Communication

On the Multiplicity of Rat Liver Glutathione S-Transferases*

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Rat liver glutathione S-transferases have been pu-
tified to apparent electrophoretic homogeneity by S-
hexylglutathione-linked Sepharose 6B affinity chro-
matography and CM-cellulose column chromatog-
raphy. At least 11 transferase activity peaks can be
resolved including five Yb size homodimeric isozymes,
two Yc size homodimeric isozymes, one Yc homodimeric
isozyme, one Yb homodimeric isozyme, and two Yc-Yb
heterodimeric isozymes. Distribution of the GSH per-
oxidase activity among the CM-cellulose column frac-
tions suggests the existence of further multiplicity in
this isozyme family. Substrate specificity patterns of
each of the five Yc-containing isozymes is composed of a
different homodimeric Yc size subunit composition. Our
findings on the increasing multiplicity of glutathion-
e S-transferase isozymes are consistent with the no-
tion that multiple isozymes of overlapping substrate
specificities are required to detoxify a multitude of
xenobiotics in addition to serving other important
physiological functions.

The glutathione transferases (GST, EC 2.5.1.18) are a
family of dimeric proteins that are multifunctional in drug
biotransformation and in xenobiotic metabolism (see Ref. 1
for a review). Historically, rat hepatic GSTs, which are abun-
dant cytosolic proteins, have been the most extensively stud-
ied. But GSTs from other rat tissues also have been purified
and analyzed (1). The expression of rat GSTs has been shown
to be tissue-specific (2–4). Two major classes of liver subunits,
Yb (M, = 25,600) and Yc (M, = 28,000), are expressed in
kidney GSTs but not in heart, lung, spleen, seminal vesicles,
and testis GSTs (5, 6), while the liver Yc class subunits are
not expressed in kidney GSTs (2, 3). Improved purification
procedures and advanced molecular cloning experiments in
this area have enabled us to develop a concept of the multi-
plicity of GSTs that far exceeds the three-subunit hypothesis
(Ya, Yb, and Yc) proposed by Bass et al. (7). Recent results
from several laboratories, including ours, have revealed that
Yb and Yc each may represent a family of closely related subunits. We have reported on the Yb subunit sequence mi-
icroheterogeneity in the two cDNA plasmids, pGTR281 and
pGTB38 (6, 8). Sheehan and Mantle (9) have reported the presence of a second Yc dimer GST (GST-F) in addition to
the basic Yc, dimer GST (GST-L) (10). Mannervik and Jens-
son (11) have purified three basic Yc-containing GST iso-
zymes that they suggest are products of two different Yc
subunits as two homodimers and one heterodimer. Hayes'
results (12) on peptide fingerprinting and subunit resolution
reconstitution support the proposal that GST-C may be a
heterodimer of GST-D and GST-A.

In this communication, we present results on the multiplicity of Yc- and Yb-containing GSTs from rat liver, and we
suggest that high multiplicity of GSTs with overlapping sub-
strate specificities may be essential to their multiple roles in
xenobiotics metabolism, drug biotransformation, and protec-
tion against peroxidative damage.

EXPERIMENTAL PROCEDURES

Chemicals and Enzyme Reagents—Chemicals and substrates for
GST and GSH peroxidase assays were as described in previous
publications (2, 13).

Enzyme Assays—GSH peroxidase activity was measured spectro-
photometrically by Faglia and Valentine’s procedure (14) as modified
by Reddy et al. (13), with cumene hydroperoxide as the substrate.
The GST activities were determined with 1-chloro-2,4-dinitrobenzene
(CDNB) and other compounds according to published procedures
(15).

Purification of GSTs from Rat Liver Cytosol—Livers (200 g) from
male Wistar rats (~500 g, body weight) were used in this enzyme
preparation. The procedures are those used to purify GSTs from
sheep liver and other rat tissues as published earlier (2, 13), except
that two linear gradients, 0–50 mM KC1 and 50–200 mM KC1, in 10
mM NaP04, pH 6.0, were used in succession to elute the various
isozymes from the CM52 column. The peak fractions of each GST
activity peak against CDNBr were analyzed by NaDodSO4-polyacyr-
lyamide gel electrophoresis as described (16).

RESULTS AND DISCUSSION

Multiplicity of Yc Subunit GST Isozymes—Ten GST activity
peaks are resolvable by the CM-cellulose chromatography with
the 0–50 mM KC1 buffer gradient. They are represented by
fractions 15, 32, 44, 76, 89, 97, 118, 124, 146, and 162 in
Fig. 1 and designated as peaks I–X. An additional isozyme
activity eluted by 50–200 mM KC1 buffer gradient, fraction
210, has been reported as the Yc-subunit GST isozyme of rat
liver (17). From the NaDodSO4-polyacrylamide gel elec-
rophoresis pattern in Fig. 1, fractions 15, 32, 44, 89, and 124
represent isozymes containing Yc size subunit(s). Peak III
(fraction 44) can be further resolved into two homodimeric
isozymes by DEAE-cellulose chromatography: an isozyme of
Yc size subunit(s) and an isozyme of Yb size subunit(s).2 Peak
I isozyme (fraction 15), which we referred to as the anionic
GST isozyme(s), is a mixture of microheterogeneous GST
isozymes because of its asymmetric GSH peroxidase activity
peak. The leading fractions have GST peroxidase activities
against cumene hydroperoxide. The asymmetric pattern remains after subsequent DEAE column
chromatography (18). Two distinct GST peaks with only one

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1 The abbreviations used are: GST, glutathione transferase; CDNB,
1-chloro-2,4-dinitrobenzene; NaDodSO4, sodium dodecyl sulfate;
DCNB, 1,2-dichloro-4-nitrobenzene.

2 C. C. Reddy and C.-P. D. Tu, unpublished results.
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Fig. 1. Resolution of rat liver GST isozymes by CM52 column chromatography. Each GST activity peak fraction was analyzed by NaDodSO4-polyacrylamide gel electrophoresis relative to the three major subunit families (MIX): Yα (Mr = 25,600), Yβ (Mr = 27,000), and Yγ (Mr = 28,000). Fraction 210 has a faster mobility than Yα subunits and is referred to as the Yγ subunit.

containing GSH peroxidase activity, however, were obtained by high performance liquid chromatography on a sulphopropyl cation exchange column. The other three peaks represented by fractions 32 (peak II), 89 (peak V), and 124 (peak VIII) are also isozymes of Yβ subunits. Although the specific activity of fraction 32 against CDNB is intermediate between that of fraction 89 and 124, fractions 89 and 124 are not directly comparable with those of the three “AC type” of GSTs reported by Mannervik and Jensson (11). According to these authors, GST-Aγ has the highest specific activity toward CDNB and DCNB. Among the six isozymes resolved by chromatofocusing between pH 8 and 10, GST-C2 has the lowest activity against CDNB, while GST-AC has intermediate CDNB activity between GST-Aγ and -C2 (11). Activities against DCNB, bromosulfophthalein, trans-4-phenyl-3-buten-2-one, [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxyl] acetic acid (ethacrynic acid), p-nitrophenyl acetate, cumene hydroperoxide, and Δ5-androstene-3,17-dione, however, are not consistent with Mannervik and Jensson’s interpretation that GST-AC is a heterodimer of GST-Aγ and GST-C2 because of additivity of subunit activities.

We would suggest an alternative explanation that each of the isozymes in Peaks II, III, V, and VIII may be a homodimer of different Yβ subunits with similar structural and immunological properties. The peak I (fraction 15) GST is composed of a mixture of isozymes with similar chromatographic properties but very different GSH peroxidase activities. This peak may contain GST-D activities and fractions N1 to N3 as described by Hayes (12). No direct comparisons can be made, however, since Hayes did not report any GSH peroxidase activities for any of his purified GSTs. The proposed Yβ multiplicity in rat liver GST isozymes can be supported by results from different laboratories. For example, reconstitution of urea or guanidine HCl denatured GST-C (presum-
ably a Yb-Yb heterodimer) did not result in a 1:2:1 (Yb1,Yb:Yb2,Yb:Yb1,Yb) ratio of CDNB conjugation activity after CM-cellulose chromatography. Tryptic peptides fingerprinting experiments revealed more peptides for the putative heterodimeric isozyme than the combination of peptides from the two corresponding putative homodimeric isozymes (12, 19). Two-dimensional gel electrophoretic separation of GST subunits revealed at least four spots of Yb size mobility. Their levels of expression are perturbed during chemical hepatocarcinogenesis (20). These results are consistent with our observed Yb subunit multiplicity.

Multiplicity of Yb Family Subunits—The results in Fig. 1 reveal two isozyme peaks of Yb, homodimers (fractions 146 and 162) and at least two Yb-Yb heterodimers (fractions 97 and 118). The Yb size subunits in the two Yb-Yb heterodimeric isozymes may not be of identical electrophoretic mobilities because fraction 110, which is at the common region of peaks VI and VII, has three bands after electrophoresis. Substrate specificity patterns of fractions 76, 97, 118, 146, and 162 in Table I did not reveal additivity of single subunit activities as assumed by Mannervik and Jansson, although the majority of the heterodimeric isozyme activities are intermediates of the Yb and Yb homodimeric isozymes. It is possible that the Yb and Yb subunits in the heterodimeric and homodimeric isozymes may not be identical.

The two Yb homodimeric isozymes have different substrate specificity patterns. For example, peak IX (fraction 146) has higher activities than Peak X (fraction 162) against CDNB, ethacrynic acid, and t-butyl hydroperoxide, but lower activities than Peak X against DCNB, 1,2-epoxy-3-(p-nitrophenoxyl)propane, 4-nitropyridine N-oxide, Δ4-androstene-3,17-dione, cumene hydroperoxide, and linoleic acid hydroperoxide. They are equally active against p-nitrobenzyl chloride and p-nitrophenyl acetate. Our resolution of two homodimeric Yb isozymes and two heterodimeric Yb-Yb isozymes may also explain the CDNB activity patterns in chromatofocusing separation observed by Mannervik and Jansson (11). They did not show the corresponding protein profile probably due to peptides used for elution. But, their peak III (GST-B1), designated as Yb,Yb homodimer, is unusually broad and contains Yb size subunit as shown in their NaDodSO4-gel electrophoresis pattern. We suggest that the broadness of their peak III is consistent with the existence of multiple isozymes and that their peak III may contain a second Yb,Yb heterodimeric isozyme and a second homodimeric Yb isozyme in addition to the assigned GST-B2 (or GST-AA according to Jakoby (21)).

The substrate specificity patterns of the three "B.L type" isozyme showed more deviation from the assumption of subunit activity than the corresponding A.C type isozymes. This is again consistent with our observed isozyme multiplicity. Our CM-cellulose chromatographic pattern of liver GST isozymes may be compared with Jakoby et al.'s (21) scheme of six isozymes, E, D, C, B, A, and AA, according to their order of elution of the CM-cellulose column. Recently, Meyer et al. (22) reported that GST-E (another Yb-containing isoyme) did not bind to the S-hexyl-GSH-linked Sepharose-4B column. Therefore, we should not have GST-E in Fig. 1. The GST-D could be contained in Peaks I, II, or III, and Peak IV (Yb,Yb) could represent GST-Ld (11) or GST-L (10), an isozyme not included in Jakoby et al.'s original six isozymes. Our observed pattern fits that of Jakoby et al. with the addition of one extra Yb,Yb heterodimer and one extra Yb homodimeric isozyme. In other words, fraction 89 should be at the position of GST-C; peak fraction 97 and/or 118 should be GST-B; peak fraction 124 should be GST-A; and peak fraction 146 and/or 162 should be GST-AA. Fraction 210 (Yb subunit) represents a new isozyme not reported by Jakoby or any other laboratory. Due to the relatively constant distribution of homodimeric and heterodimeric Yb-Yb-containing isozymes and considerable deviation from additivity of subunit activities, we cannot rule out the possible nonidentity of Yb (and Yb) in homodimeric and heterodimeric isozymes.

**Further Multiplicity of Rat Liver GSTs—**Since the GSTs are more than 95% pure after the S-hexyl-GSH affinity column chromatography as judged by their NaDodSO4-gel electrophoresis patterns, subsequent resolution on a CM-cellulose column should be considered a more efficient resolution procedure than chromatofocusing. In addition to the 11 major activity peaks, several fractions in Fig. 1 suggest the existence of minor isozymes or isozymes not completely resolved by the current condition. Two such examples can be identified around fraction 65 and between fractions 100 and 110. The former contains both GST and GSH peroxidase activities, but the latter fractions contain only GST activity against CDNB. Some other isozymes could be "hidden" be-

### Table I

**Substrate specificity of the rat liver glutathione S-transferases**

Values are expressed as specific activity (μmol/min/mg of protein). Numbers in parentheses are fraction numbers.

| I      | II     | III    | IV     | V      | VI     | VII    | VIII   | IX     | X      | XI     |
|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
| Yb     | Yb     | Yb     | Yb     | Yb     | Yb     | Yb     | Yb     | Yb     | Yb     |
|        |        |        |        |        |        |        |        |        |        |        |
| 10.15  | 25.18  | 17.20  | 1-Chloro-2,4-dinitrobenzene | 28.45 | 35.90  | 24.50  | 29.15  | 20.13  | 24.63  | 13.11  | 1.80   |
| 0.35   | 1.12   | 1.13   | 1,2-Dichloro-4-nitrobenzene | 0.53  | 1.83   | 0.27   | 0.36   | 2.30   | 0.09   | 0.14   | 0.06   |
| 0.59   | 0.27   | 0.00   | 1,2-Epoxy-3-(p-nitrophenoxyl)propane | 0.21  | 0.35   | 0.12   | 0.53   | 0.15   | 0.00   | 1.20   | 0.40   |
| 1.35   | 1.80   | 0.90   | p-Nitrobenzyl chloride | 0.92  | 1.39   | 0.29   | 0.42   | 0.81   | 0.30   | 0.32   | 0.01   |
| 0.63   | 0.24   | 0.21   | 4-Nitropyridine N-oxide | 0.07  | 0.22   | 0.33   | 0.73   | 0.38   | 0.05   | 0.08   | 0.05   |
| 0.03   | 0.08   | 0.40   | Δ4-Androstene-3,17-dione | 1.82  | 0.05   | 1.03   | 0.59   | 0.06   | 0.03   | 0.06   | 0.05   |
| 0.01   | 0.06   | 0.07   | Bromosulfophthalein | 0.23  | 0.00   | 0.06   | 0.09   | 0.00   | 0.00   | 0.00   | 0.00   |
| 0.37   | 0.11   | 0.09   | Ethacrynic acid | 0.49  | 0.06   | 0.58   | 0.99   | 0.14   | 1.15   | 0.30   | 0.34   |
| 0.14   | 0.31   | 0.48   | p-Nitrophenyl acetate | 0.48  | 0.32   | 0.26   | 0.27   | 0.41   | 0.16   | 0.18   | 0.18   |
| 0.02   | 0.33   | 0.40   | Cumene hydroperoxide | 3.11  | 0.37   | 5.90   | 8.00   | 0.89   | 8.90   | 11.90  | 0.12   |
| 0.06   | 0.09   | 2.50   | t-Butyl hydroperoxide | 0.94  | 0.32   | 1.23   | 2.15   | 0.09   | 2.33   | 1.36   | 0.05   |
| 0.08   | 0.05   | 0.58   | Linoleic acid hydroperoxide | 3.06  | 0.09   | 1.58   | 1.75   | 0.18   | 0.76   | 1.22   | 0.07   |
neath the many partially resolved peaks between fractions 76 and 140 in Fig. 1.

The protein purification and substrate specificity patterns presented here, together with results from many other laboratories, suggest a much higher multiplicity of liver GST isozymes than previously conceived. We can classify liver GST subunits into at least four classes, \( Y_a, Y_b, Y_c, \) and \( Y_d \), based upon the electrophoretic mobilities observed by Na-DodSO\(_4\)-polyacrylamide gel electrophoresis. The cDNA cloning results have probably revealed a level of microheterogeneity not resolvable by conventional protein purification techniques (5, 8). Considering the non-liver GST subunits that are tissue-specific, the multiplicity of GST isozyme subunits may well approach that of other drug-metabolizing enzymes. Each organism has to cope with many environmental xenobiotics and has to evolve adequate defense mechanisms. Being a conjugation isozyme family, GSTs have been classified as one of the phase II detoxification enzymes. Each of the GST isozymes has a limited substrate specificity pattern against many synthetic chemicals such as CDNB, DCNB, etc. Therefore, it is essential to have evolved a large number of different subunits with partially overlapping substrate specificity patterns for efficient metabolism of xenobiotics.

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