Heterologous expression of the allelic variant Mu-class glutathione transferases μ and ψ

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cDNA encoding the more acidic form, glutathione transferase (GST) ψ , of the polymorphic Mu-class GSTs discovered in liver, was mutated in the 5'-end to create an *NcoI* site, facilitating cloning into the expression plasmid pKK233-2. The protein expressed from this construct has a point mutation Pro-2→Ala-2, but gives a catalytically functional protein. Back-mutation of the codon for amino acid residue 2 gave rise to a plasmid expressing the wild-type enzyme GST ψ , or GST Mulb-1b. A variant cDNA, differing only in specifying lysine rather than asparagine in position 173 of the coding region, was generated by site-directed mutagenesis. The variant sequence corresponds to another cDNA clone isolated from a human liver cDNA library and expresses the near-neutral GST μ , or GST Mula-1a. The two recombinant proteins GST Mula-1a and GST Mulb-1b, by physicochemical as well as kinetic criteria, were found to be indistinguishable from GST μ and GST ψ respectively, isolated from human liver. It is therefore concluded that the recombinant proteins correspond to the allelic variants observed in the human population. The two forms have different isoelectric points and their protein subunits can be separated by h.p.l.c. on a reverse-phase column. With standard substrates and inhibitors no differences in kinetic parameters between the two variants were detected. The mutated GST Mulb-1b (Pro-2→Ala) was not significantly different in catalytic properties from the wild-type enzyme, even though Pro-2 is a well-conserved amino acid residue in the known Mu-class GSTs.

INTRODUCTION

Glutathione transferase (GST) μ was discovered as an enzyme present in high concentration in some, but not all, human livers [1]. It was demonstrated that, in comparison with other known GSTs, GST μ was particularly active in the conjugation, and thereby the inactivation, of various mutagenic and carcinogenic epoxides [2,3]. It was consequently proposed that individuals lacking GST μ would be more susceptible to certain genotoxic chemicals [2,3]. This proposal received support from the finding that smokers lacking the enzyme appear to be subject to a greater risk of contracting lung cancer than those who expressed the enzyme [4]. Studies of a large number of individuals indicated that the polymorphic expression of GST μ [1] was genetic in nature [5,6], and more recent studies on genomic DNA suggest that the null genotype is due to a gene deletion [7,8]. It has also been shown at the DNA level that the inheritance of the GST μ gene is Mendelian [9].

Another Mu-class GST [10] in human liver with a lower isoelectric point has also been identified [5,6] and purified under the name GST ψ [11]. It is generally believed that this is an allelic variant of the enzyme originally identified as GST μ . This interpretation is based on the similarities in enzymic properties [12] and non-coding sequences of corresponding cDNAs [7,13], as well as genetic analysis of the expression of the variant forms [5]. GST μ corresponds to the product of the type 2 allele of the *GST1* locus, and GST ψ corresponds to the type 1 allele of the *GST1* locus. These two allelic variants were distinguished on the basis of their mobility in starch-gel electrophoresis, where the product of the type 2 allele (GST μ) is more basic [5].

The two enzyme forms appear to correspond to different cDNA clones [7,13], which encode proteins with identical sequences except in amino acid position 173, where one sequence contains asparagine and the other one contains lysine. This

variation in charge is consistent with the difference in electrophoretic mobility.

In the present investigation, recombinant expression clones have been constructed for the production and characterization of these two variants of the polymorphic human Mu-class GSTs.

MATERIALS AND METHODS

Materials

S-Hexylglutathione was synthesized by the method of Vince *et al.* [14].

All restriction and DNA-modifying enzymes used were commercially available and used according to the manufacturers' recommendations.

Calf intestinal phosphatase was purchased from Boehringer Mannheim (Mannheim, Germany) and the vectors pKK233-2 [15] and M13mp18 from Pharmacia LKB Biotechnology (Uppsala, Sweden).

Radioactive nucleotides and mutagenesis kits were supplied by Amersham International (Amersham, Bucks., U.K.).

Oligodeoxynucleotides were purchased from Operon Technologies (Alameda, CA, U.S.A.).

Growth medium was prepared with tryptone and yeast extract from Difco Laboratories (Detroit, MI, U.S.A.). Antibiotics, 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside and isopropyl β -D-thiogalactopyranoside were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), and all other reagents used were of *pro analysi* purity.

Nomenclature of recombinant Mu-class GSTs

GST Mula-1a and GST Mulb-1b correspond to GST μ and GST ψ purified from human liver. The corresponding subunits are denoted as Mula and Mulb.

The numbering of amino acid residues in the protein sequences

Abbreviation used: GST, glutathione transferase.

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presented includes the initiator methionine residue. This residue is cleaved off in the mature proteins.

Plasmid constructions

The plasmid pGTH411, a pEMBL19 derivative of the cDNA encoding the human GST subunit Mu1b (also designated ψ or GST1*1 [5]), ligated into the *Eco*RI site, was digested with *Eco*RI and *Pst*I to yield a 1068 bp fragment including the coding and parts of the non-coding region of the cDNA. The fragment was excised from an agarose gel after electrophoresis and staining in ethidium bromide and purified by adsorption on silica gel (Geneclean; Bio101, La Jolla, CA, U.S.A.).

The purified fragment was ligated to *Eco*RI-*Pst*I-digested M13mp18 and used to transform competent *Escherichia coli* DH5 α F'. The bacteria were plated out in 2 ml of H-top agar [1% (w/v) tryptone, 0.8% (w/v) NaCl and 0.8% (w/v) agar] together with 200 μ l of lawn culture of *E. coli* DH5 α F' and 10 μ l of 0.1 M-isopropyl β -D-thiogalactopyranoside and 50 μ l of 2% (w/v) 5-bromo-4-chloroindol-3-yl β -D-galactopyranoside. After the top agar had set, the plates were incubated at 37 °C overnight.

Colourless plaques were picked out and single-stranded DNA was purified for site-directed mutagenesis by a method based on that of Eckstein and co-workers [16] by using the kit supplied by Amersham International. After preparation of double-stranded DNA from plaques obtained after the mutagenesis, the replicative-form DNA was digested with *Nco*I for confirmation of mutation. The 310 bp fragment present in mutant clones was isolated as above and ligated into pKK233-2 prepared by *Nco*I digestion followed by treatment with calf intestinal phosphatase. The ligation mixture was transformed as before into *E. coli* DH5 α F' and plated out on LB broth plates containing 50 μ g of ampicillin/ml.

Transformants were screened for plasmid content and insert orientation by restriction analysis with *Pst*I and *Sac*II after DNA mini-preparation [17].

pGTH411 was digested with *Pst*I and *Sac*II, and the 1020 bp fragment was purified as above and ligated into the *Sac*II-*Pst*I-digested pKK233-2 derivative of the *Nco*I-digest fragment and transformed as above.

Transformants were screened for GST activity using 1-chloro-2,4-dinitrobenzene and GSH as substrates. Portions (2 ml) of culture medium 2TY [1.6% (w/v) tryptone, 1% (w/v) yeast extract and 0.5% (w/v) NaCl] were inoculated with a single colony and incubated in the presence of ampicillin at 37 °C on a rotary shaker until the medium was visibly turbid. Isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.5 mM and the cultures were incubated for an additional 3 h as above.

The cultures were poured into Eppendorf tubes and the cells were harvested at 10000 rev./min for 3 min in an Eppendorf centrifuge. The pellets were resuspended in 150 μ l of phosphate-buffered saline (140 mM-NaCl/3.7 mM-KH₂PO₄/11.2 mM-Na₂PO₄, pH 7.2) and sonicated twice for 5 s in a Branson Sonifier equipped with a microtip at power setting 7. Debris was then spun down at full speed in an Eppendorf centrifuge for 2 min and the supernatants were assayed for GST activity.

The plasmids in clones expressing GST activity were characterized by restriction analysis. The plasmid construct was denoted as pKHM1bP2A (Fig. 1 and Table 1).

pKHM1bP2A was digested with *Eco*RI and *Pst*I and the fragment containing the promoter and cDNA was ligated into M13mp18 digested with the same enzymes. Transformation and cloning were performed as above.

The M13mp18 derivative was subjected to site-specific mutagenesis as above to regenerate the wild-type coding sequence (Table 1) and the desired clones were identified by sequencing by

Fig. 1. Construction of the expression plasmid pKHM1bP2A

Key: N, *Nco*I; E, *Eco*RI; P, *Pst*I; S, *Sac*II; Ap, β -lactamase gene; the thick black arrow represents the isopropyl β -D-thiogalactopyranoside-inducible *Trc* promoter; the black box represents the *E. coli* *rrnB* terminator. The final construct pKHM1bP2A was further mutated to yield the original Mu1b as well as the allelic Mu1a sequences. For details see the Materials and methods section.

the DNA dideoxy chain-termination method [18]. The M13mp18 derivative was denoted as mp18TrcHM1b.

The back-mutated DNA including the promoter derived from pKK233-2 was excised from the replicative-form DNA and subcloned into pKK233-2 with the use of the *Eco*RI and *Pst*I sites. The plasmid constructed was denoted as pKHM1b (Table 1).

mp18TrcHM1b DNA was mutated as above to yield the allelic sequence coding for subunit Mu1a, also denoted as μ [1], GST1*2 [5] and H_b [13], a change resulting in replacement of asparagine-173 by lysine at the protein level (Table 1). After sequencing, the DNA was subcloned into pKK233-2 as above. The plasmid was denoted as pKHM1a (Table 1).

All DNA species generated by the mutagenesis procedure were sequenced through their entire coding regions to rule out any unwanted mutations.

Protein purification

Plasmids were transfected into *E. coli* JM103. A 35 ml portion of LB broth [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0] was inoculated with 350 μ l of an overnight culture of *E. coli* JM103 carrying the Mu-class-GST-expressing plasmid grown in the presence of 50 μ g of ampicillin/ml. The

Table 1. DNA and deduced protein sequences in created mutants of Mu-class GST sub units

Schematic representation of oligonucleotides used for site-directed mutagenesis for the construction of the Mu-class GST subunits 1bP2A (top), 1b (middle) and 1a (bottom). Asterisks represent mismatches between the oligonucleotides and the DNA to be mutated.

Amino acid no.:	1				5
Amino acid sequence:	M	P	M	I	L
DNA sequence, 1b:	5'-AACCAGCACC	ATG	CCC	ATG	ATA CTG-3'
(mp18HM1b)			*		
Mutamer: 1b(P2A):	3'-GGTCGTGG	TAC	CGG	TAC	TAT GA-5'
Amino acid no.:	1				5
Amino acid sequence:	M	A	M	I	L
DNA sequence, 1bP2A:	5'-GAAACAGACC	ATG	GCC	ATG	ATA CTG-3'
(mp18TrcHM1bP2A)			*		
Mutamer: 1b(A2P):	3'-TGTCTGG	TAC	GGG	TAC	TAT GA-5'
Amino acid no.:	170				176
Amino acid sequence:	F	E	P	N	C L D
DNA sequence, 1b:	5'-TTT GAG	CCC	AAC	TGC	TTG GAC-3'
(mp18TrcHM1b)			*		
Mutamer: 1a (N173K)	3'-A CTC	GGG	TTC	ACG	AAC CTG-5'

35 ml culture was incubated overnight at 37 °C on a rotary shaker.

Six 2000 ml Erlenmeyer flasks each containing 500 ml of LB broth and 50 µg of ampicillin/ml were inoculated with 5 ml of overnight culture and incubated as above.

When cells had reached an A_{600} value of approx. 0.3, isopropyl β -D-thiogalactopyranoside was added to a final concentration of 0.2 mM and the incubation was continued overnight.

After being harvested at 5000 *g* in a Beckman JA14 rotor, the cells were resuspended in 20 ml of 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-EDTA, 15% (w/v) sucrose and 1 mg of lysozyme/ml. The suspension was incubated for 30 min at room temperature.

Cells were disrupted by sonication three times for 20 s, with the same settings as above, and the samples were then placed on ice for 10 min followed by another sonication three times for 20 s. The sonicated cells were then centrifuged at 25000 *g* for 20 min and the supernatant was collected. The pellet, still containing intact cells, was resuspended in 10 ml of water containing 50 µM-phenylmethanesulphonyl fluoride and sonicated as above. After centrifugation as before the pellet was again resuspended and sonicated.

The supernatants from the three sonication treatments were pooled and the GST activity was measured.

The lysate was passed over a Sephadex G-25 (Pharmacia) column (4 cm × 40 cm) equilibrated with 10 mM-Tris/HCl buffer, pH 7.8, containing 1 mM-EDTA, 0.2 mM-dithiothreitol and 50 µM-phenylmethanesulphonyl fluoride (buffer A), and the fractions containing GST activity were pooled.

The pooled fractions were added to an approx. 20 ml *S*-hexylglutathione-Sepharose gel [19] in 20 ml of buffer A and incubated on a rocking platform for 1 h.

After adsorption of the enzyme, the gel was washed with several volumes of buffer A containing 0.2 M-NaCl on a sintered-glass funnel.

After being washed, the matrix was packed in a column and the GST was eluted with 5 mM-*S*-hexylglutathione in the washing buffer.

Active fractions were pooled and the pool was dialysed against 2 × 5 litres of 0.1 M-sodium phosphate buffer, pH 7.0, containing 0.2 mM-dithiothreitol.

Protein concentration was determined by the method of Peterson [20] and purity was established by SDS/PAGE [12.5% (w/v) total acrylamide, 2.7% (w/w) bisacrylamide/total acrylamide] [21] and silver staining [22].

Analytical procedures

The isoelectric points were determined by PAGE/isoelectric focusing on precast gel plates run under the conditions prescribed by the manufacturer (Pharmacia LKB Biotechnology), with parallel runs of standard pI marker proteins.

Native M_r was estimated by gel filtration on a Superose 12 column connected to an f.p.l.c. system (Pharmacia LKB Biotechnology). The chromatography was run at 0.2 ml/min in 0.05 M-sodium phosphate buffer, pH 7.0, containing 0.15 M-NaCl. Marker proteins were monitored photometrically at 280 nm and were yeast glutathione reductase (M_r 110000), BSA (M_r 67000) and chymotrypsinogen A (M_r 25000). The GST was detected by measurement of the enzyme activity with 1-chloro-2,4-dinitrobenzene.

Apparent subunit M_r was determined by SDS/PAGE, with phosphorylase *b* (M_r 94000), BSA (M_r 67000), ovalbumin (M_r 43000), carbonic anhydrase (M_r 30000), soya-bean trypsin inhibitor (M_r 20100) and α -lactoglobulin (M_r 14400) as marker proteins. The proteins were stained with Coomassie Brilliant Blue R-250.

The recombinant proteins were also analysed by h.p.l.c. with a system set up for separation of GST subunits. A 0.5 µg portion of GST Mu1-1 was applied to a Dynamax-300A C-4 column (0.46 cm × 25 cm, 0.1 nm pore size and 5 µm particle size) and chromatographed at 1 ml/min in a gradient consisting of 45% (v/v) acetonitrile in aq. 0.1% (v/v) trifluoroacetic acid for 1 min and 45–60% (v/v) acetonitrile in aq. 0.1% trifluoroacetic acid for 45 min.

The *N*-terminal amino acid sequences were determined by protein sequencing with Applied Biosystems equipment.

Kinetic measurements

Enzyme activities were determined spectrophotometrically in 0.1 M-sodium phosphate buffer, pH 6.5, with alternative electrophilic substrates as follows: 1 mM-1-chloro-2,4-dinitrobenzene in the presence of 1 mM-GSH, monitored at 340 nm; 0.05 mM-*trans*-4-phenylbut-3-en-2-one in the presence of 0.25 mM-GSH, monitored at 290 nm; 0.5 mM-1,2-epoxy-3-(*p*-nitrophenoxy)-propane in the presence of 5 mM-GSH, monitored at 360 nm.

The inhibition studies were performed by addition of either Cibacron Blue F3GA, bromosulphophthalein or *S*-hexylglutathione to a reaction mixture containing 1 mM-1-chloro-2,4-dinitrobenzene and 1 mM-GSH [23]. All measurements were made in triplicate.

Table 2. *N*-Terminal sequences of Mu-class GST subunit mutants determined by protein sequencing

	2*	10	20
μ †:	PMILGYWDIRGLAHAIRLLLEYT...		
Mutamer 1a:	(M)‡PMILGYWDIRGLAHA...		
Mutamer 1b:	(M)‡PMILGYWDIRGLAHA...		
Mutamer 1bP2A:	(M)‡AMILGYWDIRGLAHA...		

* The numbering includes the initiator methionine residue.
† From ref. [33].
‡ The initiator methionine residue is absent from approx. 90% (1a and 1b) and 95% (1bP2A) of the sequenced proteins.

Fig. 2. Reverse-phase h.p.l.c. of human GSTs

Reverse-phase (C-4) analytical h.p.l.c. of human GST subunits. π , GST π purified from placenta; Mu1a, GST Mula-1a, recombinant; Mu1bP2A, GST Mulb-1bP2A, recombinant; Mu1b, GST Mulb-1b, recombinant; ϵ , GST ϵ , recombinant [27]. A 0.5 μ g portion of each protein was added to the column. The elution of the proteins was monitored photometrically at 214 nm. For chromatographic conditions see the Materials and methods section.

RESULTS

Plasmid construction

Mutation of the second codon of the cDNA derived from pGTH411, a plasmid encoding the GST subunit Mu1b, created an extra *Nco*I recognition sequence. This allowed ligation of the coding parts of the cDNA into the expression system pKK233-2 (Fig. 1 and Table 1). The mutation in the cDNA caused a change of the proline in position 2 into alanine in the corresponding protein sequence (Fig. 1 and Table 1).

The wild-type GST Mu1b sequence was recovered, as well as the allelic GST Mula sequence, through two subsequent mutation steps (Table 1).

The expression system utilizes the *Trc* promoter, which can be induced by isopropyl β -D-thiogalactopyranoside in an *E. coli* lacI^q host cell.

Expression and purification of recombinant GST Mu1-1 enzymes

After induction, followed by overnight incubation and purification, 15–20 mg of pure enzyme was routinely recovered from a 3-litre culture. Homogeneity of the protein was established by SDS/PAGE with silver staining (results not shown). Determination of the *N*-terminal sequence of the proteins (Table 2) revealed a slight difference in the extent of the removal of the

Table 3. Physicochemical properties of recombinant GST Mu1-1 proteins

GST	Apparent subunit M_r	Apparent native M_r	Isoelectric point
μ (liver)	26700	—	6.2
1a-1a (recombinant)	26700	45000*	6.2
ψ (liver)	26600	—	5.6
1b-1b (recombinant)	26600	45000*	5.6
1b-1bP2A (recombinant)	26600	45000*	5.6

* The values given have an estimated s.d. of ± 3000 .

initiator methionine residue; the mutant carrying an alanine in position 2 was devoid of the methionine in about 95% of the sequenced protein, whereas the two wild-type proteins, having proline in position 2, had undergone methionine elimination to approx. 90%.

When the purified proteins were eluted through a C-4 reverse-phase h.p.l.c. column, they gave rise to single peaks with slightly differing retention times, caused by the differences in amino acid residues at positions 2 and 173 (Fig. 2). When the recombinant wild-type proteins were co-chromatographed with their counterparts purified from human liver, corresponding subunits were eluted in the same peaks (chromatogram not shown).

Physicochemical parameters

As judged from gel filtration on a Superose 12 column, the two wild-type recombinant GST Mu1-1 enzymes as well as the GST Mu1b-1bP2A mutant occur as functional dimers with a apparent M_r of approx. 45000 (Table 3). The apparent subunit M_r , as judged by migration in SDS/PAGE, is 26700 for the GST Mula subunit and 26600 for the GST Mu1b and Mu1bP2A subunits (Table 3 and Fig. 3).

The isoelectric points of the recombinant enzymes were determined as 6.2 for the GST Mula-1a and 5.6 for the GST Mu1b-1b and Mu1b-1bP2A enzymes. All these data are in good agreement with previous studies of the GST μ and GST ψ enzymes [12], and when the liver-derived enzymes were included in the electrophoresis their properties were indistinguishable from those observed for GSTs Mula-1a and Mu1b-1b respectively (Table 3 and Fig. 3).

Activity measurements

The specific activities determined for the purified recombinant enzymes are in good agreement with previously published results from studies on the liver GST μ [2], including high activity with 1-chloro-2,4-dinitrobenzene and less significant activities with 1,2-epoxy-3-(*p*-nitrophenoxo)propane and *trans*-4-phenylbut-3-en-2-one (Table 4). The *N*-nitrosoguanidinium compound 2-cyano-1,3-dimethyl-1-nitrosoguanidine, which is a substrate as good as 1-chloro-2,4-dinitrobenzene for the rat Mu-class GST 4-4 [24], did not give any significant activity with any of the

Fig. 3. SDS/PAGE of human GSTs

ϵ , GST ϵ , recombinant [27]; μ , GST μ purified from liver; Mu1a, GST Mu1a-1a, recombinant; ψ , GST ψ purified from liver; Mu1b, GST Mu1b-1b, recombinant; Mu1bP2A, GST Mu1b-1bP2A, recombinant; π , GST π purified from placenta. The values indicated for the marker proteins are apparent M_r values.

recombinant GST Mu1-1 enzymes. A related compound, the cytostatic drug 1,3-bis-(2-chloroethyl)-1-nitrosourea, was also not a substrate for these human Mu-class GSTs, with measurement of nitrite formation (results not shown), even though the rat Mu-class enzymes consisting of subunits 3 and 4 display significant activity [25].

Inhibition studies

The recombinant GST Mu1-1 enzymes exhibit sensitivities to the inhibitors Cibacron Blue F3GA, bromosulphophthalein and *S*-hexylglutathione that are essentially identical with those of the enzymes purified from human liver (Table 5). In one case (Cibacron Blue F3GA) the I_{50} value was higher and in another case (*S*-hexylglutathione) lower than reported earlier [26], but the I_{50} values for GST μ and GST ψ from liver determined in the present study are not significantly different from those obtained for the recombinant GST Mu1-1 enzymes.

DISCUSSION

Prokaryotic expression of mammalian GSTs has previously been achieved in several cases, including enzymes of the Alpha class [27-29], the Mu-class [13,30-32] and the Pi class (M. Widersten & B. Mannervik, unpublished work).

As a direct way of demonstrating that the cDNAs previously published by Seidegård *et al.* [7] (GTH411) and by DeJong *et al.* [13] (GTH_h) are encoding functionally similar enzymes and to elucidate whether or not the proteins are identical with GSTs μ and ψ , encoded by the polymorphic *GST1* locus, we expressed both these sequences in *E. coli*.

To accomplish the heterologous expression of these enzymes, we have utilized mutagenesis techniques to create an additional

Table 4. Specific activities of recombinant GST Mu1-1 enzymes

Abbreviations: CDNB, 1-chloro-2,4-dinitrobenzene; *t*PBO, *trans*-4-phenylbut-3-en-2-one; NPEP, 1,2-epoxy-3-(*p*-nitrophenoxy)propane; Cyano-DMNG, 2-cyano-1,3-dimethyl-1-nitrosoguanidine. All values are based on six independent measurements.

GST	Substrate...	Specific activity ($\mu\text{mol}/\text{min per mg}$)			
		CDNB	<i>t</i> PBO	NPEP	CyanoDMNG
μ (liver)		187*	0.36*	0.11*	< 0.01
1a-1a (recombinant)		174 $\pm$ 8	0.3 $\pm$ 0.1	0.2 $\pm$ 0.04	< 0.005
1b-1b (recombinant)		172 $\pm$ 16	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	< 0.005
1b-1bP2A (recombinant)		178 $\pm$ 18	0.3 $\pm$ 0.05	0.20 $\pm$ 0.01	-

* From ref. [2].

Table 5. Inhibition characteristics of recombinant GST Mu1-1 enzymes

The values given are based on four independent measurements.

GST	Inhibitor...	I_{50} (μM)		
		Cibacron Blue	Bromosulphophthalein	<i>S</i> -Hexylglutathione
μ (liver)		0.3 $\pm$ 0.1	4 $\pm$ 2	3 $\pm$ 0.3
1a-1a (recombinant)		0.4 $\pm$ 0.1	5 $\pm$ 1	3 $\pm$ 0.5
ψ (liver)		0.2 $\pm$ 0.1	2 $\pm$ 1	2 $\pm$ 0.1
1b-1b (recombinant)		0.4 $\pm$ 0.2	3 $\pm$ 1	2 $\pm$ 0.2
1b-1bP2A (recombinant)		0.3 $\pm$ 0.1	3 $\pm$ 1	3 $\pm$ 0.5

I_{50} is the concentration of inhibitor giving 50% inhibition in the standard assay with 1 mM-1-chloro-2,4-dinitrobenzene and 1 mM-GSH in 0.1 M-sodium phosphate buffer, pH 6.5.

restriction site in the 5' proximal region of the cDNA coding for the subunit encoded by the GTH411 cDNA, here denoted as subunit Mu1b. This method is easier to control than a more unspecific exonuclease treatment for trimming of the cDNA in order to optimize the distance between the ribosome-binding site (Shine-Dalgarno box) and the translation initiation site before ligation into the expression system.

Using the same mutagenesis system, we were also able to create the other variant sequence, denoted as GST Mula.

The physicochemical and catalytic properties of the expressed recombinant enzymes were indistinguishable from those of the corresponding enzymes purified from human liver, GSTs μ and ψ .

The recombinant GST Mula-1a enzyme displays the same pI, apparent subunit M_r and retention time on h.p.l.c. as GST μ . The same properties are mutually shared between the recombinant GST Mu1b-1b and the liver GST ψ (Table 3 and Figs. 2 and 3).

The catalytic properties, as measured by the enzyme specific activities with different substrates, were similar among all five enzyme preparations (Table 4). The similarity of the Pro-2→Ala mutant of GST Mu1b-1b to the other enzymes indicates a non-essential role of Pro-2 with regard to the catalytic function, even though this residue is conserved in the known Mu-class GSTs.

The studies on the sensitivity of the five Mu-class GST variants towards three different inhibitors, Cibacron Blue, bromosulphophthalein and S-hexylglutathione, also demonstrated strong similarities as expressed in I_{50} value (Table 5). Thus the residues in positions 2 and 173 do not appear to be involved in any critical way in either catalysis or the binding of inhibitors.

In the other known mammalian Mu-class GST sequences, residue 173 is lysine, in all cases but three. In the rat subunit 6 (Yb₆) it is threonine [34], in rat subunit Yb₄ it is glutamic acid [32] and in the Mu-class GST found in human muscle (denoted as GST4) [35] it is serine. The predominance of Lys-173 in the gene family suggests that, of the two human alleles GST Mula and Mu1b, Mula is the original form.

The polymorphic Mu-class GST studied in the present work has received special attention in view of its importance in the detoxication of genotoxic epoxides [2,3]. The expression of the enzyme from cDNA clones will permit structure-activity studies of its protective potential *in vitro* by an enzymological approach and intracellularly by transfection experiments.

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