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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GST (glutathione transferase) T1-1 plays an important role in the biotransformation of halogenated alkanes, which are used in large quantities as solvents and occur as environmental pollutants. Many reactions that are catalysed by GST T1-1 qualify as detoxification processes, but some reactions with dihalogenated alkanes lead to reactive products more toxic than the substrates. Murine GST T1-1 is particularly active with dichloromethane, which may explain the high carcinogenicity of dichloromethane in the mouse. Human GST T1-1 activity is considerably lower with halogenated hydrocarbons and some related substrates. Human GST T1-1 is polymorphic with a frequent null phenotype, suggesting that it is advantageous, under some circumstances, to lack the functional enzyme, which catalyses GSH conjugations that may cause bioactivation. The present study shows that amino acid residue 234 is a determinant of the differences in catalytic efficiency between

the human and the rodent enzymes. The replacement of Trp²³⁴ in human GST T1-1 by arginine, found in the rodent enzyme, enhanced the alkyltransferase activity by an order of magnitude with a series of homologous iodoalkanes and some typical GST substrates. The specific activity of the alternative mutant Trp²³⁴ → Lys was lower than for the parental human GST T1-1 with many substrates, showing that a positive charge is not sufficient for increased activity. The enhanced activity of Trp²³⁴ → Arg with alkylating agents was dependent on the substrate tested, whereas no increase of the peroxidase activity with cumene hydroperoxide was noted. Residue 234 therefore is also involved in the control of the substrate selectivity of GST T1-1.

Key words: alkyl halide, bioactivation, glutathione transferase T1-1, iodoalkane, nitrophenethyl bromide, substrate selectivity.

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. An arginine residue is also present at this position in rodent GST

T1-1, and could be a major contributor to high alkyltransferase activity distinguishing the rodent enzymes from hGST T1-1 [13]. For example, the specific activity of mGST (mouse GST) T1-1 with NPB as the electrophilic substrate is > 100 times higher than that of hGST T1-1 (A. Shokeer, A.-K. Larsson and B. Mannervik, unpublished work). Based on this information, we wanted to elucidate if an arginine residue at position 234 would influence the catalytic properties of wild-type hGST T1-1, and if 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 His₆ tag was introduced into hGST T1-1. The plasmid pKHGT1.1 [14] was used as template in a PCR with the primers hT1forEcoHis (5'-ATATGAATTCATGCATCACCATCATCATCATGGGCCTTGAGCTG-3') and revHindSalBam (5'-ATATAAGCTTGTGCGACGGATCCTTA-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*-nitrophenoxy)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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Table 1 Conditions for spectrophotometric assays of GST

The final concentration of the solvent ethanol was 1.0 % (v/v) in all assays. NaP_i, sodium phosphate buffer; KP_i, potassium phosphate buffer.

Substrate	[Substrate] (mM)	[GSH] (mM)	Buffer	pH	Temperature (°C)	λ (nm)	$\Delta\epsilon$ (mM ⁻¹ · cm ⁻¹)
EPNP	0.5	10	0.1 M NaP _i	6.5	30	360	0.5
NPB	0.1	10	0.1 M NaP _i	6.5	30	310	1.2
NBC	0.25	5.0	0.1 M NaP _i	6.5	30	310	1.9
CDNB	1.0	1.0	0.1 M NaP _i	6.5	30	340	9.6
SO	1.6	5.0	0.25 M Tris/HCl	7.2	30	234	0.77
CuOOH	1.5	1.0	0.1 M NaP _i	7.0	30	340	-6.2
1-Menaphthyl sulphate	0.5	5.0	0.1 M NaP _i	7.5	30	298	2.5
1-Iodoalkanes	1.0	1.0	0.1 M KP _i	8.0	25	226	4.5
1-Iodo-3-phenylpropane	0.05	1.0	0.1 M KP _i	8.0	25	226	4.5

recommended by the manufacturer (Fermentas, Vilnius, Lithuania). The amplified product was digested with EcoRI and HindIII, run on a 1 % (w/v) agarose gel and purified with QIAquick (Qiagen, Hilden, Germany). The digested PCR product was then ligated to the EcoRI and HindIII cloning sites of pKK-D [15]. The resulting construct (hGSTT1-His) was used to transform electrocompetent *Escherichia coli* XL1-Blue cells (Stratagene, La Jolla, CA, U.S.A.).

The hGST T1-1 mutants Trp234Arg and Trp234Lys were constructed using hGSTT1-His as template in PCR with hT1forEcoHis as forward primer and the primers W234R (5'-ATATAAGCTTTTATTAACGGATCATGGCCAGCACTCTGGGCAT-3') and W234K (5'-ATATAAGCTTTTATTAACGGATCATGGCCAGCACTTTGGGCAT-3') respectively, as reverse primers. The conditions of PCR and subcloning were as described above. The His₆-tagged hGST T1-1 variant F2:1215 was available from earlier studies [12].

Purification of His₆-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 sonication. After centrifugation at 30 000 g for 30 min at 2 °C, the supernatant fraction containing the recombinant enzyme was loaded on a column (5 cm × 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 analysed by SDS/PAGE, using a 12.5 % (w/v) polyacrylamide gel. Protein bands were visualized by staining with Coomassie Brilliant Blue R-250 [16]. The purified enzyme was stored at -80 °C for 3 months without loss of activity.

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 0.5 mM EPNP, 0.1 mM NPB, 1 mM CDBN (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 are 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 °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 °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 iodo-octane, were obtained from Sigma-Aldrich. The net molar absorption coefficient for the GSH conjugation was determined as $\Delta\epsilon_{226} = 4.5 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, by reacting a limited amount of iodoalkane with an excess of GSH.

Kinetic parameters

EPNP, NPB, iodomethane and iodoheptane were chosen as substrates for determination of the kinetic parameters K_m , k_{cat} and k_{cat}/K_m 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 10 mM. The concentration of iodoheptane varied between 0.01 and 5.0 mM at a constant GSH concentration of 2.0 mM for determination of the kinetic parameters. The GSH concentration was varied between 0.08 and 2.0 mM at a constant iodoheptane concentration of 5.0 mM. The kinetic parameters were estimated by non-linear regression analysis.

Table 2 Specific activities with conventional GST substrates of mutants Trp234Arg and Trp234Lys in relation to hGST T1-1

ND, not determined.

Substrate	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)			Activity ratios		
	hGST T1-1	Trp234Arg	Trp234Lys	Trp234Arg/hGST T1-1	Trp234Lys/hGST T1-1	Trp234Arg/Trp234Lys
EPNP	7.84 ± 0.16	26.6 ± 0.4	5.78 ± 0.61	3.4	0.74	4.6
NPB	0.79 ± 0.03	41.3 ± 0.1	3.47 ± 0.14	52	4.4	12
NBC	0.32 ± 0.01	17.5 ± 0.2	0.64 ± 0.01	54	2.0	27
CDNB	0.005 ± 0.002	0.018 ± 0.002	0.001 ± 0.0002	3.6	0.25	14
SO	0.84 ± 0.03	3.79 ± 0.08	0.42 ± 0.01	4.5	0.5	9.0
CuOOH	0.40 ± 0.01	0.35 ± 0.02	0.13 ± 0.01	0.87	0.33	2.7
1-Menaphthyl sulphate	0.03 ± 0.001	0.03 ± 0.003	ND	1.0	ND	ND

Table 3 Specific activities of hGST T1-1 variants using different 1-iodoalkanes as the electrophilic substrate

The concentration of iodoalkanes used was 1.0 mM and the concentration of iodo-3-phenylpropane was 0.05 mM. The final ethanol concentration in all assays was 1.0% (v/v). ND, not determined.

Substrate	Specific activity ($\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$)			Activity ratios		
	hGST T1-1	Trp234Arg	Trp234Lys	Trp234Arg/hGST T1-1	Trp234Lys/hGST T1-1	Trp234Arg/Trp234Lys
Iodomethane	4.96 ± 0.23	64.1 ± 1.3	2.26 ± 0.07	13	0.46	28
Iodoethane	0.586 ± 0.012	4.06 ± 0.11	0.42 ± 0.01	6.9	0.72	9.6
Iodopropane	0.24 ± 0.02	0.26 ± 0.04	0.02 ± 0.002	1.1	0.09	13
Iodobutane	0.105 ± 0.002	0.168 ± 0.001	0.023 ± 0.001	1.6	0.22	7.3
Iodopentane	0.084 ± 0.002	0.58 ± 0.02	ND	6.8	ND	ND
Iodohexane	0.089 ± 0.006	2.17 ± 0.05	0.32 ± 0.01	24	3.6	6.8
Iodoheptane	0.096 ± 0.001	1.52 ± 0.1	ND	16	ND	ND
Iodo-octane	0.064 ± 0.001	0.90 ± 0.04	ND	14	ND	ND
Iodo-3-phenylpropane	0.041 ± 0.003	0.71 ± 0.05	0.067 ± 0.003	17	1.6	11

RESULTS

Characterization of purified hGST T1-1 variants

hGST T1-1, as well as the Trp234Arg, Trp234Lys and F2:1215 variants, were expressed in *E. coli* with an N-terminal His₆ tag. The addition of a His₆ tag to hGST T1-1 allowed a rapid single-step purification through Ni-IMAC affinity chromatography, resulting in a 20-fold increased yield (75 mg/l of bacterial culture) compared with non-His₆-tagged wild-type enzyme obtained by Jemth and Mannervik [14]. Unfrozen samples of the mutants Trp234Arg and Trp234Lys had decreased stability compared with hGST T1-1. This low stability was most pronounced with mutant Trp234Lys, which partially precipitated after the purification and dialysis. Therefore all measurements reported for mutant Trp234Lys were carried out on the first day after purification. The yield of Trp234Arg and Trp234Lys variants were comparable with the yield of His₆-tagged hGST T1-1.

A single amino acid substitution of tryptophan by arginine or lysine at position 234 was shown to change the substrate specificity of hGST T1-1. Mutant Trp234Arg displayed a 50-fold increase in specific activity relative to hGST T1-1 with the substrates NPB and NBC (Table 2). The specific activities with EPNP, CDNB and SO were elevated 3–5-fold, whereas the values with 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-iodoalkanes in relation to hGST T1-1 (Table 3). With mutant

Trp234Lys, only iodoheptane 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 corresponding values for Trp234Arg. The activity with 1-iodo-3-phenylpropane 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 iodoheptane as substrates at a constant GSH concentration of 2.0 mM (see supplementary 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 conjugation between GSH and iodomethane. With iodoheptane 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} and K_m values are shown in Table 4.

When the concentration of GSH was varied at a constant iodo-methane concentration of 10 mM, all hGST T1-1 variants showed a lack of rate-saturation in the experimentally accessible range as

Figure 1 Catalytic efficiency of hGST T1-1 variants with four different substrates

The catalytic efficiencies (k_{cat}/K_m) of hGST T1-1, F2:1215, Trp234Arg and Trp234Lys were determined with (A) EPNP, (B) NPB, (C) iodomethane and (D) iodohehexane as substrates. Results are means $\pm$ S.E.M. ($n = 3$).

Table 4 Steady-state kinetic parameters of hGST T1-1 variants with alternative electrophilic substrates

The hGST T1-1 variants were assayed at varying concentrations of EPNP (0.035–0.7 mM) and NPB (0.02–0.4 mM) at a constant GSH concentration of 20 mM. Initial velocities were measured at substrate concentrations between 0.05 and 10 mM for iodomethane, and between 0.01 and 5.0 mM for iodohehexane at a constant GSH concentration of 2.0 mM. The k_{cat} values were calculated on a subunit basis.

Substrate	Parameter	Enzyme variant			
		hGST T1-1	Trp234Arg	Trp234Lys	F2:1215
EPNP	K_m (mM)	0.32 ± 0.05	0.11 ± 0.01	0.23 ± 0.02	0.14 ± 0.05
	k_{cat} (s^{-1})	7.1 ± 0.5	18.3 ± 0.5	6.6 ± 0.2	12.1 ± 1.3
NPB	K_m (mM)	0.38 ± 0.07	0.13 ± 0.03	0.11 ± 0.02	0.05 ± 0.01
	k_{cat} (s^{-1})	1.23 ± 0.14	42 ± 3.2	3.1 ± 0.2	6.2 ± 0.4
Iodomethane	K_m (mM)	0.08 ± 0.01	0.19 ± 0.02	0.24 ± 0.03	0.46 ± 0.04
	k_{cat} (s^{-1})	7.4 ± 0.1	45.5 ± 1.2	0.019 ± 0.001	43.6 ± 0.9
Iodohehexane	K_m (mM)	0.058 ± 0.011	0.029 ± 0.007	0.039 ± 0.008	0.012 ± 0.002
	k_{cat} (s^{-1})	0.068 ± 0.002	1.45 ± 0.04	0.204 ± 0.005	1.03 ± 0.03

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 RFFIT (<http://www.simfit.man.ac.uk>). In the reaction

with iodohehexane, 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 CDNB, which otherwise is a standard substrate for many GSTs. Neither was any significant activity observed with 1-menaphthyl sulphate, which is a typical substrate for hGST T2-2 [25]. Despite the similarities between arginine and lysine in size and charge,

Table 5 Steady-state kinetic parameters of hGST T1-1 variants for variable GSH concentrations of 1-iodohehexane and 1-iodomethane

The GSH concentration was varied between 0.08 and 2.0 mM at constant iodohehexane and iodomethane concentrations of 5.0 and 10.0 mM respectively.

Enzyme variant	Iodohehexane			Iodomethane (k_{cat}/K_m) ^{GSH} ($\text{mM}^{-1} \cdot \text{s}^{-1}$)	Ratio of efficiency to hGST T1-1	
	K_m^{GSH} (mM)	$k_{\text{cat}}^{\text{GSH}}$ (s^{-1})	(k_{cat}/K_m) ^{GSH} ($\text{mM}^{-1} \cdot \text{s}^{-1}$)		Iodohehexane	Iodomethane
hGST T1-1	0.09 ± 0.01	0.045 ± 0.001	0.57 ± 0.08	3.56 ± 0.04	(1)	(1)
Trp234Arg	0.35 ± 0.05	1.74 ± 0.07	5.01 ± 0.51	73.9 ± 1.0	9	21
Trp234Lys	0.13 ± 0.02	0.18 ± 0.01	1.43 ± 0.15	0.19 ± 0.02	3	0.1
F2:1215	0.51 ± 0.08	1.43 ± 0.08	2.82 ± 0.31	16.1 ± 3.3	5	5

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 $< 0.1 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$, whereas the activities of Trp234Arg and Trp234Lys initially decrease with chain length and then increase to a local maximum at iodoheptane. With the larger substrates iodoheptane, 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} 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 iodoheptane 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 (Cys¹⁴ → Ser and Thr²²⁶ → Ile) in F2:1215 attenuate the enhanced activity. Iodomethane and iodoheptane 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 iodoheptane 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 iodoheptane 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 iodoheptane. The additional mutations in F2:1215 might influence the conformational flexibility of the enzyme.

Figure 2 Schematic diagram of an activated complex between GSH and the alkyl substrate ($\text{R-CH}_2\text{-X}$)

The negatively charge migrates from sulphur on GSH to the halide (X) leaving the alkyl substrate. A positively charged active-site residue in the vicinity of X would be expected to facilitate the chemical transformation.

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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