Purification and Properties of Hamster Liver Ligandins, Glutathione S-Transferases

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ABSTRACT

Glutathione S-transferases have been purified to homogeneity from Chinese hamster liver. Three enzyme forms were separated and designated Forms I, II, and III in order of their elution from carboxymethylcellulose columns. The forms exhibit close physical similarities to glutathione S-transferases B (ligandin) of rat liver and ε of human liver. However, enzyme kinetic analysis indicates that the hamster enzymes exhibit similar $K_m$ values but higher $V_{max}$ values towards common substrates compared with the rat and human forms. These differences, which explain the increased enzymic activities of hamster glutathione S-transferases in vivo and in vitro, appear to be related to slight differences in the peptide composition of hamster liver glutathione S-transferases compared to the rat and human enzymes.

INTRODUCTION

The glutathione S-transferases are multifunctional enzymes (2, 17) that catalyze the conjugation of glutathione with a wide range of electrophilic compounds (3, 10, 21). Several rat liver glutathione S-transferase forms, notably transferase B, bind a wide range of cellular metabolites in vivo, including several chemical carcinogens (13, 19, 20).

Six transferases have been separated from rat liver and designated transferases E, D, C, B, A, and AA in order of their elution from carboxymethylcellulose columns (10). Transferase B is ligandin (9). The transferases show broad and overlapping substrate specificities and similar physical properties (10). In human liver, 5 transferases have been distinguished electrophoretically and designated transferases α, β, γ, δ, and ε on the basis of increasing isoelectric point (11). The spectrum of substrates is the same for each enzyme with, however, slightly different specific activities.

Ligandin and other rat liver glutathione S-transferases form glutathione conjugates of several polycyclic aromatic hydrocarbons and azodyes and bind several hydrocarbons and azodyes, noncovalently or covalently (for reviews, see Refs. 19 and 20). These characteristics suggest that the glutathione S-transferases may play an important part in cellular detoxification of chemical carcinogens (12, 19, 20). The mechanisms by which such detoxification might occur is an area of ongoing research (1, 7, 19).

Hamster liver exhibits much higher specific enzymatic activity than do human and rat liver, and hamster-derived cells in culture express more enzyme than do lines derived from tissues of other animals (18). This report describes the purification of 2 major glutathione S-transferase forms from hamster liver and a comparison of the properties of the forms with those from human and rat liver. The data suggest that certain fundamental differences between the hamster transferases and those of other species may account for alterations in enzyme activities towards common substrates.

MATERIALS AND METHODS

Enzyme Assays

Glutathione S-transferase activity was measured using DNCB or DCNB as substrate, as described by Habig et al. (10).

SDS Electrophoresis

Proteins were subjected to electrophoresis in slab gels containing a 10 to 20% polycrylamide gradient with SDS/Tris/glycine buffer as described previously (5). Proteins were stained with Coomassie Brilliant Blue.

Acid/Polyacrylamide Gel Electrophoresis

Electrophoresis was performed with 7.5% polyacrylamide and 3% stacking gel in 8.0- x 0.9-cm cylindrical gels. The gel buffer was acetic acid/Na$_2$CO$_3$/polyacrylamide 1:1:10 molar ratio in 0.001 M glycine buffer, pH 4.6, and the running buffer was acetic acid/Na$_2$CO$_3$/polyacrylamide 3:3:10 molar ratio in 0.02 M glycine buffer, pH 4.9. Electrophoresis was performed at 4° and 1 to 5 V/cm for 16 to 24 hr. The electrophoresis tracking dye was 5% basic fuchsin (Fisher Scientific, King of Prussia, Pa.). Proteins were stained with Coomassie Brilliant Blue R-250.

Enzyme Purification

Hamster. Twenty-one male Chinese hamsters (Chickline, Vineland, N.J.) were killed by cervical fracture, and the livers were placed in 0.9% NaCl solution. This and subsequent purification procedures were performed at 0 to 4°. Twenty-eight g of liver were homogenized in 3 volumes of 50 mm Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose. The homogenate was centrifuged for 1 hr at 9000 x g to remove debris, and the supernatant fraction was centrifuged for 1 hr more at 100,000 x g.

The supernatant fraction was loaded onto a 3- x 41-cm column of DE-52 cellulose (Whatman, Clifton, N.J.) equilibrated previously with 10 mm Tris-HCl, pH 7.5, and washed with 1.5
bed volumes of this buffer. A gradient of 0 to 1.0 M KCl in 10 mm Tris-HCl, pH 7.5, was applied to the column. All of the glutathione S-transferase activity recovered was eluted with the wash buffer.

The wash fractions from the DE-52 column were pooled, and ammonium sulfate was slowly added to a concentration of 660 g per 1000 ml of solution. After equilibration for 60 min, the resulting precipitate was dissolved in 25 ml of 10 mm potassium phosphate, pH 6.7 (Buffer A) and dialysed against a 150-fold volume of Buffer A with 3 changes.

The dialysate was loaded onto a 2- x 50.5-cm column of CM-50 equilibrated previously with Buffer A and washed with 1 bed volume of this buffer. A gradient of 0 to 0.15 M KCl in Buffer A was then applied to the column. The elution profiles of glutathione S-transferase activity towards DNCB and DCNB are shown in Chart 1. Three peaks of activity towards both DNCB and DCNB are apparent. These forms have been labeled Forms I, II, and III in order of their elution from CM-50.

Forms II and III, which represent the majority of the transferase activity from CM-50, were further purified. Each form was chromatographed on a 2- x 53-cm column of Sephadex G-100 equilibrated with 10 mm potassium phosphate buffer, pH 6.7. The fractions containing glutathione S-transferase activity were concentrated to 3 ml by using an Amicon ultrafiltration chamber and PM-10 membrane. Each preparation was then loaded onto separate 100 ml isoelectrofocusing columns (LKB, Stockholm, Sweden), pH range from 3.5 to 10.0, and focused at 100 V for 17 hr and then at 300 V for 72 hr. The column was then fractionated, and the fractions were assayed for glutathione S-transferase activity. The isoelectrofocusing profiles for Forms II and III are shown in Chart 2, A and B, respectively. Each form appeared as a single symmetrical peak with pl of 9.0.

The fractions containing glutathione S-transferase activity were combined and concentrated by Amicon ultrafiltration. Ampholines were then removed by chromatographing Forms II and III separately on a 2- x 48-cm Sephadex G-75 column equilibrated previously with 10 mm potassium phosphate buffer, pH 6.7, containing 2 mm glutathione, 0.1 M EDTA, and 10% glycerol. The glutathione S-transferase activity appeared as a single symmetrical peak for each preparation (not shown).

The preparations of Forms II and III were each concentrated to 1 mg/ml by Amicon ultrafiltration and stored at -80°.

**Rat and Human.** Glutathione S-transferase B or ligandin from rat livers and human glutathione S-transferase ε were purified to homogeneity on SDS-polyacrylamide electrophoresis using identical procedures to that described for hamster liver (not shown). The rat and human enzymes were identified on the basis of isoelectric points, which were pl 9.0 and 8.8, respectively.

**Tryptic Digestion and Peptide Mapping**

The purified glutathione S-transferase preparations were separately subjected to tryptic peptide mapping by a modification of the procedure of Nochumson et al. (15). The purified protein (0.5 mg in 500 μl of Buffer A) was denatured and precipitated by the addition of 900 μl of 95% ethanol and standing at room temperature for 24 hr. After centrifugation and washing in ethanol, the pellet was air dried and redissolved in 10 mM phosphate, pH 8.1. To this solution was added trypsin (Boehringer, New York, N.Y.) at a ligandin/trypsin molar ratio of 50/1, and the mixture was shaken at 37° for 48 hr. An equivalent amount of trypsin was added at 7, 19, 26, and 43 hr. Initially, undissolved ligandin was dissolved with this trypsin treatment. After digestion, the mixture was frozen in liquid nitrogen and freeze dried. The dried sample was dissolved in 20 μl of water and spotted onto a 10- x 20-cm plate of activated (100°, 60 min) silica gel. The gel was subjected to 2-dimensional chromatography with the first dimensional solvent, butanol/acetic acid/pyridine/H₂O (15/3/10/2, v/v), and the second dimensional solvent, butanol/acetic acid/H₂O (200/30/75, v/v). The plates were developed with ninhydrin.

**Antiserum Preparation**

Antisera to purified rat liver ligandin, purified human glutathione S-transferase ε, and purified hamster liver glutathione S-transferase II, pl 9.0, were prepared in male New Zealand White rabbits by the method of Daniel et al. (5) and purified by the method of Livingston (14). Ouchterlony double-diffusion analysis has been described previously (16).
RESULTS AND DISCUSSION

Habig et al. (8) have shown a marked similarity of amino acid composition within the glutathione S-transferases of human and rat liver and also between these 2 species.

The hamster liver glutathione S-transferases II and III have identical pI values of 9.0 (Chart 2) which are very similar to those of rat liver ligandin, pI 9.0, and human glutathione S-transferase ε, pI 8.8 (not shown).

The data of Table 1 indicate that a highly purified monospecific antibody to purified rat liver ligandin (5) cross-reacts with purified rat liver ligandin, human liver glutathione S-transferase ε, and hamster liver glutathione S-transferases II and III. Antiserum to the human and hamster liver forms do not show such wide cross-reactivity (Table 1). Fleischner et al. (6) have not observed such cross-reactivity between the glutathione S-transferases of different species. A mixture of the purified proteins from each species exhibit identical mobility in 7.5% acida/polyacrylamide gels (not shown). These results suggest that the proteins from different species possess considerable physical similarity.

A summary of selected kinetic constants for transferases II and III from hamster liver is presented in Table 2. The apparent Km values towards DNCB and DCNB are similar to the constants for purified rat liver ligandin (10); however, the hamster forms show substantially higher Vmax values towards these substrates and may explain the higher rates of enzyme activity reported for hamster liver glutathione S-transferases (18).

In view of the physical similarities between the transference forms of human, rat, and hamster liver that are demonstrated above, further work was performed in order to determine differences in the proteins which might result in altered catalytic activities towards common substrates.

Table 1

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Rat</th>
<th>Human</th>
<th>Hamster</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiserum</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antirat</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Antihuman</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Antihamster</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</table>

* Forms II and III.

Table 2

<table>
<thead>
<tr>
<th>Transferase</th>
<th>II</th>
<th>III</th>
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</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Vmax (μmol/min/mg)</td>
<td>Apparent Km (mM)</td>
</tr>
<tr>
<td>DNCB</td>
<td>86.8</td>
<td>0.33</td>
</tr>
<tr>
<td>DCNB</td>
<td>4.3</td>
<td>1.11</td>
</tr>
<tr>
<td>Glutathione</td>
<td>4.3</td>
<td>0.20</td>
</tr>
</tbody>
</table>

Chart 3 shows a 2-dimensional tryptic map of hamster glutathione S-transferases II and III. The forms show similar peptide composition with only one apparent peptide difference. In addition, Chart 4 indicates a similarity between the peptide maps of the hamster glutathione S-transferases II and III (Chart 3) and those of the rat and human liver forms. The proteins from the latter 2 species show a slightly more complex peptide pattern which, however, share many common features with each other and with the hamster forms. These maps are very different from that obtained with cytochrome C (not shown).

The data suggest similarity in the physical properties of the proteins from different species with, however, minor differences in peptide composition. These differences may reflect alterations in the enzyme which results in increased activity of the hamster enzyme forms to common substrates such as DNCB and DCNB compared with the enzymes from rat and human liver. A more exact definition of the differences in primary structure of the hamster liver glutathione S-transferase forms will require more detailed study than is described here. Meanwhile, systems such as hamster and mouse liver transferases which exhibit different enzyme kinetics compared with...
other species (18) may contribute to studies of the interaction between the binding and catalytic sites of the glutathione S-transferase proteins (4). Furthermore, these systems may be useful in studies of the roles of the glutathione S-transferases in the detoxification of chemical carcinogens (1, 7, 12, 19).

REFERENCES


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