Characterization of Atrazine Biotransformation by Human and Murine Glutathione S-Transferases

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Atrazine is one of the most widely used herbicides in the United States and has been detected, occasionally, at low levels in drinking water sources. The biotransformation of atrazine in humans has not been fully characterized. Rodent studies suggest Phase I-dominated biotransformation with minor Phase II-mediated biotransformation by glutathione S-transferase(s) (GST). In human urine, mercapturates of atrazine are significant metabolites, yet the specific GST form(s) responsible for glutathione (GSH) conjugation have not been identified. Using recombinant alpha, mu, pi and theta class human GSTs, we demonstrated that only hGSTP1-1 displays significant activity toward atrazine (7.1 nmol/min/mg protein). We also confirmed that mouse GST Pi (π) protein is responsible for the GSH-dependent biotransformation of atrazine in mouse liver; recombinant mGSTP1-1 had a specific activity of 7.3-nmol/min/mg protein. Furthermore, cytosolic fractions from mouse and human liver conjugated atrazine with glutathione at rates of 282.3 and 3.0 pmol/min/mg, respectively. Docking studies of the atrazine-GST conjugate in the hGSTP1-1 substrate-binding site were used to elucidate a basis for the dramatic difference in activity between mouse GSTP1-1 and GSTP2-2 (7.14 versus 0.02 nmol/min/mg protein). The inactivity of mGSTP2-2 appears to be caused by glutathione (GSH) conjugation have not been identified. Using recombinant alpha, mu, pi and theta class human GSTs, we demonstrated that only hGSTP1-1 displays significant activity toward atrazine (7.1 nmol/min/mg protein). We also confirmed that mouse GST Pi (π) protein is responsible for the GSH-dependent biotransformation of atrazine in mouse liver; recombinant mGSTP1-1 had a specific activity of 7.3-nmol/min/mg protein. Furthermore, cytosolic fractions from mouse and human liver conjugated atrazine with glutathione at rates of 282.3 and 3.0 pmol/min/mg, respectively. Docking studies of the atrazine-GST conjugate in the hGSTP1-1 substrate-binding site were used to elucidate a basis for the dramatic difference in activity between mouse GSTP1-1 and GSTP2-2 (7.14 versus 0.02 nmol/min/mg protein, respectively). The inactivity of mGSTP2-2 appears to be attributable to an indirect structural disruption of the G-site by Pro12. Possible effects of the hGSTP1 polymorphisms were investigated. No significant differences in catalytic-specific activity were noted among purified proteins corresponding to the four hGSTP1 variants: hGSTP1*A (most common form), hGSTP1*B (Ile105Val), hGSTP1*C (Ile105Val, Ala114Val), and hGSTP1*D (Ala114Val). Overall, this work supports a physiological role for GSTs in atrazine biotransformation and indicates a novel diagnostic substrate for human and mouse GSTP1-1 proteins.

Key Words: glutathione; transferase; atrazine; human; biotransformation; conjugation.

Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a chloro-S-triazine herbicide used for selective weed control in agricultural crops and for nonselective weed

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Atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) is a chloro-S-triazine herbicide used for selective weed control in agricultural crops and for nonselective weed
be an important route of biotransformation in humans (Buchholz et al., 1999; Jaeger et al., 1998; Lucas et al., 1993).

The GSTs are a multigene family of detoxification enzymes that biotransform a wide variety of endogenous and exogenous electrophilic substrates (for review see Hayes and Pulford, 1995). Typically these enzymes catalyze detoxification of substrates by conjugation to GSH. The human GST (hGST) family is composed of at least 8 classes (alpha, α; kappa, κ; mu, μ; omega, Ω; Pi, π; sigma, σ; theta, θ; and zeta, ζ) with multiple subfamilies per class (Board et al., 2001; Eaton and Bammler, 1999). Although the spectrum of substrates biotransformed by each hGST isoform and the degree to which catalysis occurs is unique, the spectrums overlap in most cases. Several of the human genes for GSTs are known to be polymorphic in the population. For example, 43–52% and 15–20% of Caucasians are homozygous for gene deletions in the hGSTM1 and hGSTT1 genes, respectively (Eaton and Bammler, 1999). Although the spectrum of substrates biotransformed by each hGST isoform and the degree to which catalysis occurs is unique, the spectrums overlap in most cases. Several of the human genes for GSTs are known to be polymorphic in the population. For example, 43–52% and 15–20% of Caucasians are homozygous for gene deletions in the hGSTM1 and hGSTT1 genes, respectively (Eaton and Bammler, 1999). In addition, functionally relevant single nucleotide polymorphisms (SNP) are found in the coding region of hGSTP1 (Ali-Osman et al., 1997; Harries et al., 1997; Watson et al., 1998).

The purpose of this investigation was to determine the specific role of individual human GSTs in the biotransformation of atrazine. In addition, we have further explored the involvement of GSTs in atrazine biotransformation in mice. To this end, seven recombinant cytosolic hGST isoforms (hGSTM1-1, hGSTM2-2, hGSTM3-3, hGSTM4-4, hGSTA1-1, hGSTP1-1, and his-hGSTT1-1) and two mouse GST (mGST) isoforms (mGSTP1-1 and mGSTP2-2) were expressed and purified. These proteins, along with male CD1 mouse liver and human liver cytosol, have been tested for catalysis of GSH conjugation of atrazine. A secondary purpose of this communication is to relate the generation of an effective hGSTP1-1 expression construct and purification protocol that does not require the use of a histidine tag.

**MATERIALS AND METHODS**

**Chemicals and reagents.** The ring-labeled \(^{14}C\)-atrazine used in these assays was a generous gift from Syngenta Crop Protection, Inc (Greensboro, NC). GSH and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma (St. Louis, MO). Chloroform and potassium phosphate dibasic were obtained from Fisher Scientific (Fair Lawn, NJ). Potassium phosphate monobasic was purchased from JT Baker (Phillipsburg, NJ). All other chemicals were purchased from various commercial sources and were of analytical grade or better.

**Liver cytosol preparation.** Human liver samples were obtained from the University of Washington Liver Bank (UW School of Pharmacy; Paine et al., 1997). Characteristics of the liver donors are described in Table 2. After excision the liver samples were immediately snap frozen in liquid nitrogen.
Eight male CD1 mice were purchased at approximately 88 days old (Charles River Laboratories) and maintained in our animal facility for 1 week. Subsequently, the mice were sacrificed by cervical dislocation and the livers excised and pooled, according to procedures approved by the University of Washington IACUC.

The following steps were carried out at 4°C. Frozen liver tissue was thawed in 0.9% NaCl, blotted dried, and weighed. Tissue was minced in 1.5 volumes (v/w) of homogenization buffer (0.25 M sucrose, 0.2 mM EDTA, 10 mM Tris-HCl, pH 7.4), and homogenized for 10 s. Each sample was centrifuged at 10,000 × g for 10 min; the pellet was discarded and the supernatant centrifuged at 15,000 × g for 20 min. The subsequent supernatant was centrifuged at 105,000 × g for 60 min. The resulting supernatant (cytosolic fraction) was filtered through gauze to remove lipids. These cytosolic samples were stored at −80°C until used.

**Protein Assays**

Protein concentrations were approximated using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s microtiter plate protocol. Bovine serum albumin was used as the protein standard, and spectrophotometric measurements were made at 590 nm using a Molecular Devices UV Max 96-well plate reader.

**GST expression and purification.** hGSTA1-1, hGSTM1-1, hGSTM2-2, hGSTM3-3, hGSTM4-4, mGSTP1-1, and mGSTP2-2 were expressed and purified as previously described (Bammler et al., 1995). The authors gratefully acknowledge Dr. Phillip Board (John Curtius School of Medical Research, Australian National University, Canberra ACT 2601, Australia) for providing cDNA expression constructs for hGSTs M1, M2, M3, and M4. Histidine-tagged hGSTT1-1 construct was a generous gift from Dr. John Hayes (University of Dundee, Dundee, Scotland); expression and purification was performed as previously described (Sherratt et al., 1997).

An hGSTP1 expression construct was generated that allowed purification of native hGSTP1. Briefly, the cDNA for hGSTP1 was generated by PCR of an existing hGSTP1 cDNA-containing vector (gift from Dr. William Atkins, University of Washington); PCR amplification primers were used that allowed for addition of Ndel and BamHI sites to the 5' and 3' ends of the hGSTP1 cDNA, respectively (forward primer: 5'-GAG AGA GGA GCA TAT GCC GCC CTA CAC CTT GCC TTG ACC ATT-3'; reverse primer: 5'-GAG GAG GAG GAG TCC TCTA GTG CTT CCC GTT ACC ATT-3'). Subsequently, this PCR product was ligated into the pCR2.1 vector, using a TA cloning kit (InVitrogen Life Technologies, Carlsbad, CA). The vector was propagated in InvPlaR cells. Following BamHI digestion for construct linearization, an Ndel partial digestion allowed for collection of the full-length hGSTP1 cDNA with Ndel and BamHI sticky ends for ligation into the pET17b expression vector (Novagen, Madison, WI).

Four genotypes for hGSTP1 have been described: hGSTP1*A, hGSTP1*B, hGSTP1*C, and hGSTP1*D (Ali-Osman et al., 1997; Harries et al., 1997; Watson et al., 1998). The hGSTP1 expression construct described above corresponded to the hGSTP1*A genotype. Expression constructs for the remaining 3 genotypes were generated using the hGSTP1*A construct and the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The B and D alleles were created by using the primers (5'-CCT CCG CCT CAA ATA CGT CCT CCT CAT CTA C-3', 5'-CTA GAT GAG GAC GAC GTA TTT GCC GGA GAG G-3'), (5'-CATA CAC CAA CTA CTA GGT GGG CAA CAA TCA C-3', and 5'-GTC ACT ATC CTT GCC CAC CTC ATG GGT GGT GAT G-3'), respectively (MWG Biotech, High Point, NC). Subsequently, the C allele was created using the +B primers with the +D construct.

The hGSTP1 polymorphic variant plasmid sequences were confirmed via sequencing. The Big Dye sequencing protocol (ABI, Foster City, CA) was used and was followed by removal of unincorporated dye terminator using Centri-Sep Columns (Princeton Separations, Adelphia, NJ). The following primers were used, which targeted the T7 promoter of pET17b, the T7 terminator of pET17b, the 322–340 bp region of hGSTP1, and the 233–252 bp region of hGSTP1, respectively: 5’–TAA TAC GAC TCA CTA TAG GG-3’, 5’–GCT AGT TTAT TGCT GCA GC-3’, 5’–TAT TTG CAG CGG AGGT CTC-3’, and 5’–GCT CTA TGG GAA GGA CCA GC-3’. Following purification, sequencing was performed using an ABI 377 automated sequencer (ABI, Foster City, CA). hGSTP1-1 was then expressed as described previously (Bammler et al., 1995).

**Enzyme Assays**

**Atrazine.** The procedure for monitoring GSH-dependent biotransformation of atrazine is a modification of that described by Gaddewar and Dauterman (1979). Briefly, the assay was performed as follows: The incubation mixture consisted of 3.0 mM GSH, 0.3–1.37 mM ring-labeled [14C]-atrazine, 0.1 M potassium phosphate buffer (pH 7.4) and the appropriate amount of enzyme. The reaction was performed in a final volume of 1.0 ml and incubated for 30–60 min at 37°C. The reaction was stopped by the addition of 1.0 ml chloroform. After vortexing, the two phases were thoroughly separated by 5 min centrifugation at 2500 × g. The radioactivity from aliquots of both phases was determined by liquid scintillation counting until it was established that a vast majority of the radioactivity could be accounted for between the two phases. Further testing included scintillation counting of only the aqueous phase, which reflected the proportion of atrazine that had been GSH-conjugated. All specific activity values were generated within the linear range. Spontaneous reaction rate (no GST control rate) was subtracted during calculation of the specific activity values.

**CDNB.** The CDNB assay was performed as described previously (Habig and Jakoby, 1981). Briefly, 1 mM CDNB in ethanol, 1 mM GSH, and purified recombinant GST proteins were incubated in 100 mM sodium phosphate buffer at pH 6.5. The final ethanol concentration was 1.67%. CDNB was added to start the reaction. After mixing, the formation of GSH conjugate was monitored at 340 nm at 30°C; the reaction had to be linear for at least one min to be included in the calculation of the specific activity. The measurements were made using a Shimadzu UV-160 spectrophotometer.

**GSTM1 and GSTT1 genotyping.** The human liver samples had been previously assayed for hGSTM1 and hGSTT1 genotype by polymerase chain reaction methods (Chen et al., 1996).

**Molecular Docking Studies**

All docking studies were carried out with the molecular modeling program XP/QPLo (McMartin and Bohacek, 1997). Of the various crystal structures of hGSTP1-1 available, PDB 9GSS (Oakley et al., 1997), which is in complex with S-hexylglutathione, was chosen on the basis of resolution. The binding site model included all protein residues within 10.0 Å of S-hexylglutathione. We first created a model of the docked transition state, also known as the “Sigma” complex. A model of atrazine was covalently attached to the glutathione moiety of S-hexylglutathione (after removal of the hexyl group), with sp3 geometry at the attachment carbon atom. Subsequently, this glutathione conjugate of atrazine was submitted to 300 cycles of an extensive Monte Carlo search procedure, during which the glutathione moiety was kept fixed in its observed pose in the binding site while the fragment consisting of the atrazine moiety and the glutathione cysteine side chain were allowed full conformational flexibility. The procedure was repeated with the ethyl and isopropyl groups of atrazine swapped, since the nucleophilic attack leading to the formation of the transition state may occur on either side of the triazine ring. To investigate the binding mode of the reaction product, a second docking study was carried out in which the chlorine atomic bond was broken, and the sp3 character of the attachment carbon was restored. Subsequently, the structure was energy minimized. With respect to the transition state, a rotation of the triazine ring toward Ile 105 was seen. This is reminiscent of the rotation observed between the hGSTP1-1 crystal structures with Meisenheimer complex (glutathione with 1,3,5-trinitrobenzene) and the product p-bromobenzyl-glutathione (Prade et al., 1997).
RESULTS

Our aim was to investigate Phase-II atrazine biotransformation using cytosolic fractions and a panel of purified recombinant GST proteins. Preliminary to that aim, we expressed and purified active recombinant hGSTP1-1 protein. Recent reports in the literature indicate that the best way to express and purify hGSTP1-1 for significant yield is to use a cloning procedure that involves addition of a histidine-tag and replacement of a rare codon for proline (Chang et al., 1999). Using the pET17b expression system, we instead collected pure and active recombinant hGSTP1-1, avoiding sequence changes that could theoretically alter the enzyme specificity and activity. The yield was approximately 33 mg/l of culture, similar to the approximately 39 mg/l culture yield obtained using histidine-tagged hGSTP1-1 and metal affinity chromatography, previously (Chang et al., 1999). The protein appeared as a single band in SDS-PAGE coomassie-stained gel. The activity of our purified hGSTP1-1 toward CDNB was within the anticipated range (140.0 ± 4.1 μmol/min/mg).

To identify GST proteins with activity toward atrazine, we tested our panel of purified recombinant human and mouse GSTs for GSH-dependent biotransformation of atrazine. As shown in Figure 2, two purified recombinant GST proteins demonstrated significant activity toward atrazine. Mouse GSTP1-1 and hGSTP1-1 conjugated atrazine at rates of 7.3 nmol AT-SG/min/mg, and 7.1 nmol AT-SG/min/mg, respectively. Together with previously reported data (Egaas et al., 1995a; Egaas et al., 1993), these results provide definitive evidence of GST Pi class-mediated catalysis of GSH conjugation of atrazine in mouse liver cytosol. Other hGST isoforms were capable of catalysis of GSH conjugation to atrazine; however, the rates were miniscule in comparison to hGSTP1-1. hGSTs A1-1, M1-1, and M2-2, which had on average 1.3%, 2.9%, and 1.9% of hGSTP1-1 activity, respectively. Although mGSTP2-2 is highly homologous to mGSTP1-1, mGSTP2-2 demonstrated much less conjugation of atrazine (0.02 nmol/min/mg vs. 7.14 nmol/min/mg). We also determined the activity of our preparation of mGSTP2-2 toward CDNB. Although low, activity was within the anticipated range (mean: 0.05 μmol/min/mg).

Human liver cytosol GSH conjugation of atrazine, in comparison to mouse hepatic cytosol GSH conjugation, was also investigated. As shown in Table 1, pooled human liver biotransformation of atrazine is very low (3 pmol AT-SG/min/mg protein). The six human liver cytosolic samples used to create the pooled sample were known to be viable, since they were capable of conjugating the model GST substrate, CDNB (data not shown; values ranged from 0.7–2.3 micromoles/min/mg protein). Pooled mouse liver demonstrated relatively high activity (282 pmol AT-SG/min/mg protein), which was comparable to that demonstrated in previous studies (Egaas et al., 1993). The conjugation detected in the absence of exogenous GSH is likely due to the presence of endogenous GSH. The concentration of GSH in the liver is estimated to be between 5 and 10 mM (Kosower and Kosower, 1978), and the liver cytosol preparation protocol would not necessarily eliminate all of the reduced GSH that was present in the liver.

Mechanistic characterization of hGSTP1-1 catalysis of atrazine GSH conjugation was also attempted. Unfortunately, solubility limits prevented the calculation of Michaelis Menten enzyme kinetic parameters. Initial velocities increased in a linear manner when substrate concentrations were increased to levels approaching the solubility limit without reaching a plateau indicative of Vmax (37 μM atrazine:4.3 nmol AT-SG/min/mg protein, 53 μM atrazine:6.6 nmol/AT-SG/min/mg protein, 98 μM atrazine:12.3 nmol AT-SG/min/mg protein, and 138 μM atrazine:19.1 nmol AT-SG/min/mg protein).

Molecular docking of the transition state of hGSTP1-1 and the atrazine-glutathione conjugate shows that the atrazine moiety aromatic ring and alkyl groups make excellent use of the hydrophobic patches of the H-site, and the aromatic ring is stacked with relation to Tyr 109 (Fig. 3). The amino functions rest parallel on the surface, allowing accessibility for solvation by water.
The chlorine atom is situated in a channel known to contain a number of water molecules. Interestingly, this channel also includes the side chain of Arg 14. Both the water molecules and the electropositive potential from the Arg-14 residue might assist the chlorine atom in becoming a leaving group, the second step in the nucleophilic, aromatic substitution reaction. Another residue that may help in stabilizing the developing negative charge of the leaving group is Tyr 109, which also points into the channel. Its phenol function is located only 3.4 Å away from the chlorine atom. The model for the transition state exhibits great similarity to the crystal structure of the Meisenheimer complex (Prade et al., 1997). There, parallel stacking between the aromatic ring and Tyr 109 is seen, and the hydrogen at the sp³ carbon (equivalent to the chlorine atom of atrazine) points roughly in the same direction (i.e., toward the channel).

This similarity provides further support for the correctness of the docked model of the transition state complex of GSH-atrazine.

Four genotypes in the human GSTP1 gene have been characterized: hGSTP1*A (most common form), hGSTP1*B (Ile105Val), hGSTP1*C (Ile105Val, Ala114Val), hGSTP1*D (Ala114Val) (Ali-Osman et al., 1997; Harries et al., 1997; Watson et al., 1998). According to the crystal structure, the Ile105Val amino acid change in the hGSTP1*B genetic variant lies in the hydrophobic substrate pocket, whereas the hGSTP1*D variation (Ala114Val) lies outside that area, located on the outside of an α-helix some 20 Å away from ligand atoms in the G-site and even further from the H-site. Therefore, no effect on catalysis is expected. In contrast, residue 105 is located in the H-site (Fig. 3). Thus, while atrazine is accommodated in the hydrophobic substrate pocket of both hGSTP1*A and hGSTP1*B, the hGSTP1*B variant could have altered catalytic activity toward atrazine, relative to the common form.

In order to definitively determine whether the polymorphisms of hGSTP1 affect catalytic activity of the gene products, we expressed and purified hGSTP1*B, hGSTP1*C, and hGSTP1*D proteins in addition to the previously tested hGSTP1*A protein. The hGSTP1*B and hGSTP1*C proteins had altered activity toward the model GST substrate, CDNB, as anticipated and previously demonstrated (Fig. 4b) (Ali-Osman et al., 1997; Watson et al., 1998). However, no significant differences in the specific activity toward atrazine were noted between the genotypes (Fig. 4a). Two atrazine concentrations were utilized in order to ensure that the ability to demonstrate altered catalytic activity was not dependent upon the substrate concentration tested.

Molecular docking studies confirm these results. The polymorphism at residue 105, Val instead of Ile, was examined with the models of the transition state and the product state. In the transition state structure, the closest interaction between the Ile side chain and the atrazine moiety is 3.9 Å, a typical van der Waals interaction within the accuracy of a docking model. In computo mutation to Val followed by a rotamer energy

<table>
<thead>
<tr>
<th>Liver ID</th>
<th>Donor age (yrs)</th>
<th>Donor sex</th>
<th>Ethnicity</th>
<th>Cause of death</th>
<th>Home medication</th>
<th>ICU Medication</th>
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<tbody>
<tr>
<td>103</td>
<td>15</td>
<td>F</td>
<td>C</td>
<td>Head trauma</td>
<td>Trandate</td>
<td>Not recorded</td>
</tr>
<tr>
<td>104</td>
<td>32</td>
<td>M</td>
<td>B (NOH)</td>
<td>CVA</td>
<td>Phenobarbital,</td>
<td>Not recorded</td>
</tr>
<tr>
<td>105</td>
<td>21</td>
<td>M</td>
<td>B (NOH)</td>
<td>Head trauma</td>
<td>CVA</td>
<td>Not recorded</td>
</tr>
</tbody>
</table>
| 126     | 10             | F         | C         | Respiratory arrest | None             | Dexamethasone (1 day), insul,
| 131     | 62             | F         | C         | SAH            | Praze, lozol    | Dilantin, decador solumedrol, lasix, emerol, ancef |
| 134     | 7              | M         | C         | Head trauma    | None            | Unknown       |

Note. Abbreviation: C, Caucasian; B (NOH), black, not of Hispanic origin; SAH, subarachnoid hemorrhage; CVA, other cerebral vascular accident including strokes, head trauma, auto accident, gunshot, etc. Methods for procurement are described in Paine et al. (1997).
evaluation for Val 105 shows that Val prefers the same rotamer as Ile and that it maintains an identical interaction with atrazine. Similar calculations were performed for the product state, yielding the same conclusions. Thus, there is no significant difference in direct interaction between the two polymorphisms at position 105 and atrazine, either in the transition state or product state. Because the side chain of Val is smaller than of Ile, the model shows a somewhat enlarged water channel towards Arg 14 in the former case. Predictions as to whether this leads to a different water structure around atrazine are impossible without CPU-intensive molecular dynamics simulations. Given the similar activities of the polymorphic variants, such calculations do not seem warranted.

**DISCUSSION**

**Mouse GST-Mediated Conjugation of Atrazine**

Mice, in contrast to humans, have two GST Pi-class genes, mGstP1 and mGstP2. Utilizing purified recombinant protein, we demonstrated that mGSTP1-1, but not mGSTP2-2, bio-transforms atrazine. Only 6 residues differ between mGSTP1-1 and mGSTP2-2. In considering the 6 discrepant residues, a residue could explain the isoform specificity if it (a) was conserved between hGSTP1-1 and mGSTP1-1 but not mGSTP2-2, or (b) differed between all three, in which case no conclusions could be drawn. Using these criteria, five candidates for explaining the inactivity of mGSTP2-2 emerge: residues 11, 12, 105, 107, and 110. None of the 5 discrepant residues reside in the G-site. Residue 107 is located in an H-site helix; however, it is not anticipated to be near substrates due to its location in the helix (Fig. 5). Of the four remaining candidates, residue 110, appears to be located too distant from the active site to be in direct contact with conjugated atrazine.

Although residue 12 also appeared to be located too far from the active site, the Arg 12 main chain amide nitrogen forms a hydrogen bond with the carbonyl group of Ile 204, a residue of the C-terminal tail of the enzyme. The next three residues of this tail, Asn205–Gly206–Asn207 pack against the Tyr109 side chain, a residue that plays an important role in catalysis. Importantly, residue 12 is a proline in mGSTP2-2, which, unlike arginine, cannot form a hydrogen bond via its backbone nitrogen. As a result, with proline at residue 12, no hydrogen bond can be formed, and residue 204 is sterically pushed away by the C6, likely also involving the neighboring C-terminal residues (Fig. 5). This change should result in a disruption of the hydrogen bond between the main chain nitrogen of residue 205 and the Tyr109 phenol function and cause poorer packing between Asn 205–Gly206–Asn207 and the tyrosine ring. In this way the proline at residue 12 of mGSTP2-2 indirectly compromises the H-site, potentially contributing to the inactivity of mGSTP2-2 as compared to mGSTP1-1.

Two more potentially relevant residues remain: residues 11 and 105. Both residues are close to the atrazine-glutathione conjugate, especially in the transition state. The Ser11 in mGSTP2-2 (instead of Val) increases the hydrophilic character of the H-site but is not expected to make a dramatic difference in

![FIG. 4. Effect of SNPs in hGSTP1 on GSH conjugation of AT and CDNB: (a) purified recombinant hGSTP1-1 proteins of the four described genotypes were tested for AT biotransformation at two AT concentrations; (b) the same proteins were tested to confirm altered activity toward the model substrate, CDNB. The mean and standard error of at least 2 trials performed in triplicate are displayed.](https://academic.oup.com/toxsci/article-abstract/80/2/230/1660713)
enzyme activity. Also, Ser11 occurs together with Pro12 in the mGSTP2 subunit protein. Because Pro12 appears detrimental to catalysis, the effect of Ser11 by itself is not relevant. The absence of a side chain at position 105 (Glycine) of mGSTP2-2 (as opposed to the Valine at position 105 in mGSTP1-1) deprives the H-site of a significant area of hydrophobic surface and would be expected to have an effect on activity, certainly with larger substrates.

In conclusion, molecular modeling and substrate docking suggest that the poor activity of mGSTP2-2 occurs because of the presence of proline rather than arginine in position 12; the loss of a hydrophobic side chain at position 105 in mGSTP2-2 may also contribute to the lack of activity of mGSTP2-2 toward atrazine. Similar conclusions were reached by Bammler and colleagues (1995) with respect to CDNB activity. Furthermore, a dramatic decrease in activity toward CDNB was demonstrated when Arg12 of hGSTP1-1 was replaced by proline (Bammler, et al., 1995).

The mGSTP1-1 protein is likely responsible for most of the GSH-dependent metabolism of atrazine by mouse liver cytosol. GSTs are believed to account for up to 4.0% of total cytosolic protein in male DBA/2, C3H/He and C57BL6 mice; as much as 70% of total cytosolic GST protein is believed to be mGSTP1-1 protein (McLellan and Hayes, 1987). If similar percentage values are assumed to apply to male CD1 mouse liver cytosolic protein, then mGSTP1-1 protein would account for approximately 2.8% of cytosolic protein, and the anticipated specific activity for atrazine conjugation would be slightly more than 200 pmol/min/mg protein. This value is in reasonable agreement with the 282-pmol/min/mg protein value revealed in our study for male CD1 mouse liver cytosol catalysis of atrazine conjugation.

GST Pi class biotransformation of atrazine had been previously demonstrated only with liver fractionation studies and correlative evidence (Egaas et al., 1995a; Egaas et al., 1995b; Egaas et al., 1993). For example, most male mouse livers contain up to 10-fold higher amounts of GST pi protein as compared to female mouse livers (Hatayama et al., 1986; McLellan and Hayes, 1987); GSH conjugation of GST pi protein as compared to female mouse livers (Hatayama et al., 1986; McLellan and Hayes, 1987); GSH conjugation of atrazine is easier to detect in male livers. Furthermore, strain-related differences in hepatic mGST pi content appear to be correlated with atrazine conjugation capacity (Egaas et al., 1995a; Egaas et al., 1995b). Rat liver, which does not express high levels of GST pi protein (Satoh et al., 1985), shows little GSH conjugation of atrazine (Egaas et al., 1993). Thus, GST pi class protein content is predictive of hepatic atrazine biotransformation.

**Human GST-Mediated Conjugation of Atrazine**

We have demonstrated for the first time that human GSTP1-1 mediates biotransformation of atrazine. Unlike the mouse, hGSTP1-1 is not highly expressed under normal conditions in human hepatocytes (for review, see Awasthi et al., 1994). Therefore, it is not surprising that human liver cytosolic fraction demonstrated only low-level GSH-dependent biotransformation of atrazine. Due to the lack of GST pi class protein in rat or human liver, it is unlikely that GSH conjugation reactions would predominate following oral dosing. Although, direct comparison of previous studies of rodent versus human biotransformation is not possible since differing methodologies and routes of exposure have been employed, we predict based upon fundamental similarity in hepatic pi class protein expression that human hepatic clearance of atrazine is more similar to that of rats than that of male mice. Although hepatic GST biotransformation of atrazine

![FIG. 5. Atrazine and mGSTP1-1 versus mGSTP2-2 protein structures (left): View of human GSTP1-1 with atrazine in the active site locating the positions of the discrepant residues of mouse GSTP1 in the vicinity of the H-site. Note that residue 107 is on the wrong side of the helix to be seen by the enzyme substrate. (right). The presence of Pro12 in mGSTP2-2 leads to severe clashes with the protein backbone carbonyls of Pro203 and Ile204. This, in turn, changes the conformation of the C-terminal part of the protein that forms part of the H-site. Letters G and H denote the G- and H-sites, respectively.](https://academic.oup.com/toxsci/article-abstract/80/2/230/1660713)
would be expected to play only a minor role in atrazine clearance. hGSTP1 class protein is expressed in other relevant tissues such as skin and lung (Awasthi et al., 1994; Singhal et al., 1993). Extrahepatic (skin and lung) biotransformation of atrazine may be very important, as most occupational exposures are likely to occur via inhalation or dermal routes. In line with this idea, measurable levels of atrazine mercapturate metabolites have been detected in human urine following dermal exposure (Lucas et al., 1993). Furthermore, human placenta contains significant amounts of hGSTP1-1 and may afford metabolic protection to the fetus (for review, see Awasthi et al., 1994). Overall, our data support a biologically relevant role of hGSTP1-1 in the biotransformation of atrazine in humans, especially following dermal or inhalation exposure, where extrahepatic metabolism may contribute to overall elimination.

**Significance of the Human GSTP1 Polymorphism in Atrazine Conjugation**

Single nucleotide polymorphisms of the hGSTP1 gene have been described at codon positions 105 and 114 (Ali-Osman et al., 1997). In the referenced study, allele frequencies of the resulting genotypes were as follows: $hGSTP1^*$A (0.70), $hGSTP1^*$B (0.19), $hGSTP1^*$C (0.11) and $hGSTP1^*$D (not detected, rare). Our results suggest that the four variant hGSTP1-1 enzymes exhibit very similar activities toward atrazine. Therefore, it is unlikely that individuals carrying variant forms of the $hGSTP1$ gene are differently able to clear atrazine, following exposure on the basis of $hGSTP1$ genotype.

One interesting finding from this study is the discovery that atrazine is a very specific substrate of Pi class GSTs. The hGSTP1-1 enzymes (Eaton and Bammmler, 1999) are all metabolized by many other GSTs. The hGSTP1-1 conjugation of atrazine is unusually specific to that isoform; therefore, atrazine could be a useful marker substrate for detection of hGSTP1-1 activity in tissues where multiple GST isoforms are expressed.

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