Residue 234 in glutathione transferase T1-1 plays a pivotal role in the catalytic activity and the selectivity against alternative substrates

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INTRODUCTION

Various alkyl halides are commonly used in industrial tasks, agriculture and food processing. Their utilization in large quantities presents a problem because of their potential toxic and carcinogenic effects [1]. Conjugation of alkyl halides with GSH, catalysed by GST (glutathione transferase), is generally considered to be a detoxification mechanism. The reactions are catalysed by a GST from the Theta class, i.e. GST T1-1, which is present in human erythrocytes and other tissues, with the highest concentrations in liver and kidney [2,3].

In its catalytic activity, GST T1-1 is known to favour relatively small molecules as substrates. Halogenated organic compounds such as methyl chloride, methyl iodide [4], NPB (4-nitrophenethyl bromide) and NBC (4-nitrobenzyl chloride) [5], as well as epoxides exemplified by ethylene oxide [6], 1,2,3,4-diepoxybutane [7] and EPNP (1,2-epoxy-3-(p-nitrophenoxy)propane) [5] are known as GST T1-1 substrates. GST T1-1 also shows some peroxidase activity with organic hydroperoxides [5,8]. In contrast with the above reactions, the conjugation of dichloromethane, 1,2-dibromoethane, 1,2-dichloroethane and other dihalogenated alkanes results in bioactivation via the metabolic pathway that normally serves as a detoxification process [9–11].

In a previous study, we screened a recombinant library of Theta class GSTs for increased alkyltransferase activity [12]. Mutant F2:1215, the enzyme with the highest activity found in this screening, was an hGST (human GST) T1-1 variant differing from the wild-type by only three amino acid residues [12]. One of the changes compared with wild-type hGST T1-1 was the replacement of a tryptophan residue by arginine at position 234. This was the key to enhanced alkyltransferase activity of mutant F2:1215 found in our previous screening. In the present paper, two hGST T1-1 mutants, Trp234Arg (Trp234 → Arg) and Trp234Lys (Trp234 → Lys), were constructed and characterized with various substrates for comparison with hGST T1-1 and F2:1215.

An increased understanding of structure–function relationships in GST T1-1 would facilitate the redesign of GST T1-1 to obtain altered substrate selectivities, as well as increased catalytic efficiency with targeted substrates. Novel GST T1-1 variants with enhanced properties may become valuable tools in bioanalytical and therapeutic applications.

EXPERIMENTAL

Construction of recombinant hGST T1-1

An N-terminal His₉ tag was introduced into hGST T1-1. The plasmid pKHGT1.1 [14] was used as template in a PCR with the primers hT1forEcoHis (5'-ATATGAATTTCATGCATCACA-TCATACATACATGGCCCTTGACCTG-3') and revHindSalBam (5'-ATATAAGCTTGGTCGAGGATCCTAATT-3'). The reaction took place in a total volume of 100 µl containing 0.2 mM dNTPs, 1.5 mM MgCl₂, 5 units/ml Taq DNA polymerase and buffer as

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; CuOOH, cumene hydroperoxide; EPNP, 1,2-epoxy-3-(p-nitro-phenoxy)propane; GST, glutathione transferase; hGST, human GST; mGST, mouse GST; NBC, 4-nitrobenzyl chloride; NPB, 4-nitrophenethyl bromide; SO, styrene 7,8-oxide.

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were pooled and dialysed against 10 mM Tris/HCl, pH 7.8, containing 500 mM imidazole. The enzyme-containing fractions were analysed by SDS/PAGE, using a 12.5% gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 [16]. The purified enzyme was stored at \(-80^\circ\text{C}\) for 3 months without loss of activity.

**Purification of His\textsubscript{6}-tagged hGST T1-1 variants**

The clones of hGST T1-1 as well as Trp234Arg, Trp234Lys and F2:1215 variants were heterologously expressed as described by Jemth and Mannervik [14], with the exception that the concentration of ampicillin in the growth medium was raised to 100 μg/mL. The bacterial cells were harvested, resuspended in buffer A (20 mM sodium phosphate, pH 7.4, containing 0.5 M NaCl, 85 mM imidazole and 10 mM 2-mercaptoethanol), and lysed by treatment with lysozyme (0.2 mg/ml) on ice for 30 min and sonicating. After centrifugation at 30,000 g for 30 min at 2\(^{\circ}\text{C}\), the supernatant fraction containing the recombinant enzyme was loaded on a column (5 cm \(\times\) 3.2 cm internal diameter) packed with Ni-IMAC (Amersham Biosciences, Uppsala, Sweden) with a flow rate of 1.5 ml/min, and the unbound protein was washed away with buffer A. The recombinant GST T1-1 was eluted with buffer A containing 500 mM imidazole. The enzyme-containing fractions were pooled and dialysed against 10 mM Tris/HCl, pH 7.8, 1.0 mM 2-mercaptoethanol, 20% (v/v) glycerol and 0.02% (w/v) sodium azide. The homogeneity of the pooled material was estimated by non-linear regression analysis.

**Measurement of GST activity with standard substrates**

All substrates for activity measurements and kinetic studies were purchased from Sigma–Aldrich (Steinheim, Germany), except CuOOH (cumene hydroperoxide) from Merck (Darmstadt, Germany) and EPNP from Acros Organics (Geel, Belgium).

1-Menaphthyl sulphate was a gift from Dr Brian Gillham, synthesized as described by Clapp and Young [17].

The specific activities of the purified hGST T1-1 and the two variants Trp234Arg and Trp234Lys were measured with EPNP, 0.1 mM NPB, 1 mM CDNB (1-chloro-2,4-dinitrobenzene) and 0.25 mM NBC [18]. Activity with 1-menaphthyl sulphate was determined as described by Gillham [19]. The specific activity with racemic SO (styrene 7,8-oxide) was also measured [20]. The glutathione peroxidase activity of hGST T1-1 variants was determined with CuOOH as a substrate [21]. The assay conditions for the different substrates were summarized in Table 1.

**Novel GST assay based on monitoring iodide anion release**

The conjugation of GSH with straight-chain 1-iodoalkanes catalysed by hGST T1-1 variants was measured spectrophotometrically by monitoring iodide ion release at 226 nm. The GST assay was similar to the dehalogenase assay described by Stourman et al. [22]. The reaction was initiated by the addition of 1-iodoalkane to the mixture of GSH and GST in presence of 0.1 M potassium phosphate, pH 8.0, at 25\(^{\circ}\text{C}\). 1-Iodo-3-phenylpropane (50 μM) was also used as alternative substrate, and, in this case, the substrate was pre-incubated in 0.1 M potassium phosphate buffer, pH 8.0, at 25\(^{\circ}\text{C}\) for 10 min before the reaction was initiated by addition of enzyme. The iodine-containing substrates, 1-iodoalkanes, including all homologues from iodomethane to iodooctane, were obtained from Sigma–Aldrich. The net molar absorption coefficient for the GSH conjugation was determined as \(\Delta F_{226} = 4.5 \text{mM}^{-1} \cdot \text{cm}^{-1}\), by reacting a limited amount of iodooalkane with an excess of GSH.

### Kinetic parameters

EPNP, NPB, iodomethane and iodohexane were chosen as substrates for determination of the kinetic parameters \(k_{cat}\), \(k_{cat}/K_m\) and \(k_{cat}/K_m/K_a\) for the hGST T1-1 variants. The \(k_{cat}\) values were calculated on a subunit basis. The assay conditions are described in Table 1. Kinetic parameters for EPNP and NPB were determined by varying the concentration of electrophile between 0.035 and 0.7 mM for EPNP, and between 0.02 and 0.4 mM for NPB at a constant GSH concentration of 20 mM. The iodomethane concentration was varied between 0.05 and 10 mM at a constant GSH concentration of 2.0 mM. The GSH concentration was varied between 0.08 and 2.0 mM at a constant iodomethane concentration of 5.0 mM. The kinetic parameters were estimated by non-linear regression analysis.

### Table 1  Conditions for spectrophotometric assays of GST

<table>
<thead>
<tr>
<th>Substrate</th>
<th>[Substrate] (mM)</th>
<th>[GSH] (mM)</th>
<th>Buffer</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>(\lambda) (nm)</th>
<th>(\Delta\varepsilon) (mM(^{-1}) · cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPNP</td>
<td>0.5</td>
<td>10</td>
<td>0.1 M NaPi</td>
<td>6.5</td>
<td>30</td>
<td>360</td>
<td>0.5</td>
</tr>
<tr>
<td>NPB</td>
<td>0.1</td>
<td>10</td>
<td>0.1 M NaPi</td>
<td>6.5</td>
<td>30</td>
<td>310</td>
<td>1.2</td>
</tr>
<tr>
<td>CDNB</td>
<td>0.25</td>
<td>5.0</td>
<td>0.1 M NaPi</td>
<td>6.5</td>
<td>30</td>
<td>310</td>
<td>1.9</td>
</tr>
<tr>
<td>SO</td>
<td>1.0</td>
<td>1.0</td>
<td>0.1 M NaPi</td>
<td>6.5</td>
<td>30</td>
<td>340</td>
<td>9.6</td>
</tr>
<tr>
<td>1-Menaphthyl sulphate</td>
<td>0.5</td>
<td>5.0</td>
<td>0.1 M NaPi</td>
<td>7.0</td>
<td>15</td>
<td>340</td>
<td>6.2</td>
</tr>
<tr>
<td>1-iodoalkanes</td>
<td>1.0</td>
<td>1.0</td>
<td>0.1 M KP</td>
<td>8.0</td>
<td>25</td>
<td>226</td>
<td>4.5</td>
</tr>
<tr>
<td>1-iodo-3-phenylpropane</td>
<td>0.05</td>
<td>1.0</td>
<td>0.1 M KP</td>
<td>8.0</td>
<td>25</td>
<td>226</td>
<td>4.5</td>
</tr>
</tbody>
</table>

The final concentration of the solvent ethanol was 1.0% (v/v) in all assays. NaPi, sodium phosphate buffer; KPi, potassium phosphate buffer.
1-Menaphthyl sulphate 0.03
+−
CuOOH 0.40
+−
Iodo-3-phenylpropane 0.041
SO 0.84
+−
Iodo-octane 0.064
+−
Iodoheptane 0.096
+−
CDNB 0.005
+−
Iodohexane 0.089
+−
Iodopentane 0.084
+−
NBC 0.32
+−
Iodobutane 0.105
+−
Iodopropane 0.24
+−
NPB 0.79
+−
Trp234Arg has activity ratios
different ratios of the specific activities for all three variants of
but decreased values with the other substrates investigated. The
increase in activity with NPB and a 2-fold increase with NBC,
changed. In contrast, mutant Trp234Lys showed a moderate 4-fold
with CuOOH and 1-menaphthyl sulphate were essentially un-
EPNP, CDNB and SO were elevated 3–5-fold, whereas the values
strates NPB and NBC (Table 2). The specific activities with
increase in specific activity relative to hGST T1-1 with the sub-
substrates NPB and NBC (Table 2). The specific activities with
EPNP, CDNB and SO were elevated 3–5-fold, whereas the values
CuOOH and 1-menaphthyl sulphate were essentially unchanged.
In contrast, mutant Trp234Lys showed a moderate 4-fold increase in activity with NPB and a 2-fold increase with NBC,
but decreased values with the other substrates investigated. The
different ratios of the specific activities for all three variants of
hGST T1-1 are shown in Table 2.
The specific activities determined showed that mutant
Trp234Arg has activity ratios > 1.0 with all homologous 1-iodo-
alkanes in relation to hGST T1-1 (Table 3). With mutant
Trp234Lys, only iodohexane and 1-iodo-3-phenylpropane were
better substrates for the mutant than for hGST T1-1 (Table 3).
None of the activities of mutant Trp234Lys reached the corres-
ponding values for Trp234Arg. The activity with 1-iodo-3-phenyl
propane was 11-fold higher for mutant Trp234Arg than for mutant
Trp234Lys.
The effect on catalytic efficiency ($k_{cat}/K_m$) of the substitution of
arginine or lysine for tryptophan at position 234 was investigated by
determining the steady-state kinetic parameters using EPNP
and NPB as electrophilic substrates (Figure 1 and Table 4). The
ratio of catalytic efficiency compared with the value for hGST T1-
1 varied among the mutants (Figure 1). Mutant Trp234Arg showed
the highest efficiency ratio (7.9) with EPNP as a substrate. For
NPB, the efficiency ratios were all higher than for EPNP, with
values of 95 for mutant Trp234Arg, and 39 for mutant F2:1215.
Mutant Trp234Lys showed much a lower ratio compared with
F2:1215 and Trp234Arg with both of the alternative substrates
EPNP and NPB (Figure 1).
The effect of the substitutions in position 234 on the catalytic
efficiency was also investigated with iodomethane and iodohexane
as substrates at a constant GSH concentration of 2.0 mM (see sup-
plementary Figures S1 and S2 available at http://www.BiochemJ.
org/bj/388/bj3880387add.htm). Mutant Trp234Arg was approx.
2.7-fold more efficient than hGST T1-1 in catalysing the conju-
gation between GSH and iodomethane. With iodohexane as a
substrate, F2:1215 and Trp234Arg showed 75- and 41-fold
increases respectively in catalytic efficiency compared with the
parental hGST T1-1 (Figure 1). Apparent $k_{cat}/K_m$ values are
shown in Table 4.
When the concentration of GSH was varied at a constant iod-
omethane concentration of 10 mM, all hGST T1-1 variants showed
a lack of rate-saturation in the experimentally accessible range as

### Table 2 Specific activities of mutants Trp234Arg and Trp234Lys in relation to hGST T1-1

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Specific activity (µmol min⁻¹ mg⁻¹)</th>
<th>Activity ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hGST T1-1</td>
<td>Trp234Arg</td>
</tr>
<tr>
<td>Iodomethane</td>
<td>4.96 ± 0.23</td>
<td>64.1 ± 1.3</td>
</tr>
<tr>
<td>Iodoethane</td>
<td>0.586 ± 0.012</td>
<td>4.06 ± 0.11</td>
</tr>
<tr>
<td>Iodopropane</td>
<td>0.24 ± 0.02</td>
<td>0.26 ± 0.04</td>
</tr>
<tr>
<td>Iodo-butane</td>
<td>0.105 ± 0.002</td>
<td>0.168 ± 0.001</td>
</tr>
<tr>
<td>Iodopentane</td>
<td>0.084 ± 0.002</td>
<td>0.58 ± 0.02</td>
</tr>
<tr>
<td>Iodohexane</td>
<td>0.089 ± 0.006</td>
<td>2.17 ± 0.05</td>
</tr>
<tr>
<td>Iodoheptane</td>
<td>0.096 ± 0.001</td>
<td>1.52 ± 0.01</td>
</tr>
<tr>
<td>Iodo-octane</td>
<td>0.064 ± 0.001</td>
<td>0.90 ± 0.04</td>
</tr>
<tr>
<td>Iodo-3-phenylpropane</td>
<td>0.041 ± 0.003</td>
<td>0.71 ± 0.05</td>
</tr>
</tbody>
</table>
shown in supplementary Figure S3 at http://www.BiochemJ.org/bj/388/bj3880387add.htm. The catalytic efficiency was determined from the gradient at the origin of the curve, using a rational function fitted to the experimental data by the SIMFIT program RF/ FIT (http://www.simfit.man.ac.uk). In the reaction with iodohexane, all tested enzymes showed typical Michaelis–Menten (hyperbolic) behaviour with respect to GSH concentration (supplementary Figure S4 at http://www.BiochemJ.org/bj/388/bj3880387add.htm and Table 5).

**DISCUSSION**

GST T1-1 is an enzyme that catalyses the conjugation of GSH with various xenobiotics, many of which are haloalkanes or epoxides. The crystal structure of the other Theta class enzyme known, hGST T2-2, is available [24]. A homology model of hGST T1-1 based on this structure [13] presents a smaller active site in hGST T1-1 compared with the corresponding cavity in the hGST T2-2 structure, in agreement with the experimentally established preference of hGST T1-1 for small substrate molecules. Of special significance for the present study was the proposal that residue 234 plays an important role in the catalytic function, since it is situated near the active site [13].

The hGST T1-1 and mGST T1-1 sequences are 82 % identical at the amino acid level, and most of the residues that are predicted to be part of the active site are conserved. An important difference is found at residue 234, where arginine in mGST T1-1 is replaced by tryptophan in hGST T1-1. The two homologous enzymes display a 100-fold difference in specific activity with NPB as the electrophilic substrate (A. Shokeer, A.-K. Larsson and B. Mannervik, unpublished work). The importance of residue 234 was also indicated when mutant F2:1215 with arginine in position 234 was found to have the highest alkyltransferase activity with NPB. Mutant F2:1215 was identified in a recombinant Theta-class library screened for enhanced activity with alkylating substrates [12].

In the present study, we wanted to elucidate the role of residue 234 in hGST T1-1 and find out if the simple replacement of tryptophan with arginine, or lysine, would confer higher catalytic activity to the enzyme.

**Specific activities with conventional GST substrates**

Specific activities were determined for hGST T1-1, Trp234Arg, and Trp234Lys with different Theta-class substrates. Table 2 shows that Trp234Arg is a more efficient enzyme than hGST T1-1 with all tested substrates except CuOOH. The alkyltransferase activities with NPB and NBC represent the largest enhancements compared with hGST T1-1, apparently verifying the importance of Arg234 for the increased activity of mutant F2:1215. NPB and NBC are the only substrates with which the second mutant, Trp234Lys, shows higher activities than hGST T1-1. Like the wild-type hGST T1-1, the mutants show very low activity with CuOOH, which is found at residue 234, where arginine in mGST T1-1 is replaced by tryptophan in hGST T1-1. The two homologous enzymes display a 100-fold difference in specific activity with NPB as the electrophilic substrate (A. Shokeer, A.-K. Larsson and B. Mannervik, unpublished work). The importance of residue 234 was also indicated when mutant F2:1215 with arginine in position 234 was found to have the highest alkyltransferase activity with NPB. Mutant F2:1215 was identified in a recombinant Theta-class library screened for enhanced activity with alkylating substrates [12].

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there are significant differences between the mutants in their activities with various substrates (Table 2).

Specific activities with 1-iodoalkanes and steady-state-kinetic parameters

Specific activities with nine different 1-iodoalkanes were also determined for hGST T1-1, Trp234Arg and Trp234Lys. All hGST T1-1 variants showed highest activity with iodomethane as substrate (Table 3). Although hGST T1-1 prefers small substrates, the specific activity does not monotonously decrease with increasing chain length of the 1-iodoalkanes. The activities of the parental hGST T1-1 decrease to an approximately constant level < 0.1 µmol·min⁻¹·mg⁻¹, whereas the activities of Trp234Arg and Trp234Lys initially decrease with chain length and then increase to a local maximum at iodohexane. With the larger substrates iodohexane, EPNP and NPB, a lower $K_m$ value indicates a higher affinity for the mutants than for the wild-type enzyme. This may be due to an increased volume of the active-site cavity created by replacing tryptophan with the less bulky residues arginine or lysine. For a small substrate, such as iodomethane, the $K_m$ value change suggests less tight binding. On the other hand, the main effect of the mutations is noted in the $k_{cat}/K_m$ values, which increase markedly with arginine in position 234. In reactions catalysed by GST T2-2, product release is rate-limiting [26], suggesting that, also for hGST T1-1, $k_{cat}$ reflects the dissociation of the product from the enzyme. A conformational change, which is linked to catalytic turnover, may be facilitated in the enzyme when tryptophan is replaced by a residue with higher mobility.

The catalytic efficiency, $k_{cat}/K_m$, is a measure of the approach to the transition state of the chemical reaction. In the present case, the kinetic data demonstrate an approx. 100-fold increase in stabilization of the transition state with the alkyl substrates NPB and iodohexane as a result of the substitution of arginine for tryptophan. The structure of the activated complex in the nucleophilic substitution reaction includes a negative charge migrating from the sulphur of GSH to the leaving halide of the alkyl substrate (Figure 2). Therefore a positively charged active-site residue close to the leaving group could be expected to promote the catalytic process. However, the limited effect of the lysine substitution suggests that a positive charge at position 234 is insufficient for enhanced activity, even if the side chain of arginine is longer and could reach further than that of lysine.

The catalytic efficiencies ($k_{cat}/K_m$) with EPNP and NPB are higher for mutant Trp234Arg than for F2:1215. These results suggest that amino acid 234 is key to the increased alkyltransferase activity displayed in mutant F2:1215, but also that the additional two mutations (Cys<sup>14</sup> → Ser and Thr<sup>226</sup> → Ile) in F2:1215 attenuate the enhanced activity. Iodomethane and iodohexane give catalytic efficiency profiles that differ both from the results with NPB and EPNP, and from each other. With iodomethane as substrate, the catalytic efficiency is significantly higher (2.7-fold) for Trp234Arg than for mutant F2:1215, which has the same catalytic activity as the wild-type enzyme. In contrast, the F2:1215 mutant has a 75-fold higher $k_{cat}/K_m$ value than hGST T1-1 with iodohexane as substrate, whereas the catalytic activity increased 41-fold for mutant Trp234Arg, i.e. the $k_{cat}/K_m$ for F2:1215 is 1.8-fold higher than that for Trp234Arg. An explanation of this difference in kinetic properties between F2:1215 and Trp234Arg in the reactions with iodomethane and iodohexane requires further investigation. A working hypothesis could be that the higher rate obtained with iodomethane is limited by a conformational change linked to product release, which is smaller or absent with the slower reacting iodohexane. The additional mutations in F2:1215 might influence the conformational flexibility of the enzyme.

It is noteworthy that the increased activity of Trp234Arg with alkylating agents is dependent on the substrate tested, whereas no increase of the peroxidase activity with CuOOH was observed. Thus residue 234 has an important influence on the magnitude of the catalytic activity of GST T1-1, and also plays a pivotal role in determining the differential substrate selectivity of the enzyme.

Possible significance of differential alkyltransferase activity of GST T1-1 in humans

There are many examples of genetic polymorphisms that affect the human metabolism of xenobiotics. The first null GST phenotype was discovered in the Mu class GST M1-1 [27–29], later identified with a gene deficiency [30]. In contrast, it has been found that some Saudi Arabian individuals have more than two copies of the gene encoding GST M1-1 in their genomes [31]. In the same population, one of the cytochrome P450 (CYP2D6) genes has up to 13 copies in some individuals. However, humans with gene duplications that lead to overexpression of Theta class GSTs have not yet been identified.

Dependent on ethnic group, 10–65 % of the human population is lacking GST T1-1 owing to a gene deletion [32]. Although hGST T1-1 is an important catalyst of detoxification reactions, it could probably be beneficial to be devoid of a functional gene, since the enzyme also catalyses the bioactivation of some substrates. GST T1-1 activity in mouse tissues is high in comparison with the activities in rat and human tissues [33,34], and this difference in the capacity of bioactivation appears to explain why dichloromethane is a potent carcinogen in mice, but not in rats and humans. A similar GST T1-1-catalysed activation of a human toxicant, of unknown nature, may be a cause of the high frequency of the null allele. Thus the marked differences in the appearance of the null allele might reflect the notion that humans of different ethnicities are exposed to different toxic compounds.

Another way to minimize the adverse consequences of bioactivation could be to express an enzyme that is less efficient and thereby limit the formation of toxic products. Compared with hGST T1-1 having tryptophan at residue 234, the rodent GST T1-1 with arginine in the same position displays high alkyltransferase
activity (A. Shokeer, A.-K. Larsson and B. Mannervik, unpublished work), but the expression of the enzyme in the target tissues is lower in the rat than in the mouse [33–35]. The diversity in cellular GST T1-1 activity may have evolved as a result of differences in the dietary and environmental substances to which the different species are exposed. The beneficial effect of detoxification of some substances obviously needs to be balanced against the adverse effect of activation of other compounds to which the organism is exposed.

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