Clarification of the role of key active site residues of glutathione transferase Zeta/maleylacetoacetate isomerase by a new spectrophotometric technique

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INTRODUCTION

GSTZ1-1 (glutathione S-transferase Zeta 1-1) is widely distributed in nature and is found in a range of species from mammals to insects, plants and fungi [1]. hGSTZ1-1 (human GSTZ1-1) is also known as maleylacetoacetate isomerase (MAAI, EC 5.2.1.2) and catalyses the glutathione-dependent isomerization of MAA (maleylacetoacetate) to FAA (fumarylacetoacetate), the penultimate step in the catabolism of tyrosine [2]. This is an important metabolic pathway, as deficiencies of its component enzymes are associated with severe disorders, such as phenylketonuria and hypertyrosinemia type I. The metabolic significance of this pathway may explain the conservation and expression of GSTZ1 throughout such a diverse evolutionary range. In previous studies, the reaction mechanism of the Zeta-class GSTs has been limited by the absence of a convenient assay for kinetic analysis. We have now developed and validated a new spectrophotometric assay with a novel substrate [([±])-2-bromo-3-(4-nitrophenyl)propionic acid]. The assay has been used to rapidly assess the potential catalytic role of several residues in the active site. Despite its less favourable orientation in the crystal structure, Ser-14 was the only residue found to be essential for catalysis. It is proposed that a conformational change may favourably reposition the hydroxyl of Ser-14 during the catalytic cycle. The Cys16→Ala (Cys-16 mutated to Ala) mutation caused a dramatic increase in the Km for glutathione, indicating that Cys-16 plays an important role in the binding and orientation of glutathione in the active site. Previous structural studies implicated Arg-175 in the orientation of α-halo acid substrates in the active site of hGSTZ1-1. Mutation of Arg-175 to Lys or Ala resulted in a significant lowering of the kcat in the Ala-175 variant. This result is consistent with the proposal that the charged side chain of Arg-175 forms a salt bridge with the carboxylate of the α-halo acid substrates.

Key words: catalysis, glutathione transferase (GST), maleylacetate isomerase (MAAI), site-directed mutagenesis, spectrophotometry, Zeta-class glutathione transferase.

hGSTZ1-1 (human glutathione transferase Zeta 1-1) catalyses a range of glutathione-dependent reactions and plays an important role in the metabolism of tyrosine via its maleylacetoacetate isomerase activity. The crystal structure and sequence alignment of hGSTZ1 with other GSTs (glutathione transferases) focused attention on three highly conserved residues (Ser-14, Ser-15, Cys-16) as candidates for an important role in catalysis. Progress in the investigation of these residues has been limited by the absence of a convenient assay for kinetic analysis. In this study we have developed a new spectrophotometric assay with a novel substrate ([([±])-2-bromo-3-(4-nitrophenyl)propionic acid]. The assay has been used to rapidly assess the potential catalytic role of several residues in the active site. Despite its less favourable orientation in the crystal structure, Ser-14 was the only residue found to be essential for catalysis. It is proposed that a conformational change involved and the reaction mechanism is important for the rational design of selective inhibitors that may find use in the clinical treatment of hypertyrosinemia type I. In most other GST classes, the pKᵣ of the cysteine thiol group in enzyme-bound glutathione is reduced to around 6.5, and the thiolate anion is stabilized by a hydrogen bond from a tyrosine, serine, or arginine residue within the N-terminal domain [13–15]. Exceptions occur in the Omega- and Beta-class GSTs that have an active-site cysteine that can form a disulphide with glutathione [16,17]. The Zeta-class GSTs from a range of species contain a characteristic motif [SSC(X/W/H)RVLAL, using one-letter symbols to denote amino acids] in the N-terminal region [1]. The first three residues of this motif line the active-site pocket. Each of these residues (Ser-14, Ser-15, Cys-16) could play a role in catalysis, and there are examples in other GSTs where different residues contribute to catalysis with different substrates. In addition, the crystal structure of hGSTZ1-1 contains a bound sulphate molecule that is co-ordinated by Arg-175 [11]. Molecular modelling suggests that Arg-175 forms a salt bridge with the carboxylate of α-haloacid substrates and plays a critical role in maintaining their correct orientation in the active site.

Progress in the investigation of active site residues and the reaction mechanism of the Zeta-class GSTs has been limited by the absence of a convenient assay for kinetic analysis. We have now developed and validated a new spectrophotometric assay with a novel α-haloacid substrate, BNPP ([([±])-2-bromo-3-(4-nitrophenyl)propionic acid]. This new method has been used to probe the active site and the reaction mechanism of hGSTZ1-1.

Abbreviations used: BCPP, 2-bromo-3-(4-chlorophenyl)propanoic acid; BNPP, ([([±])-2-bromo-3-(4-nitrophenyl)propionic acid; CFA, chlorofluoroacetic acid; GS-CPP, 2-(glutathion-S-yl)-3-(4-chlorophenyl)propanoic acid; GS-NPP, 2-(glutathion-S-yl)-3-(4-nitrophenyl)propanoic acid; GST, glutathione transferase; hGSTZ1-1, human GST Zeta 1-1; MA, maleylacetone; SA, salicylic acid; TCHQ, tetrachlorohydroquinone.

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**EXPERIMENTAL**

**Materials**

MA (maleylacetone), a surrogate substrate for MAA [18], was synthesized by a method described previously [19]. FA (fumarylacetone) was obtained from the Chemistry Centre (Perth, Australia). CFA (chlorofluoacetic acid, 99% pure) was prepared by hydrolysis of CFA ethyl ester (Lancaster Synthesis, Windham, NH, U.S.A.), as described previously [20]. BNPP was obtained from the Chemistry Centre (Perth, Australia).

**Enzyme assays**

Activity with DCA or CFA as substrates was determined by assaying the formation of glyoxylate, as described previously [4]. The isomerization of MA to FA was determined by a HPLC method modified from previously described procedures [21]. The reaction mixture contained 0.01 M sodium phosphate (pH 7.6), 500 μM glutathione, 500 μM MA, and 0.1–1.0 μg/ml enzyme in a final volume of 500 μl. The reaction was incubated at 25 °C and was stopped after 30 s by addition of 100 μl of ice-cold stop solution, which contained a 1:1 mixture of 1 M HCl and 5 μM SA (salicylic acid) as an internal standard. The samples were chilled to 4 °C and analysed by HPLC to determine the quantity of FA produced. MA, FA, and SA were separated on a Waters µBondapak C18 column (3.9 mm × 300 mm), eluted with a mobile phase at 1.5 ml/min. The mobile phase consisted of 40% acetonitrile, 1% triethylamine, 59% MilliQ water. The pH of the mixture was brought to 3.1–3.2 with phosphoric acid, and the mixture was sparged with helium. The absorbance of the eluate was monitored at 312 nm. Under these conditions, the solvent peak eluted at 1.6 min, MA at 2.4 min, FA at 3.4 min, and the internal standard (SA) at 4 min. FA formation was quantified by reference to a standard curve prepared with pure FA. The concentration of MA used in these studies was determined by conversion of a sample of MA to FA in the presence of glutathione and hGSTZ1c-1c.

For the determination of kinetic constants, activities were determined in a 5 × 5 matrix of glutathione and MA concentrations. Glutathione concentrations ranged from 1–500 μM and MA concentrations ranged from 30–300 μM. The data were fitted to the Michaelis–Menten equation by MacCurveFit, version 1.1.2 (Kevin Raner, Mt Waverly, Victoria, Australia).

**Spectrophotometric assay of GSTZ1-1 activity**

Because the previously developed assays for GSTZ1-1 activity are cumbersome endpoint procedures, we developed a new method that allows the continuous spectrophotometric recording of enzyme activity, which simplifies the kinetic characterization of this enzyme. The method is based on the previously noted activity of GSTZ1-1 with α-halopropionates [3]. Both BNPP and BCPP [2-bromo-3-(4-chlorophenyl)propanoic acid] were tested as substrates, but BNPP provided the greatest spectral change after conjugation with glutathione. The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.4), 1 mM glutathione, and 0.75 mM BNPP and was incubated at 37 °C. Because of the high absorbance of BNPP, it was necessary to use a dual-beam spectrophotometer with a blank reaction mixture that lacked enzyme in the reference cuvette.

Repetitive scanning of the reaction mixture between 400 and 250 nm revealed a maximum absorbance peak at 310 nm. The molar absorptivity of the reaction product GS-NPP [2-(glutathionyl-S-yl)-3-(4-nitrophenyl)propanoic acid] at 310 nm was 420. The pH optimum of the hGSTZ1-1-catalysed reaction of glutathione (1 mM) with BNPP (0.75 mM) as the substrate was determined with a range of buffers that have been described previously [22] for the characterization of glucose-6-phosphate dehydrogenase variants. The enzyme reaction rates were corrected for the non-enzymatic reaction rate at each pH. For the determination of kinetic constants, glutathione concentrations ranged from 0.05–1 mM and BNPP concentrations ranged from 0.025–0.5 mM. The data were fitted to the Michaelis–Menten equation by non-linear regression with MacCurveFit version 1.1.2.

**LC-MS/MS analysis**

Glutathione, substrates, and glutathione S-conjugates were analysed by tandem LC-MS/MS analysis with an Agilent 1100 Series LC/MSD-Trap SL controlled with the Agilent Chemstation software (version A.09.01) and the Agilent LC/MSD Trap 4.1 software (Agilent Technologies, Palo Alto, CA, U.S.A.). The instrument was equipped with a Nova-Pak® (2.0 mm × 150 mm, C18-column (Waters, Milford, MA, U.S.A.). The analytes were separated with a gradient mobile phase at a flow rate of 400 μl/min; solvent A: 10 mM ammonium formate (pH 3.2) was prepared in ultra-pure water (EM Sciences, Merck KGaA, Darmstadt, Germany); solvent B: 100 % HPLC-grade acetonitrile (J.T. Baker, Phillipsburg, NJ, U.S.A.). The gradient was as follows: 5% solvent B for 2 min, increasing to 50% solvent B over 5 min, and then held at 50% solvent B for 3 min, with a total run time of 15 min.

MS/MS analyses were run in the positive APIC mode, and ionization conditions were optimized by continuous infusion of glutathione with a syringe pump. Interface conditions for optimal ionization were: dry-gas flow rate, 5.0 l/min; dry gas temperature, 350 °C; APCl temperature, 400 °C; nebulizer pressure, 60 psig; capillary voltage, 3270 V; capillary current; 95 nA; current end plate, 3314 nA; and corona current, 3931 nA. Conditions for the ion optics and CID trap were optimized with glutathione over the 50–600 m/z scan range; smart parameter settings were used for fragmentation in auto-MS/MS mode, and all ions of abundance greater than 10 000 were fragmented. Fragmentation voltages of 0.2, 0.6, and 1.0 V were used for manual MS/MS analysis. The MS/MS data were analysed with the data analysis software for version 4.1. The glutathione and the substrates were not retained on the column and eluted in the column dead volume. The retention times for GS-NPP and GS-CPP [2-(glutathionyl-S-yl)-3-(4-chlorophenyl)propanoic acid] were 8 and 9 min respectively. Both conjugates absorbed strongly at 254 nm.

**Synthesis of GS-NPP and GS-CPP**

Glutathione (0.36 mmol, 112 mg) (Sigma–Aldrich, St. Louis, MI, U.S.A.) was dissolved in 2 ml of ultra-pure water (EM Science, Merck KGaA, Darmstadt, Germany), and BNPP (0.18 mmol, 50 mg) or BCPP (0.18 mmol, 50 mg), dissolved in 1 ml of methanol, was added dropwise to the glutathione solution with stirring at room temperature; the final reaction volume was 3 ml. The pH of the solutions was brought to 9.0 with a few drops of 10 M NaOH. Product formation was monitored every 15 min by LC-MS/MS analysis, and the reaction was allowed to proceed to completion with stirring at room temperature for approx. 1 h. The reaction mixture was then concentrated under vacuum, and the dry
Enzymatic synthesis of GS-NPP and GS-CPP

hGSTZ1b-1b (5 µg) was added to 950 µl of 0.1 M phosphate buffer (pH 7.4) containing 1 mM glutathione and incubated at 37 °C for 5 min. The reaction was started by addition of 50 µl of a 100 mM solution of either BNPP or BCPP in methanol. After incubation for 10 min at 37 °C, the reaction was stopped by addition of 50 µl of concentrated HCl, and 50 µl samples were used for analysis by LC-MS/MS.

Recombinant enzymes and mutagenesis

Recombinant variants of hGSTZ1-1 were expressed in Escherichia coli with an N-terminal poly-histidine fusion and purified as described previously [23]. Specific mutations were made with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.) and oligonucleotides obtained from Genset (Lismore, Australia). All mutations were made in the hGSTZ1c, the most common allelic variant, and each mutant cDNA was checked by DNA sequence analysis.

RESULTS

A new spectrophotometric assay

To facilitate the kinetic characterization of GSTZ1-1, we developed a novel spectrophotometric assay with BNPP as the substrate. During the development of this assay, we confirmed by LC-MS/MS that the hGSTZ1-1-catalysed conjugation of BNPP with glutathione gave GS-NPP (Figure 1). As observed with other α-halopropanoic acids [6], the α-bromo group of BNPP was displaced by glutathione to give GS-NPP. To determine if there was a spectral change associated with the formation of the GS-NPP, we recorded absorbance spectra between 400–250 nm of the hGSTZ1c-1c-catalysed reaction of glutathione with BNPP at 2 min intervals (Figure 2). The results showed that the greatest change in absorbance occurred at 310 nm. The molar absorptivity at 310 nm was 420. Because of the relatively low absorptivity of GS-NPP compared with BNPP, the assay lacks the sensitivity needed to determine GSTZ1-1 activity in crude tissue extracts. Nevertheless, the method allows the rapid determination of GSTZ1-1 activity in purified preparations. The optimum pH for the hGSTZ1c-1c-catalysed reaction of BNPP with glutathione was relatively broad with a peak at pH 10.5–11 (Figure 3). For subsequent kinetic analysis, a more physiological pH of 7.4 was used.

The specific activities of several naturally occurring allelic variants of hGSTZ1-1 are shown in Table 1. The rates obtained compare well with values obtained previously with other α-halo acids, including CFA. It is notable that among these naturally occurring variants, hGSTZ1a-1a has the highest activity and is significantly higher (P = 0.0014) than the value obtained for hGSTZ1c-1c, the most common variant. As shown in Table 1, the kcat as well as the K_mBNPP and K_mGSH determined for hGSTZ1a-1a are all elevated in contrast to the other variants. In a previous study, we found that hGSTZ1a-1a shows selectivity between (R)- and (S)-2-chloropropionate [23]. The BNPP used in this study is a racemic modification, and the elevated activity of hGSTZ1a-1a may reflect a similar enantiomer selectivity.
Table 1  Specific activities and kinetic parameters of naturally occurring GST Zeta variants with BNPP and other α-halo acid substrates

Results are the mean ± S.D. of at least three determinations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>BNPP (µmol/min/mg)</th>
<th>CFA* (µmol/min/mg)</th>
<th>(R)-Z-Chloropropionate* (µmol/min/mg)</th>
<th>(S)-Z-Chloropropionate* (µmol/min/mg)</th>
<th>kcat (s⁻¹)</th>
<th>KₘBNPP (µM)</th>
<th>kcat/Kₘ (M⁻¹·s⁻¹)</th>
<th>KₘGSH (µM)</th>
<th>kcat/Kₘ (M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGSTZ1a-1a</td>
<td>2.3 ± 0.15</td>
<td>1.35 ± 0.05</td>
<td>1.11 ± 0.04</td>
<td>0.07 ± 0.02</td>
<td>1.3 ± 18</td>
<td>108 ± 43</td>
<td>10.2 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>hGSTZ1b-bb</td>
<td>1.5 ± 0.03</td>
<td>1.34 ± 0.03</td>
<td>0.28 ± 0.009</td>
<td>0.21 ± 0.04</td>
<td>0.82 ± 06</td>
<td>96 ± 19</td>
<td>8.5 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>hGSTZ1c-1c</td>
<td>1.6 ± 0.04</td>
<td>1.29 ± 0.05</td>
<td>0.29 ± 0.009</td>
<td>0.22 ± 0.05</td>
<td>0.59 ± 03</td>
<td>44 ± 8</td>
<td>13 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>hGSTZ1d-1d</td>
<td>1.2 ± 0.08</td>
<td>1.27 ± 0.025</td>
<td>0.26 ± 0.002</td>
<td>0.25 ± 0.04</td>
<td>0.55 ± 03</td>
<td>29 ± 7</td>
<td>19 × 10³</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Data from reference [29].

Table 2  Specific activity and kinetic parameters for mutants of hGSTZ1-1 with CFA, BNPP and MA as substrates

Results are the mean ± S.D. for at least three determinations. ND, not detectable; S14A, Ser¹⁴→Ala mutant etc.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Specific activity BNPP (µmol/min/mg)</th>
<th>CFA (µmol/min/mg)</th>
<th>BNPP kcat (s⁻¹)</th>
<th>BNPP Kₘ (µM)</th>
<th>kcat/Kₘ (M⁻¹·s⁻¹)</th>
<th>MA Kₘ (µM)</th>
<th>kcat/Kₘ (M⁻¹·s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>hGSTZ1c-1c</td>
<td>0.91 ± 0.13</td>
<td>1.6 ± 0.04</td>
<td>0.56 ± 03</td>
<td>44 ± 7</td>
<td>1.3 × 10⁴</td>
<td>134 ± 25</td>
<td>5.8</td>
</tr>
<tr>
<td>S14A</td>
<td>0.007 ± 0.001</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>S15A</td>
<td>0.025 ± 0.0004</td>
<td>2.7 ± 0.1</td>
<td>1.0 ± 0.14</td>
<td>319 ± 48</td>
<td>5.7 × 10⁵</td>
<td>117 ± 25</td>
<td>3.4</td>
</tr>
<tr>
<td>C16A</td>
<td>0.63 ± 0.03</td>
<td>3.7 ± 0.29</td>
<td>2.55 ± 0.44</td>
<td>417 ± 120</td>
<td>6.1 × 10⁴</td>
<td>287 ± 34</td>
<td>50</td>
</tr>
<tr>
<td>R175A</td>
<td>0.216 ± 0.003</td>
<td>0.45 ± 0.012</td>
<td>0.19 ± 0.01</td>
<td>23 ± 3.9</td>
<td>8.2 × 10³</td>
<td>64 ± 16</td>
<td>10</td>
</tr>
<tr>
<td>R175K</td>
<td>0.68 ± 0.04</td>
<td>1.41 ± 0.11</td>
<td>0.61 ± 0.027</td>
<td>27 ± 5.3</td>
<td>2.3 × 10⁴</td>
<td>47 ± 16</td>
<td>3.2</td>
</tr>
</tbody>
</table>

Mutagenesis of active site residues

Based on the crystal structure of hGSTZ1-1 and alignment of the sequences of other GSTs and related enzymes [1, 11, 24], we identified a number of residues that could potentially effect catalysis and substrate binding. Ser-14, Ser-15 and Cys-16 were considered the most likely to be involved in catalysis. These residues were mutated to alanine in hGSTZ1c. The specific activities of these mutant enzymes with different substrates are shown in Table 2. The Ser¹⁴→Ala mutant was essentially inactive with all substrates and could not be subjected to further kinetic analysis. In contrast, the Ser¹⁵→Ala mutant showed variable activity with different substrates when compared with hGSTZ1c-1c. Although the Ser¹⁵→Ala mutant exhibited low isomerase activity with MA as a substrate (≈15 % of wild-type) and low activity with CFA, it showed elevated activity with BNPP as the substrate. This elevated activity appears to be driven by a high kcat and occurs despite an elevated Kₘ for MA observed in the Arg¹⁷⁵→Ala mutant compared with the Arg¹⁷⁵→Lys and hGSTZ1c-1c (wild-type) variants.

Discussion

Because of the cumbersome nature of the enzyme assays previously used to assay GSTZ1-1 activity with α-halo acids, we explored the possibility of developing a direct rate assay that could be easily followed spectrophotometrically. We tested both BNPP and BCP as potential substrates, as we had shown previously that 2-chloropropionate was a substrate for hGSTZ1-1 and because we considered that the addition of a nitrophenyl or chlorophenyl substituent could provide a chromophore whose spectral properties would change after conjugation with glutathione. In preliminary studies, we found that BCP showed negligible spectral changes after reaction with glutathione and hGSTZ1-1 and was not studied further. Our studies with BNPP confirmed that GSTZ1-1 catalysed the formation of the expected conjugate GSTZ1-1. The optimal activity of hGSTZ1c-1c was relatively broad but had a peak at pH 10.5, which is considerably higher than the optimum with CFA as a substrate (pH 7.8–8.2) [25]; most other GSTs appear to have optima around pH 8.5 [13]. It was notable that among the naturally occurring allelic variants of hGSTZ1-1, we found that hGSTZ1a-1a had significantly higher activity with BNPP than the other variants. hGSTZ1a has an Arg residue in position 42 in comparison with a Gly in the other variants.
[21]. We have previously reported that hGSTZ1a-1a is resistant to inactivation by DCA and shows high enantiomer selectivity with (R)-2-chloropropionate compared with (S)-2-chloropropionate [23,26]. It is possible that the elevated activity of hGSTZ1a-1a with BNPP may reflect similar enantiomer selectivity. The Arg or Gly residue at position 42 occurs at the tip of the so-called Mu loop region adjacent to helix α2 and together with helices α4 and α5 forms a gateway to the active site. The loop-α2 helix region is a mobile segment in the crystal structure [11], and it is possible that substitutions at this site modify the flexibility of the region. Such a change may modulate conformational changes required in converting between the apo, substrate bound, and product release states, thereby influencing catalysis with a variety of substrates.

The availability of this new direct spectrophotometric assay has facilitated the further investigation of critical residues involved in substrate binding and catalysis in the Zeta class GSTs. Alignment of Zeta class sequences with other members of the GST superfamily shows that Ser-14 in hGSTZ1 aligns with the active-site serine in mammalian Theta-class and insect Delta-class GST sequences and with Ser-11 in TCHQ (tetrachlorohydroquinone) dehalogenase from Sphingomonas chlorophenolica [1,4]. However, the crystal structure of hGSTZ1 suggests that the hydroxyl of Ser-14 is pointing away from the cysteiny1 sulphur of glutathione and cannot form a hydrogen bond. In contrast, the crystal structure of hGSTZ1-1 indicates that the hydroxyl of Ser-15 is in a favourable position to form a hydrogen bond with the cysteiny1 sulphur of enzyme-bound glutathione [11]. This suggests that Ser-15, rather than Ser-14, is likely to be a critical active-site residue. TCHQ dehalogenase catalyses the isomerization of MA to FA and appears to be phyleogenetically related to the Zeta-class GSTs [24]. TCHQ dehalogenase has an active-site motif (Ser-11,Ile-12,Cys-13) that aligns well with the SSC motif in the Zeta-class GSTs, but lacks a Ser residue equivalent to Ser-15 in hGSTZ1. Nevertheless, Cys-13 appears to play a significant role in catalysis by TCHQ dehalogenase [27]. As well as being a substrate, DCA is a mechanism-based inactivator of rat and human Zeta-class GSTs [26]. In previous studies, we demonstrated that inactivation results from the covalent modification of Cys-16 by the 5-(α-carboxymethyl)glutathione carbonium-sulphonium intermediate that results in the inactivation of hGSTZ1-1 during the reaction with DCA [28]. Since the Cys16→Ala mutant is active, it is clear that the thiol of Cys-16 is not required for catalysis in the reactions studied here, and it is likely that the inactivation that occurs with the formation of the Cys-16 adduct is based on steric hindrance. The cysteiny1 thiol of glutathione is within hydrogen bonding or van der Waals distance of the amide of Cys-16 (3.5 Å) [11]; thus this interaction may be important for the binding and orientation of glutathione in the active site.

The crystal structure of hGSTZ1 indicates that the cysteiny1 thiol of enzyme-bound glutathione is located almost directly over the N-terminal end of helix α1 [11]. The proximity of a Cys side chain to the positive end of a helix dipole has been shown to substantially lower the pK of the thiol [33]. The potential influence of a helix dipole on the activation of enzyme-bound glutathione and consequently on catalysis has been proposed in relation to other GSTs, and it is possible that it may also play a role in the Zeta-class GSTs [16,34].

Of the three potential catalytically active-site residues that we have investigated, the Ser14→Ala mutation clearly had the greatest detrimental effect on activity. Thus despite its apparently inappropriate orientation in the active site, Ser-14 appears to be essential for catalysis. Although it is possible that this mutation caused a structural alteration that indirectly eliminated the enzyme’s catalytic capacity, it is also possible that the active site undergoes a conformational change so that Ser-14 can interact with glutathione during the catalytic cycle. Such a change appears to occur in the Pi-class GSTs, where protonation of the bound glutathione causes a conformational change and loss of direct interaction of the catalytic tyrosine with glutathione [35]. In its protonated form, the thiol sulphur points in a different direction to that seen in other glutathione complexes. The crystal structure of the Zeta-class GST from Arabidopsis thaliana does not include glutathione and thus does not provide any further information in this regard [12]. The mutation of Ser-17 in the A. thaliana Zeta-class GST (equivalent to Ser-14 in hGSTZ1) reduces its
activity to <2% and <6% of the wild-type activity with DCA and MA respectively. Consequently, the available evidence indicates that Ser-14 or its equivalent may be of primary importance in catalysis with the currently known substrates of the Zeta-class GSTs. This is consistent with the mechanism proposed for the MAA isomerization reaction [12].

The crystal structure of hGSTZ1 indicated that Arg-175 may form a salt bridge with the carboxylate of α-haloacid substrates and may play a critical role in orientating the substrate so that glutathione can attack the α-carbon and displace the halide [11]. We evaluated this proposed role by constructing and expressing Arg175 → Ala and Arg175 → Lys mutants. The activity of the Arg175 → Ala mutant was significantly diminished with all substrates tested. In contrast, wild-type activities were largely maintained in the Arg175 → Lys mutant, thereby confirming the importance of the charged side chain. The K_m and K_cat/K_m were lower in the Arg175 → Ala mutation for both the BNPP and MA isomerase reactions, again confirming the significance of Arg-175 in orientating the substrate. In addition, the K_m for MA was substantially elevated in the Arg175 → Ala mutant, which supports the contention that Arg-175 contributes to substrate binding and orientation. It is of interest that the K_m for glutathione for both the Arg175 → Ala and Arg175 → Lys mutants was reduced in reactions with both BNPP and MA, indicating that Arg-175 has an additional indirect influence on glutathione binding.

Concluding remarks

In this study, we have developed a new assay that has facilitated the exploration of the potential catalytic roles of several residues previously highlighted by structural studies of the Zeta-class GSTs. Although the enzyme has a range of catalytic activities, the displacement and isomerase reactions are dependent on the first of two conserved serine residues in the active site. Understanding the residues involved in catalysis and the reaction mechanisms will be of benefit in the design of inhibitors that could be used clinically to block the formation of FAA, a toxic intermediate in the tyrosine catabolic pathway. FAA accumulates in patients with FAA hydrolase deficiency (hypertyrosinemia type I) and is thought to be responsible for many of the severe consequences of this disorder [36]. This therapeutic possibility has increased in importance since the recent observation that GSTZ1−/− mice are not severely incapacitated [37].

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Active-site residues of GST Zeta/maleylacetoacetate isomerase


