Mu-class glutathione transferase from *Xenopus laevis*: molecular cloning, expression and site-directed mutagenesis

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A cDNA encoding a Mu-class glutathione transferase (XlGSTM1-1) has been isolated from a *Xenopus laevis* liver library, and its nucleotide sequence has been determined. XlGSTM1-1 is composed of 219 amino acid residues with a calculated molecular mass of 25359 Da. Unlike many mammalian Mu-class GSTs, XlGSTM1-1 has a narrow spectrum of substrate specificity and it is also less effective in conjugating 1-chloro-2,4-dinitrobenzene. A notable structural feature of XlGSTM1-1 is the presence of the Cys-139 residue in place of the Glu-139, as well as the absence of the Cys-114 residue, present in other Mu-class GSTs, which is replaced by Ala. Site-directed mutagenesis experiments indicate that Cys-139 is not involved in the catalytic mechanism of XlGSTM1-1 but may be in part responsible for its structural instability, and experiments *in vivo* confirmed the role of this residue in stability. Evidence indicating that Arg-107 is essential for the 1-chloro-2,4-dinitrobenzene conjugation capacity of XlGSTM1-1 is also presented.

Key words: amphibian, arginine residue, cysteine residue, thermostability.

INTRODUCTION

The name glutathione transferase (GST; E.C. 2.5.1.18) refers to a large group of ubiquitous proteins which catalyse the nucleophilic attack of GSH on electrophilic substrates [1–3]. GSTs are important parts of an organism's detoxification system and, it has been suggested, in the protection of cells against reactive oxygen metabolism [4]. Mammalian GSTs have been investigated intensively. By analysis of their structural and functional properties soluble mammalian GSTs can be divided into at least eight different classes: Alpha, Mu, Pi, Theta, Sigma, Zeta, Omega and Kappa [5–10]. Despite their low inter-class sequence identity, crystallographic analysis has indicated that the overall polypeptide folding of the different classes of soluble GSTs is very similar [5–9]. An additional GST family, designated MAPEG (membrane-associated proteins involved in eicosanoid and glutathione metabolism), has also been defined according to enzymic activities, sequence motifs and structural properties [11]. Compared with mammalian GSTs, relatively little information is available on the structural and functional properties of GSTs occurring in amphibia. We have previously purified and characterized a Pi-class isoenzyme, named bbGSTP1-1, from the common toad *Bufo bufo* in an early developmental stage [12,13]. Further studies from our laboratory revealed that bbGSTP1-1 declines to a very low level in adult liver [14]. In this tissue a GST is present that is structurally, immunologically and kinetically distinct from that found in the embryo, designated bbGSTP2-2 [15]. We have also investigated the distribution of GSTs in *Xenopus laevis*. Preliminary evidence indicated that both embryo and adult liver of *X. laevis* has the same isoenzymic pattern [16]. Although *X. laevis* is frequently used as an experimental animal, little is known about its GSTs, and none of the corresponding nucleotide sequences have been determined so far. With the aim of better understanding the structure-function relationships of GSTs from different amphibian species as well as between GSTs of amphibian and mammalian origin, the major GST isoenzyme (XlGST-7.4) present in *X. laevis* [17] was cloned, overexpressed in *Escherichia coli* and characterized further. Sequence alignment indicated that it is most closely related to mammalian Mu-class GSTs and in particular to a subfamily with 217 amino acid residues and three Cys residues in each subunit [18]. Following the recommended nomenclature [19] it was named XlGSTM1-1. Rationally designed point mutations were also introduced to define the functional importance of residues that may contribute to the characteristic properties of XlGSTM1-1.

MATERIALS AND METHODS

Animals

X. laevis were purchased from a local dealer (Rettili di Schneider Dott. Rainero, Varese, Italy), and maintained in aquaria. Animals were anaesthetized by hypothermia and killed by head concussion. Tissues were excised and frozen immediately in liquid nitrogen before being stored at −80 °C until use.

Identification of *X. laevis* partial GST cDNA

A *X. laevis* cDNA encoding a protein with an amino acid sequence related to the 29 N-terminal residues of XlGST-7.4 [16,17] was identified by a BLAST search of the expressed sequence tag (EST) database (generated by partial sequencing of random cDNA clones and assignment to gene families based on sequence similarities) through the National Centre for Biotechnology Information's website (http://www.ncbi.nlm.nih.gov/BLAST/). The EST clone (accession no. BE506028) was isolated from a *X. laevis* liver cDNA library constructed by Life Technologies.

RNA extraction and reverse transcriptase PCR

mRNA from *X. laevis* liver was obtained by using the Messenger RNA isolation kit (Sigma), according to the manufacturer's

Abbreviations used: GST, glutathione transferase; IPTG, isopropyl β-D-thiogalactoside; EST, expressed sequence tag; LB, Luria–Bertani.

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The amino acid and nucleotide sequences reported for XlGSTM1-1 have been submitted to the EMBL database under accession number AJ416998.

protocol. First-strand cDNA was synthesized using 1 μg of mRNA and the First-Strand Synthesis Kit for reverse transcriptase PCR (RETROscript™; Ambion). We used 100 units of Moloney-murine-leukaemia virus reverse transcriptase and oligo-dT_{18} primer at a concentration of 5 μM, according the manufacturer. The reaction was carried out in 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 500 μM of dNTP mix and 10 units of placental RNase inhibitor, at 37 °C for 50 min.

On the basis of the EST clone identified, a partial cDNA (534 bp) was isolated by PCR by using as primers the following sense and antisense oligonucleotides: 5′-ATGGTGGTGATACTAGGATACTGGG-3′ (forward), and 5′-AAATTTCTGAAGCCAAGTGGGATC-3′ (reverse). PCR amplification was performed using 1 μl of synthesis mix (from a total volume of 20 μl), 200 ng of each primer, 200 μM dNTP mix and 1 units of *Taq* Gold polymerase (PE Applied Biosystems) in its own buffer. The reaction mixture was subjected, according to the manufacturer of the *Taq* polymerase, to a cycle at 95 °C for 10 min, followed by 33 cycles of amplification (60 s at 95 °C, 60 s at 55 °C and 60 s at 72 °C).

A PCR product (534 bp) was recovered from an agarose gel using the Geneclean II Kit (BIO 101) as described by the manufacturer. The DNA fragment recovered from the gel was subjected to a PCR amplification by using as primers FBam (5′-GCGGATCCATGGTGGTGATACTAGGATACTGG-3′; sense) and REco (5′-CGGAATTCAAATTTCTGAAGCCAAGTGGG-3′; antisense). Both primers had 5′ recognition sites for the respective restriction enzymes *Bam*HI and *Eco*RI (underlined). In addition to the template and 200 ng of each primer, the 50 μl reaction mixture contained 200 μM dNTP mix and 1 unit of *Taq* Gold polymerase in its own buffer. The reaction mixture was subjected to one cycle at 95 °C for 10 min and six cycles of amplification (60 s at 95 °C, 60 s at 48 °C and 60 s at 72 °C), followed by 30 cycles of amplification (60 s at 95 °C, 60 s at 55 °C and 60 s at 72 °C).

The PCR product was recovered from an agarose gel using the Geneclean II Kit, digested with *Eco*RI and *Bam*HI, and cloned into the *Eco*RI/*Bam*HI sites of the *E. coli* vector pBluescript SK; the resulting plasmid was named FBam-REco BSK. The PCR product was confirmed by DNA sequencing.

Library screening and cDNA clone isolation

A DNA probe (FBam-REco) was prepared from FBam-REco BSK labelled with fluorescein using the PCR Fluorescein Labelling Mix (Roche), according the manufacturer.

The UNI-ZAP *X. laevis* liver cDNA library (Stratagene) was screened in two steps. The first round of screening was performed by PCR, using forward and reverse primers, as described by Lardelli and Lendahl [20]. The tube from which the PCR product was generated was plated at approx. 50000, 5000 and 500 p.f.u. of viral encapsulated DNA for each 150 mm culture plate.

Hybridization using the probe, prepared as described above, was performed using the Chemiluminescent Detection System (Southern-star™; PE Applied Biosystems), according to the manufacturer's protocol. Single positive plaques were picked and pBluescript phagemids were excised by the *in vivo* excision protocol using ExAssist helper phage and *E. coli* SORL strain (Stratagene). The resulting plasmid was named XlGSTM1-pBSK. The DNA insert in pBluescript was confirmed by sequencing.

Nucleotide sequence was translated into amino acidic sequence and similarities between the primary structures of GSTs were searched using the Swiss-Prot Protein Sequence Data Bank and protein analyses were done using the proteomics tools of the ExPASY Data Bank.

Construction of an expression plasmid containing the *XlGSTM1* gene

Two oligonucleotide primers were designed as follows: fEcoXM (5′-GCGAATTCATGGTGGTGATACTAGGATAC-3′; sense) and rBamXM (5′-CGGGATCCTTATTTTTTGTTGCCCCAGG-3′; antisense). The primers contained 5′ recognition sites for *Eco*RI and *Bam*HI (underlined) respectively. To obtain the *XlGSTM1* gene, we performed PCR using XlGSTM1-pBSK as a template, and fEcoXM and rBamXM as primers at a concentration of 200 ng. In addition to the template and 200 ng of each primer, the 50 μl reaction mixture contained 200 μM dNTP mix and 1 unit of *Taq* Gold polymerase in its own buffer, and was subjected to 1 cycle at 95 °C for 10 min and 30 cycles of amplification (60 s at 95 °C, 60 s at 55 °C and 60 s at 72 °C), followed by 10 min at 72 °C. The PCR product was recovered from the agarose gel by using the Geneclean II Kit, digested with restriction enzymes *Eco*RI/*Bam*HI and ligated into the *Eco*RI/*Bam*HI sites of pBTac 1 (Boehringer Mannheim). The resulting plasmid was named XlGSTM1-pBTac. The fidelity of PCR amplification was verified by DNA sequencing.

Expression and purification of recombinant XlGSTM1-1

A single colony of *E. coli* XL1-Blue transformed with XlGSTM1-pBTac was picked and grown overnight in Luria–Bertani (LB) medium containing ampicillin (100 μg/ml). This was then diluted 1:10 and grown until the D_{600} reached 0.4. To induce *XlGSTM1* gene transcription isopropyl β-D-thiogalactoside (IPTG) was added to a final concentration of 1.5 mM and the incubation was prolonged for further 5 h, setting the temperature at 30 °C.

Cells were harvested by centrifugation (15000 ***g*** for 30 min) and the cell pellet was frozen at −20 °C overnight. The frozen bacterial pellet was allowed to thaw at room temperature before being resuspended in 10 mM potassium phosphate buffer, pH 7, containing 1 mM EDTA (buffer A) and 1 mM dithiothreitol (buffer B).

The resuspended cells were disrupted by cold sonication. The insoluble bacterial debris was removed by centrifugation (15000 ***g*** for 20 min at 4 °C) and the resulting supernatant was loaded on to a GSH–Sepharose 6B (Sigma) column that had been equilibrated with buffer A. The column was washed overnight with 500 ml of buffer A containing 200 mM KCl (more than 30 times the column volume).

The overexpressed protein, which was bound to the affinity column, was eluted at 0.5 ml/min, with 200 mM Tris/HCl buffer, pH 7.8, containing 50 mM GSH. The protein eluted by GSH was collected, dialysed against buffer B, concentrated and glycerol was added to a final concentration of 30% (v/v) before being stored at −20 °C.

Enzyme assay

GST activity with 1-chloro-2,4-dinitrobenzene (Aldrich), 1,2-dichloro-4-nitrobenzene (Fluka), *p*-nitrobenzylchloride (Aldrich), ethacrynic acid (Sigma), 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Eastman Kodak), Δ^5-androstene-3,17-dione (Sigma), *trans*-4-phenyl-3-buten-2-one (Aldrich), *p*-nitrophenyl acetate (Serva) and bromosulphthalein (Serva) was measured as described by Habig and Jakoby [21]. The selenium-independent GSH peroxidase activity of GST was measured with cumene hydroperoxide (Sigma) as reported previously [22]. GST activity

towards 4-nitroquinoline-1-oxide (Fluka) and *trans*-2-nonenal (Sigma) was measured as reported by Stanley and Benson [23]. Protein concentrations were calculated by the method of Bradford [24] using γ-globulin as a standard.

Mutagenesis of the *XlGSTM1* gene

The plasmid XlGSTM1-pBTac, carrying the *XlGSTM1* gene, was used as a template for mutagenesis using the QuikChange Site-Directed Mutagenesis Kit (Stratagene). The single mutations (underlined) Ala-114Cys and Cys-139Ala were made with the following oligonucleotides: fAla114Cys, 5′-GTCTTGTTGTAATTTGTTACAGTCCTCAATTT-3′; rAla114Cys, 5′-CAAATTGAGGACTGTAACAAATTACAACAAGAC-3′; fCys139Ala, 5′-CTGAAAAAGTTCTCTGCCTTTCTAGGAGATAG-3′, and rCys139Ala, 5′-CTATCTCCTAGAAAGGCAGAGAACTTTTTCAG-3′.

Exchange of the Ala residue at position 114 for a Cys residue using fAla114Cys and rAla114Cys as primers and the Cys-139Ala mutant as a template produced the Ala-114Cys/Cys-139Ala double mutant.

The mutation Arg-107His was made with the following oligonucleotides: fArg107His, 5′-CAAGCGATGGATTTTCACATGGGTCTTGTTGTA-3′, and rArg107His, 5′-TACAACAAGACCCATGTGAAAATCCATCGCTTG-3′. The clones used for production of all mutants were confirmed by DNA sequencing. *E. coli* XL1-Blue cells transformed by the XlGSTM1-pBTac mutants were induced by IPTG under the same conditions as for cells transformed by wild-type XlGSTM1-pBTac. The crude extracts were loaded on to GSH–Sepharose 6B (Sigma) columns.

Enzyme thermostability

The wild-type and mutant enzymes were incubated at 25 °C for 30 min in 0.1 M potassium phosphate buffer, pH 7, containing 1 mM EDTA. Appropriate aliquots were taken for activity measurement [21] and for thiol determination [25].

RESULTS AND DISCUSSION

The N-terminal amino acid sequence of XlGSTM1-1 (29 residues) has been reported previously [17]. A search of the EST database revealed the presence of a *X. laevis* cDNA (accession no. BE506028) encoding a protein sharing 100 % identity with the N-terminal sequence of purified enzyme from *X. laevis* liver. This EST, however, did not code for the whole protein because of a stop codon at 502 bp. In order to identify the complete cDNA coding the protein, two oligonucleotides were designed, and a partial cDNA was obtained by PCR amplification of liver cDNA. The PCR product was sequenced and found to encode a partial protein containing the N-terminal sequence of the purified enzyme. This PCR product was then used to screen an *X. laevis* liver cDNA library. Several cDNA clones were isolated and an insert of 893 bp was sequenced. The sequence contains an open reading frame encoding a protein of 219 amino acids with a calculated molecular mass of 25359 Da, which is compatible with the molecular mass (25231 Da without the Met residue) of the purified protein estimated by MS analysis [16]. Moreover, the deduced amino acid sequence contains the N-terminal sequence determined from the native purified enzyme [16]. The translation-termination codon TAA is followed downstream by a conventional polyadenylation signal, AATAAA (Figure 1).

Comparison of the deduced primary structure of this GST with those of proteins present in the GenBank database showed that it shares high (61–67 %) sequence similarity with GSTs of the Mu family (Figure 2) and in particular with the subset of Mu-class GSTs that have 217 amino acid residues and three Cys residues in each subunit [18]. The highest score (67 % identity) was found with human hGSTM1-1. This result indicates that the amphibian enzyme is a member of the Mu class and it was designated XlGSTM1-1, according to the guidelines adopted for GSTs nomenclature [19].

To investigate the physico-chemical properties of this protein, and to confirm its GST activity, the entire open reading frame was subcloned into an expression vector. A culture of *E. coli* XL1-Blue containing the plasmid was induced with IPTG. The supernatant of the cell extract showed overexpression of a protein of approx. 25000 Da (Figure 3). Using GSH-affinity chromatography [26] the overexpressed protein was purified. The yield of the cloned enzyme was about 30 mg/l of culture, allowing its characterization. The recombinant purified protein had an apparent molecular mass of 25000 Da, as measured by SDS/PAGE, with an electrophoretic mobility essentially identical to that of the native enzyme (Figure 3). The recombinant protein gave a single HPLC component eluting at 19.9 min, which is precisely the retention time of the native protein [16].

Multiple sequence alignments (Figure 2) also revealed that the majority of amino acid residues shown to constitute the G-site, i.e. Tyr-6, Trp-7, Leu-12, Lys-42, Trp-45, Lys-49, Asn-58, Leu-59, Pro-60, Gln-71, Ser-72 and Asp-106 (Thr-106), of mammalian Mu-class GSTs [27,28] are conserved in XlGSTM1-1. At position 106 a Phe residue rather than Asp or Thr is present in XlGSTM1-1. This suggests a very similar organization of the GSH-binding site in mammalian and amphibian GSTs. Although the precise structure of the H-site of mammalian Mu-class GSTs is not yet well defined [27,28], it appears that Tyr-6, Trp-7, Val-9, Leu-12, Ile-111, Tyr-115, Phe-208 and Ser-209 may have an important role. With the exception of Val-9, Phe-208 and Ser-209, which are replaced by Ile-9, Asn-208 and Asn-209, the other residues are conserved in XlGSTM1-1. These substitutions would indicate that the H-site of amphibian GST accommodates less hydrophobic substances compared with mammalian Mu-class GSTs, probably responding to the necessity to bind molecules of different chemical structures. It has to be noted that the presence of Val-9, Ile-111 and Ser-209 are important determinants in the stereoselectivity and catalytic efficiency of rGSTM1-1 towards enone and epoxide substrates [29,30]. In mammalian Mu-class GSTs, the 'key' and 'lock' hydrophobic motif, which partly maintains the subunit–subunit interaction of the protein, occurs between Phe-56 (lock) of one monomer and the side chains of Leu-98, Gln-102, Leu-136, Tyr-137 and Phe-140 located on the other side of the interface [27]. With the exception of Leu-136 and Tyr-137, which are replaced by Lys-136 and Phe-137, the other amino acid residues are conserved in XlGSTM1-1, suggesting a possible significant difference between amphibian and mammalian Mu-class GSTs.

The data reported in Table 1 indicate that the recombinant enzyme exhibited the highest activity with 1-chloro-2,4-dinitrobenzene. A low but significant activity was also found with the carcinogenic 4-nitroquinoline-1-oxide. Like many mammalian Mu-class GSTs the recombinant XlGSTM1-1 was found to be active against nonenal [2] compounds, suggesting it may play a key role against the toxic compounds of oxygen metabolism.

It is interesting to note that Cho et al. [31] have demonstrated that murine mGSTM1-1 can modulate stress-activated signals by

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1    AGGCTTTAGAAGCAGGACAAAGGCTCGCTGATAGTGTTGGGGTTCAAG ATG GTG GTG ATA CTA GGA TAC TGG
1                                                      M   V   V   I   L   G   Y   W

73   GAC ATC AGA GGG TTG GCT CAT TCT ATC CGT CTC CTG CTT GAA TAC ACA GGC ACG CAA TAC
9      D   I   R   G   L   A   H   S   I   R   L   L   L   E   Y   T   G   T   Q   Y

133  GAA GAA AAA CTT TAT GTA ACA GGG GAT GCC CCC AAT TAT GAT AAG AGC CAG TGG CTG AAT
29     E   E   K   L   Y   V   T   G   D   A   P   N   Y   D   K   S   Q   W   L   N

193  GAA AAA GAG AAG CTG GGA TTG GAC TTT CCC AAT TTA CCA TAC CTC GTG GAT GGT GAT GTG
49     E   K   E   K   L   G   L   D   F   P   N   L   P   Y   L   V   D   G   D   V

253  AAG CTT ACA CAG AGT AAT GCA ATT CTT CGC TAT ATT GCA CGC AAG CAT GGA CTT TGT GGA
69     K   L   T   Q   S   N   A   I   L   R   Y   I   A   R   K   H   G   L   C   G

313  GAG TCA GAA AAA GAA AAG AAC TAT GTT GAT CTA ATA GAA AAT CAA GCG ATG GAT TTT CGC
89     E   S   E   K   E   K   N   Y   V   D   L   I   E   N   Q   A   M   D   F   R

373  ATG GGT CTT GTT GTA ATT GCT TAC AGT CCT CAA TTT GAA ACA CTG AAG GGG CCA TAT TTA
109    M   G   L   V   V   I   A   Y   S   P   Q   F   E   T   L   K   G   P   Y   L

433  GAG AAG CTG CCT ATT GCT CTG AAA AAG TTC TCT TGC TTT CTA GGA GAT AGA TCT TGG TTT
129    E   K   L   P   I   A   L   K   K   F   S   C   F   L   G   D   R   S   W   F

493  GCA GGA GAT AAG ATC ACT TAT GTC GAT TTC GTG ATG TAT GAT GTA CTA GAT CAA CAC CGG
149    A   G   D   K   I   T   Y   V   D   F   V   M   Y   D   V   L   D   Q   H   R

553  ATA TTA GAT CCA ACT TGT CTG CAG AAT TTT AAG AAT TTG CAG GCC TTT CTA ACC CGA TTT
169    I   L   D   P   T   C   L   Q   N   F   K   N   L   Q   A   F   L   T   R   F

613  GAG GCC CTC CCT GCT ATT GAT GCT TAC GTG AAG TCC TCG CGT TTT ATG AAG ACA CCG ATT
189    E   A   L   P   A   I   D   A   Y   V   K   S   S   R   F   M   K   T   P   I

673  AAC AAC CGC ATG GCA TCC TGG GGC AAC AAA AAA TAAGGCATTCTACACATGGAAGACTTCACTCTTT
209    N   N   R   M   A   S   W   G   N   K   K  Stop

740  TGTTTCACACCAGAACATGTCCACTGTTCTGTTTTTGTTTGTGTAGCCATCAGATCACTTTCTCTCTCCCCTACCTGAT

819  GTCCTTTTGTTTGGTAACTCGGTTTACAATAAAGCCATCCAATAAGTAACCAGCAAAAAAAAAAAAAAAAAAAAA
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Figure 1 cDNA sequence and deduced amino acid sequence of XlGSTM1-1

The start and stop codons are shown in bold; the polyadenylation site is shown underlined and in italics. The underlined sequence perfectly matches those determined by amino acid sequencing of the purified enzyme.

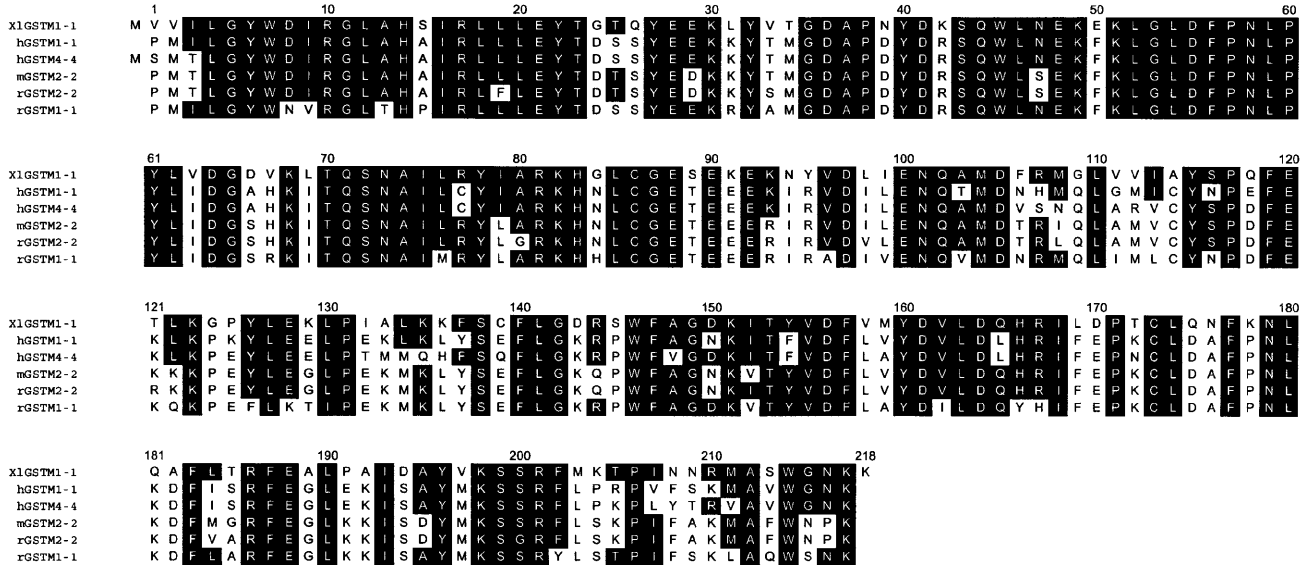

Figure 2 Sequence alignment of XlGSTM1-1 and mammalian Mu-class GSTs

Sequences were obtained with the following accession numbers: P09488 (hGSTM1-1), Q03013 (hGSTM4-4), P15626 (mGSTM2-2), P08010 (rGSTM2-2) and P04905 (rGSTM1-1). h, human; r, rat; m, murine.

Table 1 Activities of wild-type and mutant forms of XlGSTM1-1 with several substrates

The data are means from quadruplicate preparations; S.E.M. values never exceeded 10% of the mean. n.d., no detectable activity.

	k_{cat} (s^{-1})				
Substrate	Wild type	Arg-107His	Ala-114Cys	Cys-139Ala	Ala-114Cys/Cys-139Ala
1-Chloro-2,4-dinitrobenzene	6.47	0.01	4.23	4.35	3.51
Trans-2-nonenal	1.86	n.d.	0.95	1.01	0.92
4-Nitroquinoline-1-oxide	0.42	n.d.	0.25	0.23	0.24
p-Nitrophenyl acetate	0.13	n.d.	0.09	0.07	0.07
Δ^5-Androstene-3,17-dione	n.d.	n.d.	n.d.	n.d.	n.d.
Cumene hydroperoxide	n.d.	n.d.	n.d.	n.d.	n.d.
1,2-Dichloro-4-dinitrobenzene	n.d.	n.d.	n.d.	n.d.	n.d.
1,2-Epoxy-3-(*p*-nitrophenoxy)propane	n.d.	n.d.	n.d.	n.d.	n.d.
p-Nitrobenzylchloride	0.04	n.d.	n.d.	n.d.	n.d.
Bromosulphophtalein	n.d.	n.d.	n.d.	n.d.	n.d.
Trans-4-phenyl-3-buten-2-one	n.d.	n.d.	n.d.	n.d.	n.d.
Ethacrynic acid	0.04	n.d.	0.05	0.04	0.02

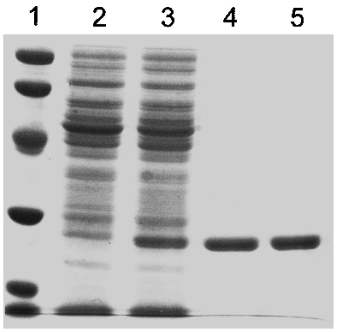

Figure 3 SDS/PAGE of *X. laevis* GST

Lane 1, molecular-mass standards (from top to bottom), phosphorylase b (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa). Lane 2, total cellular extracts of *E. coli* XL1-Blue (XlGSTM1-pBTac) before induction. Lane 3, as lane 2 but after induction. Lane 4, GSH-affinity-purified cloned XlGSTM1-1. Lane 5, native purified GST from *X. laevis*.

interacting with apoptosis-signal-regulating kinase (ASRK1). Thus XlGSTM1-1 might also have a key role in oxidative-stress tolerance, not directly derived from its ability to catalyse the GSH conjugates.

Compared with hGSTM1-1, XlGSTM1-1 has a narrower spectrum of substrate specificity and it is also significantly less effective in conjugating 1-chloro-2,4-dinitrobenzene [2]. It has been demonstrated that the exceptionally high activity of hGSTM1-1 is due to the involvement in the catalytic mechanism of His-107 residue, which is located near the protein's active-site pocket [32]. hGSTM4-4, which has a Ser residue at position 107, also displays a low 1-chloro-2,4-dinitrobenzene conjugation activity [32]. The reciprocal replacement of His-107 and Ser-107 in chimaeric hGSTM1-1/hGSTM4-4 enzymes results in reciprocal conversion of 1-chloro-2,4-dinitrobenzene catalytic properties [32]. Thus the presence of Arg-107, instead of a His-107, in XlGSTM1-1 could explain its weak ability to conjugate 1-chloro-2,4-dinitrobenzene. As can be seen in Table 1, the substitution Arg-107His in XlGSTM1-1 produces a considerable decrease in 1-chloro-2,4-dinitrobenzene activity. This result is significantly different from that obtained for hGSTM2-2, which, like XlGSTM1-1, belongs to subgroup Mu, having an Arg residue at position 107 [33]. In fact, in hGSTM2-2, the replacement of Arg-107 with His has no effect on its 1-chloro-2,4-dinitrobenzene catalytic-centre activity [33]. Thus the transition from XlGSTM1-1 to hGSTM1-1 characteristic 1-chloro-2,4-dinitrobenzene activity appears to require a more complex modification of the protein's tertiary structure than a single substitution of a functional residue. On the other hand, there are 30 amino acid residues conserved in the mammalian Mu-class GSTs reported in Figure 2 that are different in XlGSTM1-1. These residues could contribute significantly to the kinetic differences found in the amphibian isoenzyme.

A remarkable structural feature of recombinant XlGSTM1-1 is its considerable instability. The results presented in Figure 4 indicate that XlGSTM1-1 retains about 48% of its initial activity when it is incubated at 25 °C for 30 min. Figure 4 shows that at the end of incubation the number of titratable thiol groups of XlGSTM1-1 decreases, suggesting the involvement of Cys residue(s) in the inactivation process. The introduction of a Cys residue at position 114 and the replacement of a Cys-139 with Ala, which in terms of Cys residues renders XlGSTM1-1 identical to a mammalian Mu-class GST, does not avoid inactivation of the enzyme. However, the incubation of inactivated enzymes with 50 mM dithiothreitol restored the original activity and the initial number of titratable thiol groups. On the other hand, the substitution of Cys-139 with Ala prevented inactivation of the enzyme and the simultaneous loss of titratable thiol groups. The above results indicate that the Cys residues of XlGSTM1-1 undergo a reversible oxidative inactivation through the formation of intra- or inter-subunit disulphide bonds, certainly involving Cys-139. The presence of a Cys residue at position 139 instead of the conserved Glu-139 of mammalian Mu-class GSTs is one of several distinctive structural features of XlGSTM1-1.

In any case, the presence of a Cys residue at position 139 contributes greatly to making XlGSTM1-1 particularly unstable. It has to be noted that the Ala-114Cys, Cys-139Ala and Ala-114Cys/Cys-139Ala mutants bound the GSH-affinity matrix in a very similar manner and had substrate specificities very similar to the wild type (Table 1). This result suggests that the Cys residues are not involved in the enzymic mechanism of XlGSTM1-1. Chen et al. [34], using site-directed mutagenesis and chemical-modification experiments, also demonstrated that none of the

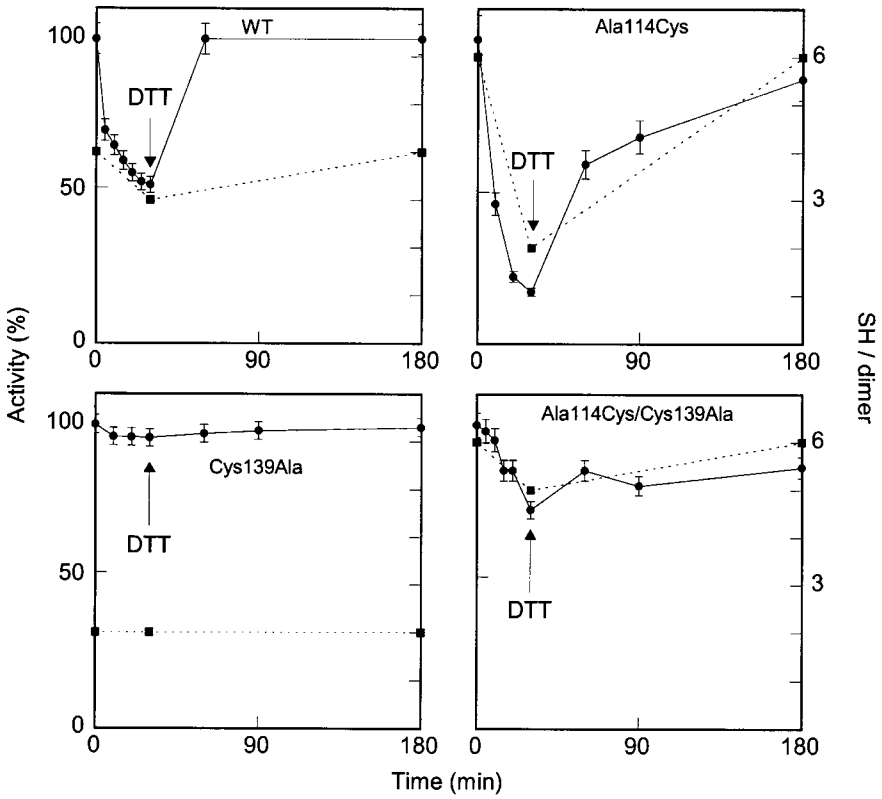

Figure 4 Time course of inactivation and thiol titration of wild-type and mutant enzymes of XlGSTM1-1 at 25 °C

Approx. 0.5 mg/ml proteins were incubated in 10 mM potassium phosphate buffer, pH 7.0, supplemented with 1 mM EDTA and 30% glycerol at 25 °C. At specific intervals adequate volumes were withdrawn for 1-chloro-2,4-dinitrobenzene activity measurements (●) and thiol-group determination (■). The arrows indicate the time of addition of 50 mM dithiothreitol (DTT). The error bars indicate the S.E.M. from five independent measurements.

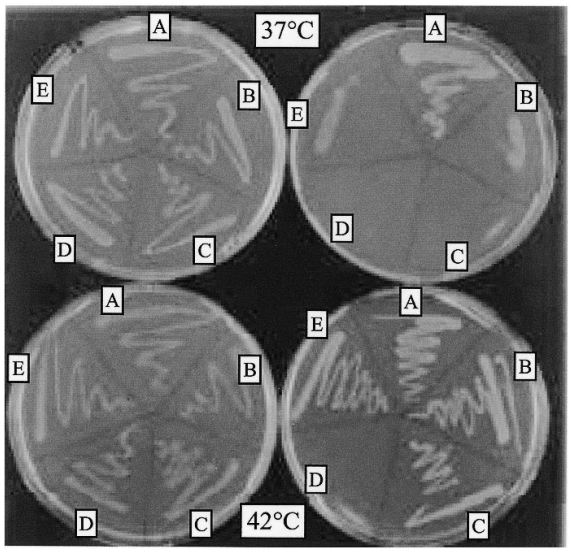

Figure 5 Activity of wild-type and mutant XlGSTM1-1 *in vivo*

E. coli strain XL1-Blue expressing wild-type or mutant enzymes are indicated by different letters: A, pBTac vector alone; B, wild type; C, Ala-114Cys mutant; D, Cys-139Ala mutant; E, Ala-114Cys/Cys-139Ala mutant. On the left-hand plates, one loopful of overnight culture was evenly streaked on to an LB agar plate containing 100 μg/ml ampicillin and placed at 37 or 42 °C overnight, to serve as a control for normal growth. On the right-hand plates, another loopful of the same culture was streaked on to an LB plate containing 100 μg/ml ampicillin and 20 μg/ml 1-chloro-2,4-dinitrobenzene and placed at 37 or 42 °C overnight.

three Cys residues, i.e. Cys-86, Cys-114 and Cys-173, present in rat rGSTM3-3 are needed for its enzymic activity.

XlGSTM1-1 activity was also tested *in vivo* using the method of Lee et al. [35]. The molecular basis of this test relies on the fact that 1-chloro-2,4-dinitrobenzene, the common substrate for GSTs, also has antibiotic activity [36]. This activity is enhanced when *E. coli* expresses a functional GST [35]. *E. coli* colonies carrying either wild-type or Cys mutants of GST were grown overnight in LB medium supplemented with 100 μg/ml ampicillin. A loopful of bacterial colony was evenly streaked across a fixed area on agar plates containing 20 μg/ml 1-chloro-2,4-dinitrobenzene. All these agar plates were grown at 37 °C. The results shown in Figure 5 indicate that, in the presence of 1-chloro-2,4-dinitrobenzene, the growth of *E. coli* carrying both the wild-type and the mutant gene were inhibited. On the other hand, when the cells were grown at 42 °C, streaked as described above, only the growth of *E. coli* carrying the Cys-139Ala mutant form was inhibited. These results confirm that the Cys-139Ala mutant is more stable than the wild type and that it can also operate *in vivo*. These data not only seem to confirm the role of free Cys residues in mechanisms that are liable to irreversible denaturation in proteins, but also suggest that, at least in some cases, the substitution of free Cys residue(s) may significantly improve protein conformational stability.

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