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Characterization of a marsupial glutathione transferase, a class Alpha enzyme from Brown Antechinus (*Antechinus stuartii*)

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Abstract The major form of glutathione transferase from the marsupial *Antechinus stuartii* has been purified and characterized as an Alpha class enzyme (Ast GST A1-1) with distant sequence relationships to other class Alpha sublines, compatible with the early origin of marsupials. Amino acid replacements toward the closest enzyme characterized (chicken, form A3) involve no less than 79 positions (36%). At the active site, as deduced from comparisons with the known tertiary structure of the corresponding human enzyme, over half of the residues (8 of 15) ascribed to substrate binding interactions are exchanged although the general character of that site is conserved, while only 1 of 11 positions ascribed to interactions with GSH is exchanged. Class variability and species variability appear to coincide, with divergent segments centering around positions 33–49, 103–130 and 205–222. The pattern is reminiscent of that in similarly multiple MDR alcohol dehydrogenases. Both these enzyme families involved in cellular defense reactions have diverged considerably.

Key words: Cytosolic glutathione transferase; Enzyme family; Amino acid sequence; Molecular evolution; Structure–function relationship

1. Introduction

Complex protein families of multiple forms is a feature characteristic of several enzymes in cellular defense systems, such as glutathione transferases [1], cytochrome P450s [2], and alcohol dehydrogenases [3]. In all cases, gene duplications at different levels have created large enzyme families in humans and other mammals, with subclasses of distinct properties and with intraclass isozymes. In the case of glutathione transferases, three classes of cytosolic enzymes (Alpha, Mu and Pi) and one microsomal form were originally distinguished [4]. In addition, classes Sigma, Theta and Kappa have now been discerned, of which Theta and Kappa appear to represent ancestral forms [5,6]. Interestingly, the apparent ancestor of MDR alcohol dehydrogenases (MDR, medium-chain dehydrogenases/reductases) also involves a glutathione-dependent enzyme, i.e. class III alcohol dehydrogenase [3], which corresponds to glutathione-dependent formaldehyde dehydrogenase [7].

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In the case of alcohol dehydrogenases, extensive studies on many different vertebrate and invertebrate lines have given much useful information [8], whereas in the case of the soluble glutathione transferases, evolutionary studies are more limited [cf. [5,6]]. We have therefore now analyzed a marsupial class Alpha enzyme, the major liver form of cytosolic glutathione transferase in *Antechinus stuartii*. In the evolution of glutathione transferases, this marsupial form constitutes a new line, illustrating extensive divergence, corresponding to the distant separation time of 130 MY from eutherian mammals [9]. In addition to demonstrating molecular evolution and divergence in the glutathione transferase family, this study establishes further structure–function relationships and promotes understanding of the ability of marsupials to detoxicate environmental chemicals. Glutathione transferase constitutes a phase II enzyme, now characterized from a small (<70 g) insectivorous marsupial of southeastern Australia. Previously, the detoxication enzyme systems of Australian marsupials have received limited attention with most earlier investigations focusing on phase I mixed function oxidases [10].

2. Materials and methods

2.1. Purification of glutathione transferases

Brown Antechinus (*Antechinus stuartii*) were collected in an area of sclerophyll forest near Powelltown, 70 km east of Melbourne, Victoria (Department of Conservation and Natural Resources permit RP-91-046). Livers were perfused in situ with ice-cold 10 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA, 2 mM DTT and 0.15 M KCl, and then excised. Homogenization of the liver (10% w/v in the perfusion buffer) was performed using three passes on an Ultra-Turrax T25 homogenizer (Janke and Kunkel, Staufen, Germany). The homogenate was centrifuged at $10\,000\times g$ for 20 min to remove cell debris, nuclei and mitochondria. The supernatant was further centrifuged at $100\,000\times g$ for 60 min.

All procedures were performed at 0–4°C unless otherwise stated. *S*-Hexyl glutathione-linked Sepharose 6B was prepared from *S*-hexyl glutathione (Sigma) and epoxy-active Sepharose 6B (Pharmacia), as described [11]. The pooled cytosolic supernatant was diluted with 10 mM Tris-HCl, pH 7.8, and applied to the *S*-hexyl glutathione column which was pre-equilibrated with this buffer. After washing, bound glutathione transferases were eluted with 50 mM Tris-HCl containing 10 mM GSH, pH 8.0, followed by 50 mM Tris-HCl containing 10 mM GSH, pH 7.6 [12]. The affinity-purified glutathione transferases were further fractionated by chromatofocusing using an LKB 2150 HPLC system fitted with a Mono P HR 5/20 column (Pharmacia, Sweden). The column was pre-equilibrated at room temperature with 25 mM diethanolamine, pH 9.6. The first elution buffer, 1 ml of Pharmalyte 8–10.5 and 5.2 ml of Polybuffer 96 diluted to 200 ml with water and adjusted to pH 8 with 1 M HCl, was run onto the column (5 ml) prior to sample application. The sample was diluted in the elution buffer prior to injection. The second elution buffer, Polybuffer 74 diluted 20 times with water and adjusted to pH 4 with 1 M HCl, was then applied at 0.5 ml/min when the pH of the eluant was

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Table 1
Enzymatic and physicochemical properties of the Brown Antechinus glutathione transferase Ast GST A1-1

Property	Ast GST A1-1 [10]	Human GST A1-1 [19]
Class	Alpha[1]	Alpha
Subunit M_r (kDa)	25.6[2]	25.7
Isoelectric point	9.7[3]	8.9
Specific activity (μmol·min^{-1}·mg^{-1})		
1-Chloro-2,4-dinitrobenzene	71	80
1,2-Dichloro-4-nitrobenzene	0.4	0.2
Ethacrynic acid	0.8	0.2
Cumene hydroperoxide	5.4	10

[1]From immunocross-reactivity with human glutathione transferases using Western blotting.
[2]From the amino acid sequence determined.
[3]From pH of elution from the chromatofocusing column.

stable at pH 8. The effluent was monitored for absorption at 280 nm, for pH by a glass combination electrode, and for glutathione transferase activity with 1 mM 1-chloro-2,4-dinitrobenzene and 1 mM GSH. Concentration and removal of GSH and buffer salts was achieved using an Amicon 8010 ultrafiltration unit with a YM 30 membrane. The sample was washed 5 times with 22 mM phosphate buffer containing 1 mM DTT, 1 mM EDTA and 20% (v/v) glycerol, pH 7.0, and was then dialysed against 20 mM ammonium bicarbonate, pH 7.5, and freeze dried.

The purified Antechinus glutathione transferase was analysed by reverse-phase HPLC [13], with modifications as described [14]. Protein concentration was measured [15] with bovine serum albumin as standard.

2.2. Structural analysis

The major protein form purified was submitted to carboxymethylation [4] although later found not to contain Cys. Different samples were then digested with Lys-specific protease, Glu-specific protease, Asp-specific protease, and CNBr [16]. Each digest was separated by reverse-phase HPLC on Vydac C4 (4.6×250 mm) or C8 (2.1×250 mm) columns in 0.1% aqueous trifluoroacetic acid with a gradient of acetonitrile [16]. Peptides obtained were analyzed for composition after acid hydrolysis, and for amino acid sequence by degradation in Applied Biosystems 477 and 470, or MilliGen 6600 instruments, with on-line detection. For amino acid analysis, a ninhydrin-based Alpha-Plus (Pharmacia) analyzer was utilized. Molecular masses were determined by MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) mass spectrometry with a Lasermat 2000 instrument. For sequence comparisons, the structure obtained was aligned with characterized glutathione transferases and phylogenetic trees were constructed with the program CLUSTAL W [17], utilizing bootstrap analysis [18] for confidence evaluation of the relationships obtained.

3. Results and discussion

3.1. Purification and enzymatic properties of the major Antechinus glutathione transferase A1-1

Glutathione transferase was purified from Brown Antechinus [10], as described in Section 2 and separated by chromatofocusing into its constituent isozymes of classes Alpha, Pi and Mu, as identified by their differential substrate specificities, inhibition characteristics and cross-reactivities with class-specific antibodies [4]. The major form was a class Alpha enzyme originally called Antechinus glutathione transferase 1-1 [10], which constituted 71% of the total glutathione transferase activity recovered (corresponding to 1 mg, after a 66-fold purification). Its enzymatic and physicochemical properties are given and compared to those of human glutathione transferase A1-1 [19] in Table 1. In accordance with the guidelines adopted for glutathione transferase nomenclature [20],

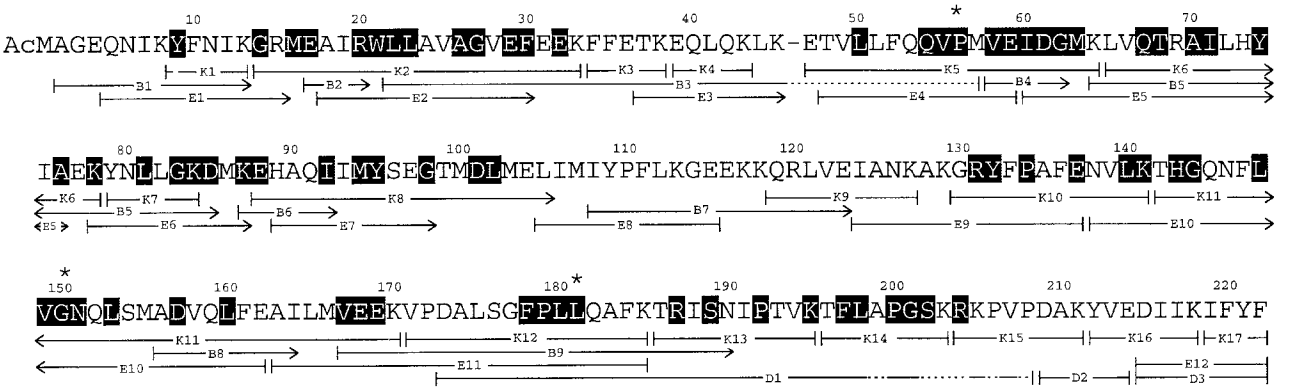

Fig. 1. Primary structure of the Brown Antechinus glutathione transferase Ast GST A1-1 purified, together with the peptides used to establish this structure. Peptide names show the cleavage method by E for fragments from the Glu-protease digest, K for those from the Lys-specific digest, D for those from the Asp-protease digest, and B for those from the CNBr treatment. Peptide regions verified by sequencer degradations are indicated by solid lines, while dashed segments show residues determined from peptide total masses and from degradation of other peptides. Ac, acyl group as indicated by blocked N-terminus, liberated by CNBr treatment. No other marsupial class Alpha enzyme structure was known before, and relationships are distant as evidenced by comparatively few residue identities (residues shown against a black background) among all class Alpha forms now characterized (16 with the present one). The strictly conserved class Alpha residues (black background) show that three segments exhibit greater-than-average species divergence, at positions 33–49, 103–130 and 205–222. Class divergence is much more extensive, and only three residues (marked by asterisks) are strictly conserved among all vertebrate glutathione transferases characterized. However, those three residues center at each of the segments with species conservation (black background), showing that overall patterns of class and species conservation are identical in cytosolic glutathione transferases. The dash sign at position 46 corresponds to a gap in the marsupial protein sequence versus that of other class Alpha proteins, but positional numbers are kept with the human residue numbering to facilitate comparisons (cf. Table 2).

Table 2
Functionally important residues at the active site of Brown Antechinus glutathione transferase Ast GST A1-1, as deduced from comparisons with the human glutathione transferase A1-1 [22]

A. stuartii residue	Human residue	Function [22]
G-site residues		Interactions with GSH:
Tyr^{9}	Tyr^{9}	H-bonding to sulfur
Gly^{14}	Gly^{14}	Unusual main-chain conformation
Arg^{15}	Arg^{15}	Interaction with sulfur
Lys^{45}	Arg^{45}	Ionic bond to Gly carboxylate
Val^{55}	Val^{55}	Main chain β-sheet with peptide bonds of GSH
Pro^{56}	Pro^{56}	*cis*-conformation, conserved in several GSH-binding domains
Gln^{67}	Gln^{67}	H-bonding to γ-Glu ammonium
Thr^{68}	Thr^{68}	H-bonding to γ-Glu α-carboxylate
Asp^{101}	Asp^{101}	Interaction with γ-Glu of GSH in neighbouring subunit
Glu^{104}	Glu^{104}	Ionic bond to Arg 15
Arg^{131}	Arg^{131}	Ionic bond to Gly carboxylate of GSH in neighbouring subunit
H-site residues		
Phe^{10}	Phe^{10}	
Ile^{12}	Ala^{12}	
Lys^{13}	Arg^{13}	
Gly^{14}	Gly^{14}	
Arg^{15}	Arg^{15}	
Glu^{104}	Glu^{104}	
Met^{107}	Leu^{107}	All H-site residues
Ile^{108}	Leu^{108}	contribute to binding
Pro^{110}	Pro^{110}	of second substrate
Phe^{111}	Val^{111}	
Pro^{208}	Met^{208}	
Val^{213}	Leu^{213}	
Ile^{216}	Ala^{216}	
Phe^{220}	Phe^{220}	
Phe^{222}	Phe^{222}	

the marsupial class Alpha enzyme is designated *Antechinus stuartii* glutathione transferase A1-1 (Ast GST A1-1).

3.2. Structural analysis

The primary structure of the pure protein was determined by peptide analysis from four sets of major proteolytic treatments, utilizing Lys-, Glu-, and Asp-specific proteases, respectively, and CNBr. In this manner, a total of 41 peptides were analyzed by sequencer degradations to finally yield a 221-residue amino acid sequence (Fig. 1). Formally, a few peptide bonds have not been passed by Edman degradations, but continuity as indicated in Fig. 1 is ascertained by total compositions, and peptide masses as obtained by laser desorption mass spectrometry (Fig. 1). The direct continuity from peptide K14 to peptide K15 was established by analysis of peptide D1 (3898.6 Da recorded for the molecular ion vs. 3897.6 Da calculated) and by identification of the subsequent K15 residues in the sequencer degradation of D1 as shown by the indications in Fig. 1. Similarly, the mass of peptide B3 (4065.5 Da recorded, 4064.8 Da calculated) shows that the residue corresponding to position 46 of the human enzyme (and other class Alpha enzymes [1]) is missing in the marsupial protein, and that this position therefore corresponds to a gap as shown in Fig. 1. For ease of comparisons, positional numbers below and in Fig. 1 follow the designation of the human enzyme, thus including a number for the gap position and therefore with the C-terminus at position 222. The N-terminus was found to be blocked and was concluded to be acetylated, as in other class Alpha glutathione transferases [16], by lack of results upon attempts at direct sequence analysis of the intact protein, and by proper recovery of the sequence starting at position 2 (peptide B1 in Fig. 1) after cleavage with CNBr, proving the preceding, blocked residue to be a methionine. Although the N-terminal structure thus deduced is not fully typical of acetyl-blocked termini [21], it does not fit non-blocked structures better and corresponds to the analytical data obtained. The C-terminus was established as Phe by identical ends of corresponding peptides from three separate digests, all with enzymes of different specificities (Lys-, Glu- and Asp-specific proteases, respectively, cf. Fig. 1). Unambiguous sequence results were obtained at all positions with exception of position 99, where Thr, although reproducibly found, was weak and recovered together with Gly. This is interpreted to represent the presence of Thr at position 99 and a carryover of Gly^{98} from the previous residue, although presence of a modified or untraditional residue or a Thr/Gly microheterogeneity should perhaps not be fully excluded at position 99.

3.3. Alignment and functional conclusions

The new structure determined is clearly but distantly related to other cytosolic glutathione transferases, in particular of the Alpha class (Fig. 1). In relation to human glutathione transferase A1-1, the structure of which has been determined [22], it is possible to identify amino acid residues of functional significance. First, a key residue, Tyr^{9}, which is believed to promote catalysis by hydrogen bonding to the sulfur of glutathione bound at the active site, is conserved. In fact, essentially all the amino acid residues in human glutathione transferase A1-1 that interact with glutathione bound to the G-site of the enzyme are present in the *A. stuartii* sequence (Table 2). In particular, it is noteworthy that Pro^{56} is conserved, since this residue occurs in the *cis*-conformation not only in other glutathione transferases, but also in proteins such as thioredoxin and selenium-dependent glutathione peroxidase, and

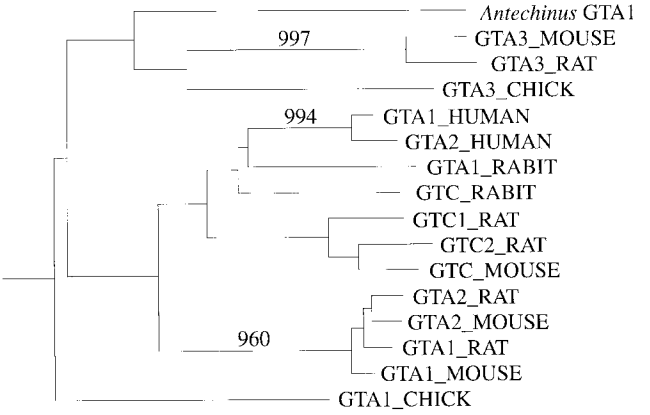

Fig. 2. Phylogenetic tree of class Alpha cytosolic glutathione transferases. Tree construction with the program CLUSTAL W [17]. Numbers show bootstrap values [18] for the only three branches that have significant confidence values (>950), showing an early origin for the marsupial form, but not significantly separated from the A3 chicken form. The designations of the sequences are those used in the SWISS-PROT data bank. Human glutathione transferase GST M1-1 was used as outgroup structure.

seems to be an important signature of a canonical glutathione-binding fold [22].

The binding site for the electrophilic substrate, the H-site, has hydrophobic character although composed of several formally polar side chains in human glutathione transferase A1-1 (Arg^{13}, Gly^{14}, Arg^{15}, and Glu^{104}, contributing, however, with their methylene groups). This character is preserved in the *A. stuartii* glutathione transferase, even though the majority of the residues have been substituted (Table 2). The nature of the H-site residues is believed to govern the substrate selectivity, and the conservation of the binding character explains the observed similarities in specific activities (Table 1).

Alignment of the novel Alpha glutathione transferase to those known before shows that overall residue variability is approximately similar to that of the active site, suggesting that the class characteristics are equally clear in relation to both overall residue replacements and active site positions. This is in marked contrast to 'mixed-class' forms detected in some lines of MDR alcohol dehydrogenases, where the deviations between overall and active site exchanges have been deduced to reflect early steps of enzymogenesis [23,24]. Hence, the present marsupial enzyme is a typical class Alpha glutathione transferase, with largely fixed properties, suggesting an early origin well before the separation of the marsupial line, 130 MY ago. Such an early origin for the class Alpha line is compatible also with its presence in avian lines [25] and with the extended phylogenetic trees previously constructed [5,6]. A more detailed phylogenetic tree, based on all presently known class Alpha structures (Fig. 2), shows the presence of several sub-branches within the same species, in agreement with the presence of multiple gene loci for class Alpha [26]. The marsupial structure now obtained appears to branch off early (before the avian/mammalian separation), but the exact point of branching in relation to the avian line is ambiguous since these lines have insignificant bootstrap values (Fig. 2).

Finally, the patterns of residue conservation are of interest (Fig. 1). In MDR alcohol dehydrogenases the variable regions show unexpected distributions and distinguish the rapidly evolving classes undergoing enzymogenesis from the constant ones with more traditional protein evolution where the variation is at non-functional sites [27]. In the glutathione transferases, particularly variable segments (33–49, 103–130 and 205–222) are also observed. Although overall variation is much larger among the glutathione transferases than among the MDR alcohol dehydrogenases, species and class variabilities appear to coincide in the transferase cases, too (Fig. 1). Hence, the glutathione transferase class evolution resembles that of the MDR alcohol dehydrogenases, but is more rapid and appears to follow a more protein-typical divergence and spread throughout. It is noteworthy that the variable regions 103–130 and 205–222 contain the main determinants of the H-site, suggesting an explanation for the evolution of distinct substrate specificities.

In conclusion, the characterization of a marsupial glutathione transferase confirms a distant separation of marsupials from the eutherian line, establishes a protein-typical evolution of glutathione transferase classes, highlights the pattern of Alpha class conservation and reveals some reminiscence between the patterns of class evolution in glutathione transferases and MDR alcohol dehydrogenases.

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