Specificity of Isozymes of Murine Hepatic Glutathione S-Transferase for the Conjugation of Glutathione with L-Phenylalanine Mustard

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ABSTRACT

Glutathione S-transferase (GST) isozymes play a central role in the protection of cells from cytotoxic chemicals and have a putative role in the intrinsic and acquired resistance of tumors to cytotoxic drugs. We have isolated and purified GST isozymes from mouse liver (M. Warholm et al., Biochemistry, 23: 4119–4125, 1986) and analyzed the metabolic products of the reaction of l-phenylalanine mustard (L-PAM) with glutathione in the presence of GST isozymes, using reverse phase high performance liquid chromatography. At pH 6.5, the spontaneous conjugation of L-PAM and glutathione is suppressed and the major product at 60 min is the monochloro, monohydroxyl derivative of L-PAM. Addition of either class μ or class π isozymes to the reaction has any effect on the metabolism of L-PAM. Only isozymes of the α GST class catalyze the conjugation of L-PAM with glutathione. In this case, the major metabolite at 1 h is the monochloro, monogluthathionyl conjugate. Increasing the amount of μ or π isozyme in the reaction mixture has no effect on the metabolism of L-PAM, whereas increasing the amount of α isozyme completely supplants hydrolysis with conjugation. Thus, increased levels of class α GST isozyme may represent a specific mechanism whereby cells can acquire resistance to nitrogen mustards.

INTRODUCTION

Cancer cells exposed to chemotherapeutic drugs either in vivo or in vitro often display resistance to further exposure to the drugs at doses that were initially effective. A number of mechanisms have been shown to account for this acquired resistance: altered membrane functions that result in decreased cellular drug uptake or enhanced transport of the drug out of the cell, altered nuclear phenomena such as enhanced DNA repair or decreased drug binding, and cytoplasmic phenomena such as decreased activation of a prodrug or increased detoxification of an active metabolite. In the case of the alkylating agents, inducible enzymic systems for detoxification of the drugs are a major mechanism whereby cells may acquire resistance (1, 2).

The GST3 system (RX:glutathione S-transferase; EC 2.5.1.18) plays an important role in the biotransformation of xenobiotics and is a natural candidate as an enzyme system that detoxifies alkylating agents. The sulfur atom of glutathione provides electrons for nucleophilic attack on an electrophilic substrate, with the formation of a thioether. The dichloroethylnitroso derivative of nitrogen mustards appears to react through an aziridinium ion intermediate (3), which has strong electrophilic properties and is a likely substrate for the glutathione conjugation. Multiple reports from our laboratory (4-7) and others (8) have confirmed the existence of glutathionyl adducts of the nitrogen mustards. Using an in vitro preparation and an immobilized enzyme system, Dulik et al. (4) demonstrated GST-dependent conjugation of glutathione and L-PAM.

There are many examples in which the development of resistance to alkylating agents is associated with increases in cellular content of glutathione (9-13) or increased expression of glutathione S-transferase activity (13-18). In addition, depletion of intracellular glutathione content is correlated with the reestablishment of drug sensitivity (9, 19-22), and use of known inhibitors of glutathione S-transferase activity has resulted in decreased cytotoxicity of a nitrogen mustard in drug-resistant cell lines (23). Data such as these have been offered as evidence for a causal relation between resistance to alkylating agents and elevated glutathione and glutathione S-transferase activity. The relationship, however, is not uniform. There are examples in which the levels of glutathione or glutathione S-transferase activity are decreased, unchanged, or only minimally elevated and are insufficient to quantitatively account for the degree of resistance observed (13, 24-28). One explanation for this finding is the measurement of total glutathione S-transferase activity. The glutathione transferases are a family of isozymes with different properties and substrate specificities (29, 30), varying concentration profiles in different tissues (30), and differential responses to inducers and inhibitors (31). The possibility exists that only one isozyme class of glutathione transferases catalyzes the conjugation of nitrogen mustards. The increased expression of such an isozyme might occur in a tumor which has acquired resistance to nitrogen mustards but does not have an increase in glutathione content or in total glutathione transferase activity, compared to a tumor which is sensitive to nitrogen mustards. Smith et al. (28) have shown this to be the case for the nitrosoureas. They found a decrease in total glutathione S-transferase activity in a BCNU-resistant rat gliosarcoma cell line, compared to the sensitive parent cell line. Immunoblot analysis showed overexpression of a μ class isozyme. In their assay system, μ class isozymes had the greatest catalytic effect on the BCNU denitrosation reaction. Addition of the GST inhibitors ethacrynic acid and triphenylmethyl chloride increased the cytotoxicity of BCNU for the resistant cell line, further supporting the hypothesis that the μ class isozymes are specific for the detoxification of BCNU.

Robson et al. have developed a Chinese hamster ovary cell line with 20-fold resistance to the nitrogen mustard chlorambucil (25) and a 3-fold increase in total glutathione S-transferase activity, which is reflected in higher levels of both μ and α class isozymes (17). Buller et al. (14) have reported increased expression of an α class isozyme in their chlorambucil-resistant Walker 256 rat mammary carcinoma cell line, compared to the sensitive parent line. It is thus possible that isozymes of the α class of glutathione S-transferase are specifically involved in the detoxification of nitrogen mustards. The experiments described below were designed to test this hypothesis.

MATERIALS AND METHODS

Materials

L-PAM was supplied by the Division of Cancer Treatment, National Cancer Institute (Bethesda, MD). L-phenylalanine mustard (specific activity,
150 mCi/mmol) was obtained from Amersham Corporation. Reduced glutathione and other chemicals were of analytical grade and readily commercially available. The pH of the buffers was measured and adjusted at the working temperature.

Methodology

Enzyme Purification. Glutathione S-transferase isozymes were purified from either male or female (BDF1) mouse liver cytosol by affinity chromatography and chromatofocusing, following the method of Warholm et al. (32). All steps were carried out at 4°C. Mouse livers were homogenized in phosphate-buffered saline, to obtain a 30% (w/v) homogenate. The homogenate was centrifuged at 15,000 x g for 20 min; the supernatant was then centrifuged at 100,000 x g for 1 h. The resulting supernatant was passed through a Sephadex G-25 column packed in 10 mM Tris-HCl buffer containing 0.2 M NaCl. Glutathione transferase activity was eluted with 5 mM S-hexylglutathione in the Tris-HCl/NaCl buffer. The enzymatically active fractions were pooled; the S-hexylglutathione and salt were removed and the proteins were transferred to the high-pH chromatofocusing buffer by passage through a Sephadex G-25 column running in 25 mM triethylamine-HCl (pH 9.5). The protein solution was concentrated in a Centricon-10 ultrafiltration cell and then applied to a Mono P HR 5/20 chromatofocusing column (Pharmacia) at 22°C, to separate the GST isozymes. The matrix was equilibrated with 25 mM triethylamine-HCl (pH 9.5); the activity was eluted using a pH gradient from pH 9.5 to pH 7.0, at a flow rate of 1 ml/min. Fractions were pooled based on activity and stored in 50% glycerol, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.4, at −20°C. Enzyme activity was measured weekly to verify the stability of the preparation under these conditions.

Glutathione Transferase Activity. Glutathione S-transferase activity was measured spectrophotometrically using CDNB as a substrate, following the method of Habig et al. (33). The assay was carried out at 25°C, pH 6.5.

Protein Determinations. Protein concentration was measured using the Bio-Rad protein assay, with bovine serum albumin as standard (34).

Western Blotting. Western immunoblotting was used to analyze pooled fractions obtained from the electrophoresing column. Aliquots of each fraction were electrophoresed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose. The methods have been described in detail in a previous publication (35).

Antibodies to the 8.7, 9.0, 9.3, and 10.3 isozymes of murine glutathione transferase were generously donated by Dr. Ann Benson at the University of Arkansas, as were samples of the purified isozymes, which were used as standards (31).

L-PAM/Glutathione Conjugation. Equivalent aliquots of each isozyme (based on either CDNB activity or protein concentration) were incubated at 37°C for 1 h with L-[3H]phenylalanine mustard (100 μM, 10 μCi/μmol) and reduced glutathione (1 mM). The incubation mixture was buffered with either sodium phosphate (0.2 M) or 2-[N-morpholino]ethanesulfonic acid buffer (0.2 M) at the desired pH (pH 6.5 or 7.4). Identical chromatograms and radiograms were obtained with each buffer system. The conjugation reaction was terminated and protein was removed from the incubation mixture by the addition of cold (4°C) perchloric acid to a final concentration of 0.5 N.

HPLC Purification. L-PAM hydrolysis products and glutathione adducts were separated by reverse-phase HPLC (C18 Altex Ultrasphere ODS column; 5 μm; 150 x 4.6 mm) with a 15-min gradient from 7 to 70% methanol/0.2% trifluoroacetic acid, followed by elution for 10 min at 70% methanol/0.2% trifluoroacetic acid. The flow rate was 2 ml/min. Fractions were collected at 0.5-min intervals, and radioactivity was measured by liquid scintillation counting. Approximately 20,000 cpm of tritiated L-PAM were contained in each 25-μl assay injected onto the HPLC column. The recovery was greater than 90% and the limit of detection was less than 0.5%, such that a component which gives a peak containing 50 cpm above background represents 0.25% of the total radioactivity. L-PAM metabolites were also detected by the absorbance at 254 nm. The graphic tracings obtained by monitoring the radioactivity closely approximated those obtained by monitoring the absorbance at 254 nm.

Mass Spectrometry. Mass spectra were obtained at the Middle Atlantic Mass Spectrometry Laboratory, a National Science Foundation Shared Instrumentation Facility at The Johns Hopkins University. Fast atom bombardment mass spectra were measured on a Kratos MS 50 mass spectrometer with a Kratos fast atom bombardment source and DS-55 data system (accelerating voltage, 8 kV; resolution, 3000). Details of the methodology have been previously published (4).

RESULTS

Purification and Identification of Isozymes. The results of the purification of three major GST isozymes from mouse liver resemble those obtained by Warholm et al. (32). The protein in the eluate from the FPLC Mono-P HR 5/20 column was monitored by its absorption at 280 nm. Three major peaks, corresponding to each of the isozymes, were identified. Fractions from each peak were pooled and analyzed by Western immunoblotting, using polyclonal antisera directed against unique epitopes of each class of isozyme. The specificity and cross-reactivity of these antibodies have been described elsewhere (36). Table 1 contains the enzyme activities of pooled fractions from the three individual peaks obtained by chromatofocusing and identified by immunoblotting. Fig. 1 shows a typical qualitative Western immunoblot. Equal amounts of protein were loaded in each lane. In Fig. 1A, the immunoblot was exposed to the μ antiserum; similarly, Fig. 1B shows the results of exposure to the α antiserum, Fig. 1C shows the results of exposure to a second μ antiserum, and Fig. 1D shows the results of exposure to the α antiserum. Pool 1 (MI) cross-reacted only with the α antiserum as seen in Fig. 1D. It did not react with either the μ antiserum (Fig. 1A or C) or the π antiserum (Fig. 1B). Pool 3 (MII) cross-reacted mainly with the π antiserum (Fig. 1B) and, to a lesser extent, the α antiserum (Fig. 1D). It did not cross-react with the μ antiserum (Fig. 1, A and C). Pool 7 (MIII) cross-reacted mainly with the μ antiserum (Fig. 1, A and C) and showed only weak reactivity with the π antiserum (Fig. 1B). Pool 9 contains the early fractions eluted in the chromatofocusing. This pool contained very little protein and is a “run-through” peak which contains only α GST isozyme.

Thus, the α class isozyme pool was not discernably contaminated by isozymes of the π or μ class. The MII pool (pool 3) contained primarily π class isozymes, with a minor component of α isozymes (<10% by densitometric analysis). The MIII pool (pool 7) similarly contained only a minor contamination with immunologically identifiable π isozymes. The presence of two bands in pool 7 (Fig. 1B) probably results from contamination of this pool with a π isozyme, which migrates separately from the band containing the μ isozyme, and from some cross-reactivity of this antiserum with the μ isozyme as well.

The data in Table 1 indicate that the three isozymes showed

### Table 1 Murine hepatic GST isozymes

<table>
<thead>
<tr>
<th>Isozyme</th>
<th>Class</th>
<th>Elution pH</th>
<th>Activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MI (pool 1)</td>
<td>α</td>
<td>9.1</td>
<td>10</td>
</tr>
<tr>
<td>MI (pool 3)</td>
<td>π</td>
<td>8.5</td>
<td>54</td>
</tr>
<tr>
<td>MII (pool 7)</td>
<td>μ</td>
<td>7.9</td>
<td>42</td>
</tr>
</tbody>
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* pmol/min/mg protein, CDNB substrate, 25°C, pH 6.5. Values from a single purification.
Fig. 1. Western immunoblot of pooled fractions obtained from the chromatofocusing column. One µg of protein was loaded in each lane. Pool 1 contains fractions from the first major protein peak, corresponding to M1; pool 3 contains fractions from the second major peak, corresponding to MII; pool 7 contains fractions from the third major protein peak, corresponding to MIII; pool 9 is an early peak that contains little protein. A, immunoblot with µ antiserum, pI 8.7; B, immunoblot with µ antiserum, pI 9.0; C, immunoblot with µ antiserum, pI 9.3; D, immunoblot with α antiserum, pI 10.3.

Fig. 2. Metabolic products of the conjugation of L-PAM and glutathione. L-[ring-3H]PAM (100 µM) and glutathione (1 mM) were incubated for 1 h at 37°C, in 2-[N-morpholino]ethanesulfonic acid buffer, pH 6.5. Where present, equal enzyme activities (45 nmol of CDNB conjugated/min) were added. A, nonenzymic conjugation; B, α GST isozyme; C, χ GST isozyme; D, µ GST isozyme.

Reactivities to CDNB comparable to those previously reported (32, 37).

L-PAM/Glutathione Conjugation. Fig. 2 represents the HPLC analysis of products resulting from the incubation of L-[ring-3H]PAM, glutathione, and purified glutathione S-transferase isozymes for 1 h at 37°C at pH 6.5. In the absence of enzyme (Fig. 2A), no glutathione conjugates were formed at pH 6.5. The major product at 1 h was the monochloro, monohydroxyl derivative of L-PAM (peak B), which results from the nucleophilic attack of water on the aziridinium ion intermediate. Continued incubation resulted in the complete disappearance of L-PAM and appearance of dihydroxy L-PAM (peak A) as the sole major product.

In the presence of the GST α isozyme (Fig. 2B), the major product at 1 h was the monochloro, monogluthathionyl conjugate (peak C), indicating that glutathione replaces water as the dominant nucleophile. The identity of this metabolite has been established by mass spectrophotometry for this system (Fig. 3) and is equivalent to the data obtained by Dulik et al. (4). Increasing the amount of GST α isozyme completely supplanted L-PAM hydrolysis by conjugation; only glutathione adducts were formed. When the incubation was extended for 6 h, all of the L-PAM was consumed. The major end product was the digluthathionyl-L-PAM adduct. This conjugate appears to be a stable end product, without further degradation to hydrolysis products. At 60 min, very little of the digluthathionyl adduct was formed (Fig. 2B).

In contrast, the addition of GST µ isozyme to the reaction
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The mixture did not catalyze the formation of glutathione-L-PAM adducts (Fig. 2D). This was the case whether equivalent amounts of isozyme as determined by weight of protein or by reactivity with CDNB were added. Increasing the amount of MIII isozyme in the reaction mixture had no effect on the rate or pattern of metabolism of L-PAM. Analysis of the reaction mixture containing this isozyme showed its composition to be barely distinguishable from that of the nonenzymic reaction (Fig. 2A). The addition of equivalent amounts of π isozyme resulted in an insignificant increase (<5%) in the amount of monochloro, monoglutathionyl adduct formed (Fig. 2C). This most likely reflects the presence of a small amount of α isozyme in pool 3 (Fig. 1D), although we cannot exclude the possibility that the π isozyme has a small amount of catalytic activity for the conjugation reaction. Increasing the amount of π isozyme in the reaction mixture resulted in little discernable difference in the rate or pattern of L-PAM metabolism. In Fig. 2, A, C, and D, an additional small peak was detected, which elutes in fractions 25 and 26. The identity of this peak is unknown.

The effect of pH on the catalytic activity of the isozymes was examined by comparing the results obtained at pH 6.5 and pH 7.4. pH 6.5 was chosen as the optimal pH to study the effects of the presence of the isozymes, since the nonenzymic conjugation of L-PAM and glutathione is suppressed and the catalytic activity of the isozymes is more easily assessed. In this pH range, the rate of formation of the aziridinium intermediate is independent of pH (38). Thus, increasing the pH enhances the nucleophilicity of the sulfhydryl moiety of glutathione. This is also the presumed function of the glutathione S-transferases (29, 30). There was a substantial amount of enzyme-dependent glutathione adduct formation at both pH 6.5 and 7.4.

DISCUSSION

Elevation of GST enzymes is associated with the development of resistance to carcinogens that induce malignant transformation (39) and with the development of cross-resistance to other structurally unrelated carcinogens (40, 41) or anticancer agents (15). In addition, elevated levels of GST activity have been described in some cell lines which appear to have innate resistance to anticancer agents (42–46). In most of these cases, the π family of isozymes accounts for the increase in GST activity. However, when complementary DNA coding for the GST π isozyme gene is transfected into cells, GST π isozyme expression is increased but neither wild-type multidrug resistance nor resistance to alkylating agents is induced (41). Thus, there does not appear to be a specific causal relationship between increased expression of GST π isozyme and resistance to anticancer drugs. It is possible that the increased expression of the GST π isozyme found in cells with innate resistance to anticancer agents and induced in cells in response to carcinogens and anticancer drugs is part of the generalized coordinated enzymic response of cells to transforming events (47). Increased expression of this isozyme would thus serve as a marker of neoplastic transformation rather than a selective mechanism whereby cells have acquired resistance. Such a model has been proposed to account for the induction of GST Ya isozymes by a wide variety of compounds which contain or acquire, by metabolism, electrophilic centers (48). In addition to being inducers of the Ya isozyme, however, these compounds are also substrates.

There are a few reports describing increases in GST isozymes in tumor cells selected for resistance to a specific alkylating agent (14, 17, 25, 28, 49). Smith and co-workers (13, 28) described a rat gliosarcoma cell line with selective resistance to BCNU and a selective increase in the expression of μ class GST isozymes. Kinetic studies showed that isozymes in this class had a 6- to 15-fold increase in catalytic efficiency, relative to α or π GST isozymes. Interestingly, the development of BCNU resistance was accompanied by a collateral sensitization to nitrogen mustard and down-regulation of other GST isozymes.

In the case of nitrogen mustards, the α family of isozymes is selectively overexpressed (14, 17, 25). This is in agreement with the hypothesis of Friling et al. (48) that inducers of GST Ya isozymes possess an electrophilic center. In an aqueous environment, the nitrogen mustards undergo spontaneous dechlorination, with formation of an aziridinium ion (38). This intermediate species contains two electron-deficient carbons, is a putative substrate, and may also be an inducer for the α GST isozyme. Robson et al. (17, 25) have developed a Chinese hamster ovary cell line with acquired resistance to chlorambucil, a nitrogen mustard analogue. The cells are also characterized by cross-resistance to mechlorethamine and L-PAM and relative sensitivity to alkylating agents of different classes (BCNU, cisplatin, and mitomycin C). The increase in nitrogen mustard resistance is accompanied by increased expression of α class GST isozymes and amplification and increased expression of the α class GST-encoding genome (49).

Puchalski and co-workers (50, 51) have shown that transfection of cDNA encoding for the rat YaYa (1-1) promoter-GST hybrid gene into a mouse cell line results in increased expression of the α class GST isozyme and confers resistance to chlorambucil. Transfection into a COS monkey cell line of complementary DNA encoding for the rat μ isozyme Yb1, and a human π isozyme was also associated with increased resistance to nitrogen mustards, but to a lesser degree. Overexpression of the μ isozyme conferred the greatest increase in resistance to cisplatin, supporting the theory that resistance to different classes of alkylating agents is associated with distinct GST isozyme classes.

Early work by Dulik et al. (4) demonstrated that immobilized microsomal and cytosolic glutathione S-transferase enzymes catalyzed the formation of L-PAM-glutathionyl thioether ad-
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ducts. The work described here, and reported initially in abstract form (5), extends these observations and identifies the α class of GST isozymes as the principal form manifesting this catalytic activity in mouse. These results have been confirmed by Ciaccio et al. (8). Their data suggest that, at very high levels (protein concentration and CDNB catalytic activities), both μ and π isozymes may have some catalytic activity for the formation of the monochloro-, monoglutathionyl-L-PAM adduct, in keeping with the known property of broad and overlapping substrate specificity of the GST isozymes.

Thus, the ability of the α class of GST isozymes to catalyze the conjugation of glutathione with L-PAM may represent a specific and physiologically important mechanism through which a cell can be rendered resistant to the effect of a nitrogen mustard.

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29. Rush, C. N., Lewis, A. D., Wolf, C. R., Hayes, J. D. A., Proctor, S. J., et al. (8). Their data suggest that, at very high levels (protein concentration and CDNB catalytic activities), both μ and π isozymes may have some catalytic activity for the formation of the monochloro-, monoglutathionyl-L-PAM adduct, in keeping with the known property of broad and overlapping substrate specificity of the GST isozymes.
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