Expression of an Enzymatically Active \( Y_{b3} \) Glutathione S-Transferase in *Escherichia coli* and Identification of Its Natural Form in Rat Brain*

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Glutathione S-transferases containing \( Y_{b3} \) subunits are relatively uncommon forms that are expressed in a tissue-specific manner and have not been identified unequivocally or characterized. A cDNA clone containing the entire coding sequence of \( Y_{b3} \) glutathione S-transferase mRNA was incorporated into a plasmid expression vector to transform *Escherichia coli*. A fusion \( Y_{b3} \)-protein containing 14 additional amino acid residues at its N terminus was purified to homogeneity. Recombinant \( Y_{b3} \) was enzymatically active with both 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene as substrates but lacked glutathione peroxidase activity. Substrate specificity patterns of recombinant \( Y_{b3} \) were more limited than those of glutathione S-transferase isoenzymes containing \( Y_{b1} \)- or \( Y_{b2} \)-type subunits. Peptides corresponding to unique amino acid sequences of \( Y_{b3} \) as well as a peptide from a region of homology with \( Y_{b1} \) and \( Y_{b2} \) subunits were synthesized. These synthetic peptides were used to raise antibodies specific to \( Y_{b3} \) and others that cross-reacted with all \( Y_{b} \) forms. Immunoblotting was utilized to identify the natural counterpart of recombinant \( Y_{b3} \) among rat glutathione transferases. Brain and testis glutathione S-transferase isoforms were rich in \( Y_{b3} \) subunits, but very little was found in liver or kidney. Physical properties, substrate specificities, and binding patterns of the recombinant protein paralleled properties of the natural isoenzyme isolated from brain.

Glutathione S-transferases (EC 2.5.1.18) catalyze the reaction of glutathione with a variety of electrophiles (1-3) and function in intracellular transport of hormones, metabolites, and xenobiotics (4-6). In the rat, subunits \( Y_{a} \), \( Y_{b} \), and \( Y_{c} \) (a class), \( Y_{b} \) (\( \mu \) class), and \( Y_{b} \) (\( \tau \) class) are encoded by a supergene class, and \( Yb \) cDNAs have been cloned and sequenced (7, 8). In the \( \mu \) family, \( Y_{\mu A} \), \( Y_{\mu D} \), and \( Y_{\mu B} \) cDNAs have been cloned and sequenced (7, 12-14). Homogeneous proteins are often difficult to resolve among subpopulations of multiple glutathione S-transferase isoenzymes present in most tissues (15-17). \( Y_{b3} \) is a relatively uncommon mRNA that is present at low levels in liver and other tissues but is a predominant transcript in brain (14, 18). In this report, an enzymatically active \( Y_{b3} \) form expressed in *Escherichia coli* is characterized and its counterpart in brain identified.

**Experimental Procedures**

Construction of \( pY_{b3} \) Vector—A 0.93-kilobase EcoRI restriction enzyme fragment, which included 18 nucleotides upstream of the site of initiation of translation, the entire coding region (654 nucleotides), and part of the 3' untranslated region (261 nucleotides) of a previously characterized \( Y_{b3} \) glutathione S-transferase cDNA (14), was ligated into the EcoRI site of the expression vector pBluescript IIISK (pBluescript IIISK, Fig. 1). The expression vector contains an lpp promoter followed by the lac promoter-operator sequence and three convenient restriction sites (19) for insertion of foreign DNA sequences. The 7.4-kilobase vector also contained the gene for ampicillin resistance and the lacI gene. Isopropyl-\( \beta \)-D-thiogalactopyranoside was used to induce the transcription of \( Y_{b3} \) driven by the lpp promoter. E. coli (W620 recA) was transformed with the \( Y_{b3} \) cDNA vector constructs.

Transformants in which the \( Y_{b3} \) cDNA had been inserted in the correct orientation were then selected on the basis of restriction enzyme analysis of the plasmid DNA, by immunoblot analysis of the proteins produced by the E. coli using anti-\( \alpha Y_{b} \) antisera, and by measurement of glutathione S-transferase enzymatic activity.

**Purification of Recombinant \( Y_{b3} \)—** A 20-ml overnight culture of the transformed E. coli grown in LB medium with ampicillin (40 \( \mu \)g/ml) at 37°C was used to inoculate 1 liter of the LB medium. The culture was grown (to an \( A_{600} \) of 0.6) in an incubator at 37°C. The cells were then centrifuged at 1000 \( \times \) g for 10 min at 4°C; resuspended in 100 ml of 2 \( \times \) LB medium, 10 \( \mu \)m isopropyl-\( \beta \)-D-thiogalactopyranoside; and incubated with shaking at 37°C for an additional 16 h. The cells were harvested, resuspended in 20 ml of E. coli lysing buffer (20 \( \mu \)m Tris-HCl (pH 8.0), 1 \( \mu \)m dithiothreitol, 1 \( \mu \)m EDTA, 5% (v/v) phenylmethylsulfonyl fluoride) (20), and sonicated. The cellular debris was pelleted (1000 \( \times \) g for 10 min at 4°C), and the DNA was then precipitated from the supernatant by addition of streptomycin sulfate (2%) with stirring at 4°C for 15 min. The supernatant was used to purify the protein.

Homogeneous preparations of \( Y_{b3} \) were obtained using two chromatographic steps. The soluble cell extract in 20 \( \mu \)m Tris-HCl (pH 8.0) was applied to a Sephadex G-100 column (2.5 \( \times \) 90 cm) and eluted with the same buffer. Fractions containing glutathione S-transferase activity were applied to a column (2.7 \( \times \) 50 cm) of DEAE-Sephacel in 20 \( \mu \)m Tris-HCl (pH 8.0); the column was then washed with buffer and eluted with a NaCl gradient (0-200 mm). Most of the glutathione S-transferase activity was eluted as a sharp peak at NaCl concentrations of about 60 mm. That component contained pure \( Y_{b3} \) (see Fig. 2). A typical purification scheme is outlined in Table I.

Glutathione S-transferases were purified from rat brain (18), testis, and liver (21, 22) by procedures described previously.

**Sequence Analysis—** \( Y_{b3} \) was treated with 3 \( \mu \)l HCl at 37°C for 2 h to remove the formyl substituent before sequencing (23). The lyophilized product was subjected to amino acid sequence analysis by an automated Edman degradation procedure using an Applied Biosystems Model 470-A Gas-Phase Sequencer. Phenylthiohydantoin-derivatives were identified using a Waters HPLC system with an Altex Ultrasphere 250 \( \times \) 4.6-mm C18 reverse-phase column (24).

**Peptide Synthesis—** Oligomeric peptides corresponding to sequences of \( Y_{b3} \) glutathione S-transferase (14) were synthesized by solid-phase methods using a Vega 250 module peptide synthesizer. p-

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The abbreviations used are: \( rY_{b3} \) recombinant \( Y_{b3} \)-type subunits of glutathione S-transferase synthesized in E. coli; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; HPLC, high pressure liquid chromatography; SDS, sodium dodecyl sulfate.
**Expression of Brain Glutathione Transferase in E. coli**

**TABLE I**

<table>
<thead>
<tr>
<th>Purification of Yb-glutathione S-transferase from E. coli</th>
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</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>E. coli extract</td>
</tr>
<tr>
<td>Gel permeation chromatography (G-100)</td>
</tr>
<tr>
<td>DEAE-Sepacel</td>
</tr>
<tr>
<td>Breakthrough fraction</td>
</tr>
<tr>
<td>GST* major peak (60 mM NaCl)</td>
</tr>
<tr>
<td>GST minor peak (70-80 mM NaCl)</td>
</tr>
</tbody>
</table>

* GST, glutathione S-transferase.

Hydroxymethyl polystyrene resin was used for the solid support and the symmetrical anhydride procedure employed for the coupling reactions, and deblocking was performed with anhydrous liquid HF. Peptides were purified by preparative HPLC using a C18 µBondapak column; purity was also determined by HPLC analysis.

**Circular Dichroism**—Spectra were obtained using a Cary Model 60 spectropolarimeter with a 6001 CD attachment. Measurements in the 260–200 nm spectral region were made at 27 °C using 1-cm path length cells and protein concentrations of 0.05–0.2 mg/ml in 10 mM sodium phosphate (pH 7.4). Bilirubin binding data in the spectral region between 520 and 400 nm were obtained with 1.0 mM glutathione S-transferase in 10 mM sodium phosphate with additions of 1.0-µl aliquots of 10−6 M bilirubin solution.

**Antisera**—The synthetic peptides containing cysteine residues at their N termini were coupled to keyhole limpet hemocyanin by the method of Liu et al. (25). New Zealand White rabbits were immunized with the peptide-keyhole limpet hemocyanin conjugates (1.0–2.0 mg) in Freund’s complete adjuvant, with equal portions injected over scapulae and backs. Booster injections were administered after 2 weeks and again after 4 and 6 weeks. The rabbits were bled and sera obtained after 8 and 10 weeks.

**Enzymatic Assays**—Measurements of enzymatic activity with 1.0 mM GSH and 1.0 mM CDNB or 1.0 mM DCNB as substrates were performed according to the method of Jensson et al. (26). Other substrates were assayed in the presence of 1.0–5.0 mM GSH under conditions described by Jensson et al. (26).

**Immunoblotting**—Proteins from 10% SDS-polyacrylamide slab gels were transferred electrophoretically to nitrocellulose membranes (Bio-Rad Trans-Blot) in 25 mM Tris-HCl, 192 mM glycerol (pH 8.3) with 20% (v/v) ethanol for 12 h (0.1 A, 2.5 watts). The nitrocellulose was blocked with 3% (w/v) bovine serum albumin in 50 mM Tris-HCl (pH 7.9) containing 0.15 M NaCl and 0.5% Tween 20 for 2 h; the membranes were incubated with the specific peptide antisera described above and 3% (w/v) albumin in the same buffer for 1 h. The blot was then incubated with 125I-protein A (106 cpm) in 3% (w/v) albumin for 1 h, washed with the same buffer, and prepared for autoradiography.

**RESULTS**

**Characterization of Recombinant Protein**—The rYb-glutathione S-transferase subunits purified from bacteria by GSH affinity or anion-exchange chromatography yielded a single component with an electrophoretic mobility on SDS-polyacrylamide gels similar to that of other liver Yb forms (Yb3 and Yb2) (Fig. 2A). The rYb3 band was also prominent among the proteins of the bacterial extract (Fig. 2A), but neither glutathione S-transferase enzymatic activity nor immunoreactive Yb subunits were detected in transformants contain-
ing the plasmid with the Yb3 cDNA in a reverse orientation. The purified recombinant protein was homogeneous in isoelectric focusing (pI 6.5) and two-dimensional gel electrophoresis (Fig. 2B). Sequence analysis showed that rYb3 was a fusion protein with a blocked N-terminal residue and 14 additional amino acids at its N terminus (Fig. 1). The results confirmed a predicted amino acid sequence of rYb3 (deduced from the known nucleotide sequence) that included the first 4 amino acids coded for by the expression vector (Met-Lys-Gly-Lys-), Glu-Phe from the EcoRI site, and Gly from the original cDNA construction, followed by 18 nucleotides of the Yb3 untranslated sequence coding for Arg, Ser, Asp, Pro, Ser, Thr, and the methionine (14) (Fig. 1). rYb3 was eluted as a dimer by gel permeation and ion-exchange chromatography (see "Experimental Procedures"). An isoelectric focusing (IEF) gel (5% acrylamide) of the first dimension is shown at the top adjacent to pI markers (7.35, 6.85, 6.50, and 5.85 bands from left to right). An isoelectric focusing gel was applied to an SDS slab gel in the second dimension (12% acrylamide), and the gel was stained with Coomassie Blue. The same molecular weight markers in A (lane 1) are shown on the left of the slab gel.

**TABLE II**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(Yb1)</th>
<th>(Yb2)</th>
<th>(Yb3)</th>
<th>(rYb3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/min/mg</td>
<td>pmol/min/mg</td>
<td>pmol/min/mg</td>
<td>pmol/min/mg</td>
</tr>
<tr>
<td>CDNB</td>
<td>27.5</td>
<td>31.0</td>
<td>27.2</td>
<td>100.6</td>
</tr>
<tr>
<td>DCNB</td>
<td>1.6</td>
<td>1.8</td>
<td>0.7</td>
<td>10.9</td>
</tr>
<tr>
<td>TPBO*</td>
<td>0.18</td>
<td>0.49</td>
<td>0.41</td>
<td>ND</td>
</tr>
<tr>
<td>BSP</td>
<td>0.25</td>
<td>0.17</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td>Δ5-Aandrostone-3,17-dione</td>
<td>0.07</td>
<td>0.08</td>
<td>0.05</td>
<td>ND</td>
</tr>
<tr>
<td>Ethacrynic acid</td>
<td>0.05</td>
<td>0.17</td>
<td>0.2</td>
<td>0.48</td>
</tr>
<tr>
<td>Comene hydroperoxide</td>
<td>1.7</td>
<td>1.2</td>
<td>3.5</td>
<td>ND</td>
</tr>
</tbody>
</table>

*TPBO, trans-4-phenylbutyl-3-ene-2-one; BSP, sulfobromophthalain; ND, not detected.

Expression of Brain Glutathione Transferase in E. coli

**FIG. 2.** Electrophoretic properties of recombinant glutathione S-transferase. A, SDS-polyacrylamide gel electrophoresis in 12% acrylamide gels stained with Coomassie Blue. Lane 1, molecular weight markers; lane 2, proteins of the E. coli extract; lane 3, rYb3 purified from E. coli; lane 4, liver glutathione S-transferases containing Yb Ym and Yb subunits. B, two-dimensional gel electrophoresis of rYb3 purified from E. coli by gel permeation and ion-exchange chromatography (see "Experimental Procedures"). An isoelectric focusing (IEF) gel (5% acrylamide) of the first dimension is shown at the top adjacent to pI markers (7.35, 6.85, 6.50, and 6.85 bands from left to right). An isoelectric focusing gel was applied to an SDS slab gel in the second dimension (12% acrylamide), and the gel was stained with Coomassie Blue. The same molecular weight markers in A (lane 1) are shown on the left of the slab gel.

**FIG. 3.** Amino acid sequences of peptides used to raise antibodies. Peptides were synthesized by methods described under "Experimental Procedures." The indicated sequences correspond to amino acid sequences of Yb3 (14) that are homologous with Yb1 and Yb2 subunits (residues 43–66) or specific for Yb3 (residues 121–136 and 202–217). Sequences of Yb1, Yb2, and Yb3 are shown for comparison.
bands of opposite signs and with large rotational strengths (4, 21, 27). These are exemplified by spectra obtained for bilirubin complexes with liver glutathione S-transferase isoenzymes containing Yb1 and Yb2 subunits (Fig. 5). In contrast, the spectrum for glutathione S-transferase with Yb3 subunits featured a lower intensity single broad positive ellipticity band centered near 455 nm. Similar spectra were previously obtained with glutathione S-transferases lacking primary high affinity binding sites (29).

**DISCUSSION**

An enzymatically active glutathione S-transferase consisting of Yb3 subunits has been produced in *E. coli* by recombinant DNA methods. The rYb3 subunits dimerized spontaneously, and circular dichroism spectra showed that the secondary structure of the dimer was similar to that of other glutathione S-transferases containing Yb-type subunits (27). Since mammalian glutathione S-transferases do not undergo post-translational processing, the other properties of the fusion protein expressed in *E. coli* are probably representative of those of the natural rat protein. The 14 additional amino acid residues at the N terminus of rYb3 do not suppress its enzymatic activity (Table II); in fact, its activities with DCNB and CDNB as substrates were more than 3 times greater than those of the other Yb glutathione S-transferases. The isoelectric point of the recombinant fusion protein (pI 6.5), which contains 3 additional basic residues counterbalanced by 2 acidic residues and a blocked N terminus, was higher than that of natural Yb3 from brain (pI 6.0).

To distinguish between rYb3 and Yb3 or Yb2 subunits and to identify its counterpart among brain glutathione S-transferases, Yb3-specific antisera were produced on the basis of its unique peptide sequences (Fig. 3). Yb3 is a minor glutathione S-transferase form in liver (which is rich in Yb1 and Yb2 mRNA), but is a predominant glutathione S-transferase transcript in brain and is also found in testis and heart (14, 18). Although mRNAs for this glutathione S-transferase have been characterized, it was not clear until now which previously identified protein subunit corresponded to Yb3. Earlier SDS-polyacrylamide gel electrophoresis of glutathione S-transferase isoenzymes of rat brain resolved two components in the Yb region (18, 30–33). The faster moving band, previously designated as Yb4 or Yb5 (30–33), is now identified as Yb3 (Fig. 4). Hayes (34) reported that Yb4 from testis cross-reacted with anti-Yb antisera. Likewise, anti-Yb antisera raised against testicular Yb1-type subunits also cross-reacted with liver Yb-type subunits (32). The specific antisera used in this study clearly distinguish between the faster moving Yb3 component and the Yb5 component with electrophoretic mobility similar to that of liver Yb4, which is largely Yb3, in brain (14) (Fig. 4). The presumptive Yb3 glutathione S-transferase (Yb3) protein was isolated from rat brain (Fig. 4), and its properties were found to resemble those of rYb3; this was true particularly with regard to its lack of high affinity bilirubin binding capacity (Fig. 5) and its substrate specificity patterns (Table II and Ref. 32).

The particular properties of glutathione S-transferase isoenzymes containing Yb3 subunits may be related to the functions of those subunits in tissues in which they occur specifically. Brain and testis are rich in Yb3, whereas liver, which has the highest overall glutathione S-transferase activity, has very low levels of Yb3. Accordingly, this form lacks the high affinity bilirubin binding capacity associated with the liver glutathione S-transferases containing Yb1, Yb5, and Yb-type subunits (21, 27–29). rYb3 showed no glutathione S-transferase/peroxidase activity, and its substrate specificity was more limited than that of either Yb4 or Yb5 forms of liver (Table II). Indeed, the major GSH peroxidase activity of rat brain, where Yb3 predominates, is associated with the selenium-dependent enzymes rather than with glutathione S-transferases (35, 36).

Enzymatically active maize (37) and parasitic (38) as well as human (39) glutathione S-transferases have recently been expressed in *E. coli*. Earlier kinetic and structural analyses
have frequently been confusing because of cross-contamination of closely related glutathione S-transferase isoenzymes (15). The availability of glutathione S-transferases from recombinant sources will permit preparation of large quantities of homogeneous protein to facilitate additional structural studies and allow mutagenic experiments to define catalytic and binding sites of glutathione S-transferases that remain obscure at present. For $V_{max}$, earlier observations that glutathione S-transferases with $V_0$ subunits of brain are localized in astrocytes and ependymal cells and the proposal that they function in the uptake, transport, and detoxication of certain hormones, drugs, and neurotransmitters (13) may now be approached from a molecular standpoint.

REFERENCES