminoethyl (DEAE)-non-adsorbed fraction of rabbit liver 20000 × *g* supernatant.[2] Seven isozymes could be purified to homogeneity and their subunit compositions were determined to be Y1Y1 (R1a), Y2Y4 (R1b, R1c, R1d), Y2Y2 (R2), Y1Y3 (R3a) and Y3Y3 (R3b) by SDS/polyacrylamide-gel electrophoresis.[1,2] Although our nomenclature used to define these subunits has been based only on the molecular weight, a subunit with a given molecular weight may be further divided as observed for Ya,[17] Yb[15,16] and Yc[18] subunits in the rat isozymes. In the present study, we present the properties and the observed combinations of the subunits.

Experimental

Materials——Female Japanese white rabbits (body weight, about 3 kg) were purchased from Sankyo Labo. and fed a standard diet (100 g/head/d, Clea Japan Inc.) and tap water *ad libitum*. Pharmalytes (pH 10.5—8 and pH 9—6.5) and GelBond PAG film (114 × 229 × 0.2 mm) were from Pharmacia Fine Chemicals. 1-Chloro-2,4-dinitrobenzene (CDNB), urea and guanidine hydrochloride were obtained from Wako Pure Chemical Industries Ltd. All other chemicals were of the highest purity available.

Enzyme Assay——Enzyme activity was spectrophotometrically determined by measuring the rate of conjugation of glutathione with CDNB according to the report of Habig *et al.*[7]

Protein Concentration——This was determined by measurement of the absorbance at 280 nm or by the method of Lowry *et al.*[19] with bovine serum albumin as a standard.

Purification of Glutathione S-Transferases from Rabbit Liver——Purification of glutathione S-transferases was carried out as reported.[1,2] In brief, the DEAE-non-adsorbed fraction of rabbit liver 20000 × *g* supernatant was resolved into four active fractions (R1, R2, R3, R4) by CM-cellulose chromatography. R1a, R1b, R1c and R1d were purified from R1 fraction by affinity (S-hexylglutathione-linked Sepharose 6B) chromatography and isoelectric focusing. R2 was purified from R2 fraction by affinity and hydroxylapatite chromatography. The partially purified R3 fraction obtained by affinity chromatography was resolved into four activity peaks toward CDNB by hydroxylapatite chromatography (Fig. 1). The peaks eluted with 112 and 132 mM potassium phosphate buffer (pH 6.7) corresponded to R3a and R3b in the previous report,[1] respectively. Two other peaks, designated as R3o and R3c, were newly detected in the present study. The following experiments were performed using mainly R1c, R2, R3o, R3a and R3b as purified isozymes.

SDS/Polyacrylamide-Gel Electrophoresis——This was performed to determine the subunit compositions of the purified isozymes as previously described.[20]

Determination of the pI Values of the Subunits——To determine the pI values of Y1, Y2, Y3 and Y4 subunits, the purified isozymes were subjected to ultrathin-layer isoelectric focusing on a polyacrylamide-gel plate (0.2 mm in thickness) containing 7.5 M urea. A mixture of Pharmalytes with pH ranges of 10.5—8 and 9—6.5 (3 : 1, v/v) was used at 2.4% final concentration as the carrier ampholyte. The gel plate was pre-focused for 30 min at a constant voltage of 400 V. Then purified isozymes (about 2 μg) were applied. Focusing was performed for 1 h at 700 V and further for 1.5 h at 1400 V. A section of the gel plate not containing isozymes was cut into slices. Pharmalytes in the slices were extracted with distilled water and the pH value of each extract was measured at 24 °C. The remainder of the gel plate was fixed, stained and destained as previously described.[1]

Reconstituted Glutathione S-Transferases——R1c, R2, R3o, R3a and R3b (about 100 μg) were separately

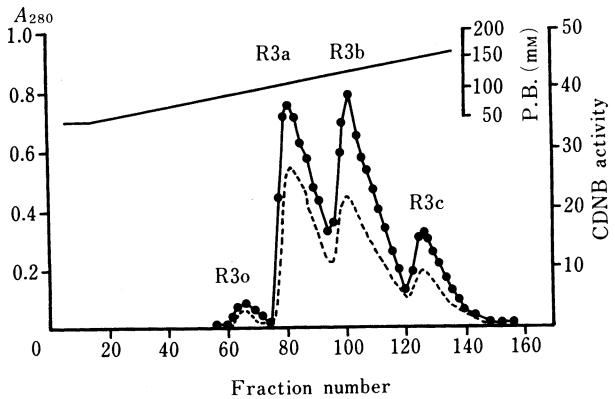

Fig. 1. Hydroxylapatite Chromatography of R3 Fraction

The further purified R3 fraction (179 A_{280} protein) obtained by affinity chromatography was applied to a hydroxylapatite column (2.8 × 26 cm) equilibrated with 50 mM potassium phosphate buffer (pH 6.7). The column was eluted with a linear gradient (——) of 50 to 200 mM potassium phosphate buffer (pH 6.7) in a total volume of 1.6 l. The eluate was collected in 10 ml fractions, which were monitored at A_{280} (------) for protein concentration and assayed with CDNB (●) for transferase activity.

incubated for 20 min at 2 °C in 4 M guanidine hydrochloride containing 5 mM 2-mercaptoethanol. The guanidine hydrochloride was removed by dialysis against three changes, each of 5 l, of 5 mM potassium phosphate buffer (pH 6.7) containing 5 mM 2-mercaptoethanol for 18 h at 4 °C. After removal of the precipitated materials by centrifugation (20000 × *g*, 20 min, at 4 °C), each dialyzed sample was concentrated to about 50 μl in a Centricon-10. To identify the products, the concentrated samples were subjected to normal ultrathin-layer isoelectric focusing on polyacrylamide-gel plate.

Mixtures of two isozymes (R2 and R3b, R2 and R3o, R1c and R3b, R1c and R3o) were treated similarly and their products were identified as described above.

Formation of Y1Y1 and Y3Y3 Homodimers from Glutathione S-Transferase R3a (Y1Y3)——In order to dissociate dimer to monomers, Y1Y3 heterodimer (R3a, about 5 mg) was treated with 4 M guanidine hydrochloride containing 5 mM 2-mercaptoethanol as described above. The reconstitution was performed by removing the guanidine hydrochloride by dialysis. As a control, Y1Y3 heterodimer was treated in the absence of 4 M guanidine hydrochloride under the above conditions. Both samples were separately subjected to isoelectric focusing in columns. The subunit compositions of active fractions toward CDNB were identified by SDS/polyacrylamide-gel electrophoresis.

Results

Subunit Compositions of Purified Glutathione S-Transferases

The subunit compositions of the purified isozymes were determined by SDS/polyacrylamide-gel electrophoresis (Fig. 2). R1a, R1b, R1c, R2, R3a and R3b were found to be Y1Y1, Y2Y4, Y2Y4, Y2Y2, Y1Y3 and Y3Y3, respectively. This result was in complete agreement with those of the previous studies.[1,2] R3o and R3c, which were newly detected in the present study (Fig. 1), were Y1Y1 and Y3Y3, respectively.

Isoelectric Focusing of the Purified Isozymes on a Polyacrylamide-Gel Plate Containing 7.5 M Urea

The subunits in rabbit hepatic glutathione S-transferases can be classified four species (Y1, Y2, Y3, Y4) according to the molecular weight. Furthermore, the subunit with the same molecular weight may be subdivided, as observed for the subunits in rat isozymes,[15-18] because the same subunit composition was found in the purified isozymes, *i.e.* Y1Y1, R1a and R3o; Y3Y3, R3b and R3c; Y2Y4, R1b and R1c. Therefore, the pI values of subunits were compared among the various isozymes by isoelectric focusing on a polyacrylamide-gel plate containing 7.5 M urea. As shown in Fig. 3, the subunits with the same molecular weight were found to have the same pI values (Y1, pI 8.49; Y2, pI 7.86; Y3, pI 8.41; Y4, pI 7.15). R3c and R1d showed focusing patterns similar to those of R3b and R1c, respectively (data not shown).

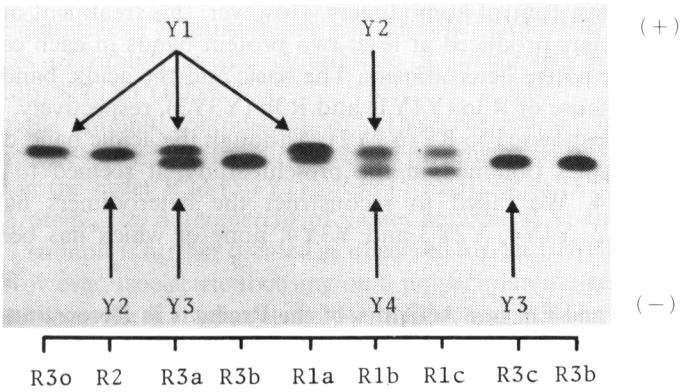

Fig. 2. SDS/Polyacrylamide-Gel Electrophoretic Pattern of Purified Glutathione S-Transferases

A portion (about 10 μg) of each purified isozymes was loaded onto polyacrylamide-gel.

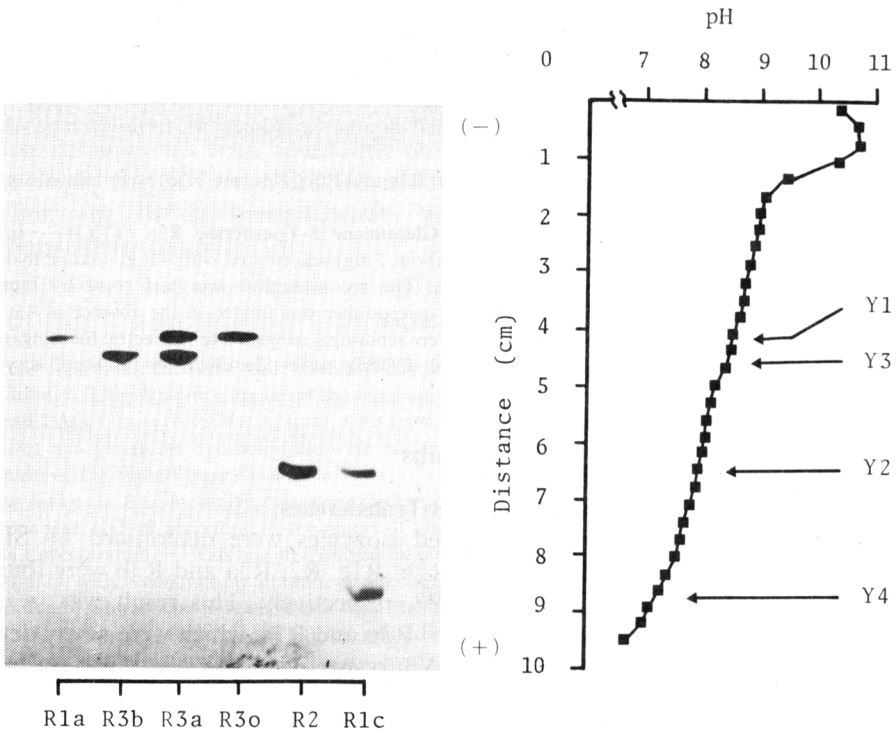

Fig. 3. Determination of the pI Values of the Subunits in Glutathione S-Transferases

A portion (about 2 μg) of each purified isozyme was loaded onto a polyacrylamide-gel plate containing 7.5 M urea. The pI value of each subunit was estimated from the pH values (■) of the solutions which were extracted from slices of gel plate not containing isozymes. For details, see Experimental.

Properties of Reconstituted Glutathione S-Transferases

The glutathione S-transferases, dimeric proteins, were dissociated to monomers with 4 M guanidine hydrochloride. After removal of guanidine hydrochloride by dialysis, the products in reconstituted isozymes were identified by normal isoelectric focusing on a polyacrylamide-gel plate (Fig. 4). The focusing patterns of treated homodimers (R2, R3o, R3b) were identical to those of the respective control homodimers. However, this treatment of Y1Y3 (R3a) and Y2Y4 (R1c) heterodimers produced at least two protein bands in each case, in addition to those of the respective native heterodimers. The basic and the acidic bands of reconstituted R3a corresponded to those of R3o (Y1Y1) and R3b (Y3Y3), respectively. The basic band in reconstituted R1c corresponded to R2 (Y2Y2). Although the acidic band did not correspond to either of the isozymes obtained in the present study, it seemed to have the subunit composition of Y4Y4. We failed to reconstruct the heterodimers having the subunit compositions of Y1Y2, Y1Y4, Y2Y3 and Y3Y4, none of which has been identified from rabbit liver.

Subunit Compositions and Enzyme Activities of the Products in Reconstituted R3a

This experiment was performed to examine whether the new protein bands of reconstituted R3a observed in Fig. 4 have the transferase activity. Control and reconstituted R3a were separately subjected to isoelectric focusing in a column (Fig. 5). The control R3a was focused at pH 10.36 as a single activity peak. On the other hand, the reconstituted R3a was resolved into three activity peaks with pIs of 10.88, 10.42 and 10.10. By SDS/polyacrylamide-

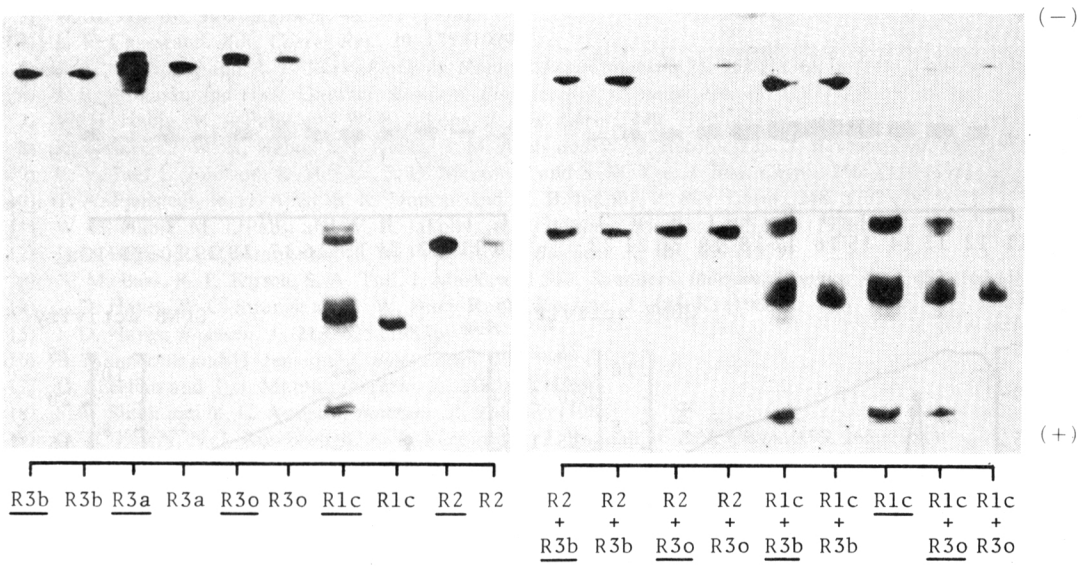

Fig. 4. Isoelectric Focusing Patterns of Reconstituted Glutathione S-Transferases on a Polyacrylamide-Gel Plate

The isozymes or mixtures of two isozymes were treated with 4 M guanidine hydrochloride. A portion (2—8 μg) of each sample was loaded onto a polyacrylamide-gel plate. In order to detect new proteins formed during this treatment, non-treated samples (2—8 μg) were subjected to electrophoresis as controls. The treated samples are marked with underlines. For details, see Experimental.

gel electrophoresis of active fractions, all the active fractions in the control R3a were found to have the subunit composition of Y1Y3. The peaks with pI 10.88, 10.42 and 10.10 in the reconstituted R3a showed the subunit compositions of Y1Y1, Y1Y3 and Y3Y3, respectively. They corresponded to R3o, R3a and R3b, respectively, with regard to pI value and subunit composition.

Discussion

It has been experimentally shown that the pI values of homodimers and heterodimers are in the order one homodimer > heterodimer > another homodimer in the case of rat[15,21] and human[22] hepatic glutathione S-transferases. On the other hand, our previous study showed that the pI values of rabbit hepatic glutathione S-transferases are in the order Y1Y3 (R3a) > Y3Y3 (R3b) > Y1Y1 (R1a).[1]

R3o, having the subunit composition of Y1Y1, corresponding to that of R1a, was obtained in the present study. R3o has a more basic pI value than R3a. Namely, the pI values of isozymes were in the order R3o (Y1Y1) > R3a (Y1Y3) > R3b (Y3Y3) > R2 (Y2Y2) > R1c (Y2Y4) > Y4Y4 protein. The difference of pI values between R3o and R1a may suggest the possibility that Y1 subunit is further divided as observed for Ya,[17] Yb[15,16] and Yc[18] subunits in rat isozymes. However, isoelectric focusing on a polyacrylamide-gel plate containing 7.5 M urea demonstrated that the Y1 subunits in R1a, R3o and R3a have the same pI values. Moreover, the reconstitution experiment with R3a (Y1Y3) showed that R3a is a hybrid of R3o (Y1Y1) and R3b (Y3Y3).

R1c seemed to be a hybrid of R2 (Y2Y2) and Y4Y4 protein by isoelectric focusing on a polyacrylamide-gel plate of reconstituted R1c. Although the Y4Y4 protein has not been identified from the DEAE-non-adsorbed fraction, this protein probably exists in the DEAE-

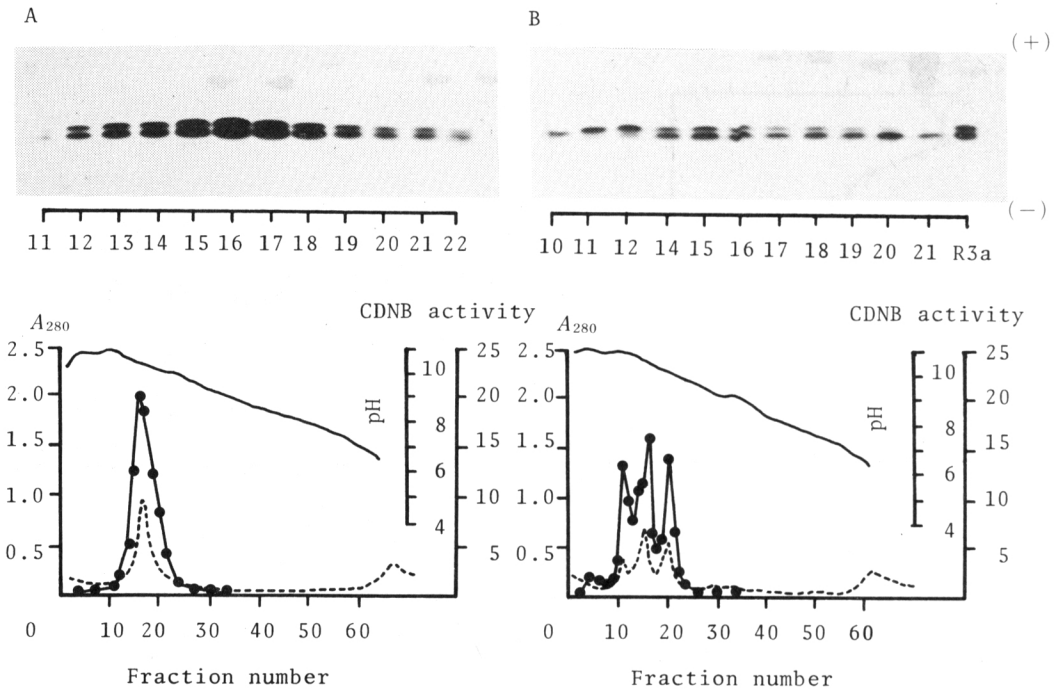

Fig. 5. Identification of the Product in Reconstituted Glutathione S-Transferase R3a

A, control R3a. B, reconstituted R3a. A mixture of Pharmalytes with pH ranges of 10.5—8 and 9—6.5 (3 : 1, v/v) was used at 1% final concentration. Focusing was performed in a 0—50% sorbitol density gradient at 3 W for 46 h at 2 °C with a 110 ml column. The contents were collected in 1.5 ml fractions, which were monitored at A_{280} (------) for protein concentration and assayed with CDNB (●) for transferase activity. The pH values (——) in the fractions were measured at 2 °C. SDS/polyacrylamide-gel electrophoretic patterns of active fractions are shown in the upper part.

adsorbed fraction because the pI value of the Y4 subunit was the most acidic among the four subunits. The isozymes having the subunit compositions of Y1Y2, Y1Y4, Y2Y3 and Y3Y4 also have not been identified. The failure of *in vitro* formation of these isozymes in reconstitution experiment suggests that such combinations of subunits essentially can not occur *in vivo*, in the same way that the combinations of subunits are limited in rat isozymes.

In the present study, two additional acidic proteins which possess the transferase activity and the subunit composition of Y2Y2 were generated when the partially purified R2 was stored at 4 °C for 2.5 months (unpublished data). Most of the two acidic proteins was converted into native R2 by addition of a reductant, 2-mercaptoethanol, at 1% final concentration. Some isozymes showed the same subunit compositions, as follows: Y1Y1 (R1a, R3o), Y2Y4 (R1b, R1c, R1d) and Y3Y3 (R3b, R3c). However, these isozymes were not affected with the reductant. Therefore, they may be generated by irreversible oxidation or some other process different from that in the case of R2.

Acknowledgments This work was supported in part by a Grant-in-Aid for Scientific Research (No. 62771927) from the Ministry of Education Science and Culture of Japan.

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