A sensitive core region in the structure of glutathione S-transferases

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A variant form of an *Anopheles dirus* glutathione S-transferase (GST), designated AdGSTD4-4, possesses a single amino acid change of leucine to arginine (Leu-103-Arg). Although residue 103 is outside of the active site, it has major effects on enzymic properties. To investigate these structural effects, site-directed mutagenesis was used to generate mutants by changing the non-polar leucine to alanine, glutamate, isoleucine, methionine, asparagine, or tyrosine. All of the recombinant GSTs showed approximately the same expression level at 25 °C. Several of the mutants lacked glutathione (GSH)-binding affinity but were purified by S-hexyl-GSH-based affinity chromatography. However the protein yields (70-fold lower), as well as the GST activity (100-fold lower), of Leu-103-Tyr and Leu-103-Arg purifications were surprisingly low and precluded the performance of kinetic experiments. Size-exclusion chromatography showed that both GSTs Leu-103-Tyr and Leu-103-Arg formed dimers. Using 1-chloro-2,4-dinitrobenzene (CDNB) and GSH substrates to determine kinetic constants it was demonstrated that the other Leu-103 mutants possessed a greater K_m towards GSH and a differing K_m towards CDNB. The V_{max} ranged from 44.7 to 87.0 μmol/min per mg (wild-type, 44.7 μmol/min per mg). Substrate-specificity studies showed different selectivity properties for each mutant. The structural residue Leu-103 affects the active site through H-bond and van-der-Waal contacts with six active-site residues in the GSH binding site. Changes in this interior core residue appear to disrupt internal packing, which affects active-site residues as well as residues at the subunit–subunit interface. Finally, the data suggest that Leu-103 is noteworthy as a sensitive residue in the GST structure that modulates enzyme activity as well as stability.

Key words: *Anopheles dirus*, non-active site, structure–function.

INTRODUCTION

Glutathione S-transferases (GSTs, EC 2.5.1.18) are well known as dimeric detoxification enzymes that catalyse a wide variety of conjugations of glutathione (GSH) to hydrophobic toxic compounds. Each subunit of 25 kDa contains a catalytic pocket of the GSH binding site (G-site) and the nearby hydrophobic substrate-binding site (H-site) [1–3]. In general, the N-terminal domain of GST enzymes comprises most of the G-site and is quite conserved across classes, whereas the C-terminal domain that forms part of the H-site is more divergent. Variations in hydrophobic residues of the H-site, between different GSTs, are proposed to play a role in substrate selectivity.

Previously we have reported *adgst1AS1* as an *Anopheles dirus* alternatively spliced GST gene [4]. This gene encodes four Delta class (insect class 1) GST enzymes: adGST1-1, adGST1-2, adGST1-3 and adGST1-4, which share 61–77 % amino acid identity [5]. The four splicing products were named according to the insect GST nomenclature in use. However, to be in alignment with a proposed universal GST nomenclature, we have renamed the enzymes AdGSTD1-1, AdGSTD2-2, AdGSTD3-3 and AdGSTD4-4 [6]. The 'D' refers to the GST Delta class and the subunit number remains the same since the subunits were enumerated as they were initially discovered. The double number, GSTD3-3 for example, signifies that the enzyme is a homodimer. These four GSTs share an untranslated exon 1 and a translated exon 2 that codes for 45 amino acids at the N-terminus. These two exons are spliced to one of four alternative versions of exon 3 (3A, 3B, 3C or 3D) to generate four different mature transcripts. A homologous GST gene has also been reported in *Anopheles gambiae* as *aggst1α* [7]. The *A. dirus* and *A. gambiae* genes have a conserved exon arrangement with approximately 79 % nucleotide identity. The four GST proteins from each of the two mosquito species have 85–92 % amino acid identity between the homologous enzymes. These two mosquito species are both malaria vectors, although they are geographically distant, with *A. dirus* in Southeast Asia and *A. gambiae* in Africa. The two Anopheline species must have diverged several million years ago; therefore the high conservation and type of gene organization suggests this gene is very important. In fact, the four splice products are active GSTs possessing distinct enzyme-kinetic properties. The insecticide-interaction properties, the available crystal structure for AdGSTD4-4, and the canonical nature of GST tertiary structure make AdGSTD4-4 an ideal candidate for further structure–function studies [5,8].

As well as allelic variants (i.e. variants from a single gene locus), it has been shown that site-directed mutagenesis variants with single residue changes could influence the kinetic property, thermal stability, substrate affinity, substrate specificity, and/or catalytic activity of a GST enzyme [9–14]. Studies of allelic variants provide useful information for elucidation of structure–function relationships. Several variant forms of AdGSTD4-4, obtained by reverse transcriptase PCR, yielded amino acid residue changes distributed throughout the tertiary structure. One of these proteins, GST Leu-103-Arg, markedly differed from the other GSTs. This substitution, at position 103, was of particular interest since the change from the non-polar leucine to a positively charged arginine reduced most of the enzymic activity. In addition, this protein could not be purified using GSH-based affinity chromatography. An available X-ray crystal structure showed that the 103

Abbreviations used: GST, glutathione S-transferase; GSH, glutathione; G-site, glutathione binding site; H-site, hydrophobic substrate-binding site; CDNB, 1-chloro-2,4-dinitrobenzene; Leu-103-Arg, denotes substitution of one amino acid residue with another, e.g. Leu-103 with Arg-103.

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position does not form part of the active site pocket of the enzyme, thus making this residue of interest for further studies [8].

To elucidate the importance of this residue position, site-directed mutagenesis was employed to change the leucine to six other residues: alanine, glutamate, isoleucine, methionine, asparagine, or tyrosine, respectively. The mutant isoform types generated for the Leu-103 residue included: hydrophobic, positively charged, negatively charged, polar uncharged, as well as those possessing various sizes and/or lengths of functional group. Together with available tertiary structure [8,15], enzyme kinetic properties were investigated to provide a clearer understanding of the structure–function relationship.

EXPERIMENTAL PROCEDURES

Expression and purification of recombinant AdGSTD4-4 protein

Cloning and heterologous expression of the *A. dirus* GSTs were performed as described previously [5]. The mutant construction method was based on the Stratagene Quick Change™ Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). The oligonucleotide primers were designed to introduce a single residue mutation into AdGSTD4-4 to change leucine at the 103 position to alanine, glutamate, isoleucine, methionine, asparagine or tyrosine. In the primers shown below, altered nucleotides are shown in bold and additional recognition sites of restriction endonucleases (*Sac*II for the Leu-103-Ala and Leu-103-Ile sets; *Eco*RI for the Leu-103-Glu set; *Hinc*II for the Leu-103-Met and Leu-103-Tyr sets; *Xmn*I for the Leu-103-Asn set) are underlined: Leu-103-Ala reverse, 5′-CAC ATC GAA GAA C**GC** CCG CTG GTG GAC GAC GGC ACG GCG CCG CGG ATC GCT TG-3′; Leu-103-Ala forward, 5′-CAA GCG ATC CGC G**G**C GCC GTG CCG TCG TCC ACC AGC GG**G** **C**GT TCT TCG ATG TG-3′; Leu-103-Glu reverse, 5′-CAC ATC GAA GAA **TTC** CCG CTG GTG GAC-3′; Leu-103-Glu forward, 5′-GTC CAC CAG CGG **GAA** TTC TTC GAT GTG-3′; Leu-103-Ile reverse, 5′-CAC ATC GAA GAA **GAT** CCG CTG GTG GAC GAC GGC ACG GCG CCG CGG ATC GCT TG-3′; Leu-103-Ile forward, 5′-CAA GCG ATC CGC G**G**C GCC GTG CCG TCG TCC ACC AGC GG**A** **TC**T TCT TCG ATG TG-3′; Leu-103-Met reverse, 5′-GAA GAA CAT CCG CTG GTG GAC GAC GGC ACG **TCG AC**-3′; Leu-103-Met forward, 5′-GTC GAC GTG CCG TCG TCC ACC AGC GGA TGT TCT TC-3′; Leu-103-Asn reverse, 5′-CCA CCA GCG G**AA** **C**TT CTT CGA TGT GGC C-3′; Leu-103-Asn forward, 5′-GGC CAC ATC GAA GAA **GTT** CCG CTG GTG G-3′; Leu-103-Tyr reverse, 5′-GAA GAA ATA CCG CTG GTG GAC GAC GGC ACG **TCG AC**-3′; Leu-103-Tyr forward, 5′-GTC GAC GTG CCG TCG TCC ACC AGC GGT ATT TCT TC-3′. The expression construct of AdGSTD4-4 was used as template DNA. The soluble recombinant GST proteins were purified by GSTrap affinity chromatography (Amersham Biosciences, Piscataway, NJ, U.S.A.) or S-hexylglutathione agarose (Sigma) affinity chromatography, in the case of low affinity towards the GSH ligand [5,9].

Enzyme characterization and protein assay

The substrate-specificity studies and steady-state kinetics were performed as described previously [5,16,17]. The data shown are means ± standard deviations from at least three independent experiments. Protein concentration was determined by the Bradford method [18] using the Bio-Rad Laboratories (Hercules, CA, U.S.A.) protein reagent with bovine serum albumin as standard protein.

Size-exclusion chromatography

An *Escherichia coli* lysate of expressed enzymes was filtered through a 0.45 micron Acrodisc® syringe filter (Pall Life Sciences, Sydney, Australia) and fractionated through a Superdex 75 HR 10/30 column (Amersham Biosciences) at a flow rate of 0.5 ml/min with a pressure of 1 MPa on an ÄKTA™ purifier liquid chromatography system (Amersham Biosciences). Chymotrypsinogen A (25 kDa ± 25 %) and ovalbumin (43 kDa ± 15 %) were used as molecular mass standards. The buffer used contained 50 mM K_2HPO_4/KH_2PO_4 and 150 mM NaCl, pH 7.0. The elution was collected as 1 ml fractions.

Heat inactivation assay

Enzymes derived from the different isoforms, each at a concentration of 0.05 mg/ml [in 0.1 M phosphate buffer (pH 6.5) containing 5 mM DTT and 1 mM EDTA], were incubated at various temperatures for 10 min. The temperatures used were between 25 °C to 60 °C in 5 °C increments. An appropriate amount of incubated mixture was taken to assay for remaining GST activity [19].

Half-life determination

Enzymes derived from the different isoforms [each at a concentration of 0.05 mg/ml in 0.1 M phosphate buffer (pH 6.5) containing 5 mM DTT and 1 mM EDTA] were incubated at 45 °C. An appropriate amount of incubated mixture (adjusted for the specific activity of each mutant enzyme, e.g. an amount of approx. 10 μl of incubated mixture was used for the mutant GSTs in this study) was taken to assay for remaining GST activity at various time points ranging from 0 to 15 minutes. Log percentage of original activity was plotted versus pre-incubation times. Slopes from linear regression analysis using GraphPad Prism 2.01 software were employed in the half-life calculation.

RESULTS AND DISCUSSION

Recombinant protein expression and purification

All mutant isoforms were successfully generated by site-directed mutagenesis. DNA sequencing was used to confirm each single nucleotide at least twice. Protein expression at 37 °C showed that Leu-103-Asn and Leu-103-Tyr were expressed as insoluble inclusion bodies within the *E. coli* cells and this created difficulties in the purification process. The temperature of induction was lowered to 25 °C and then all mutant isoforms were expressed as soluble proteins of approximately the same amount (Figure 1). Temperature-sensitive GST mutants have been observed previously, where the mutations impact upon important structural motifs [19–23]. The temperature-sensitive folding mutants were obtained when expressed at a permissive temperature such as 25 °C. It was suggested that these mutants fail to attain the native conformation at higher temperatures, such as 37 °C, and are therefore expressed in lower amounts or as insoluble inclusion bodies. The expression of two mutant (at residue 103) proteins as inclusion bodies suggests that these residue changes impact significantly upon the structure during the initial folding process.

GST activity was also undetectable in the lysates from Leu-103-Arg and Leu-103-Tyr mutants, therefore these lysates were subjected to size-exclusion chromatography to investigate whether these mutants were expressed in an active dimeric, or inactive monomeric, form. The result showed that the GST was

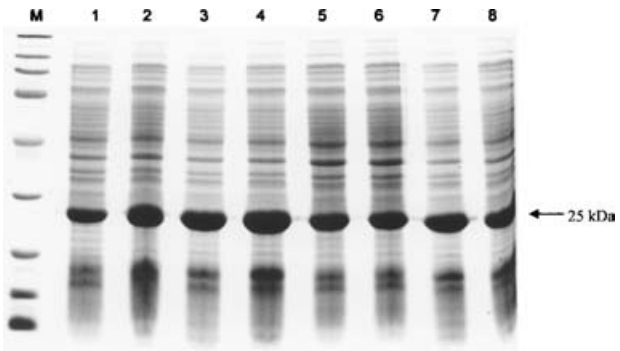

Figure 1 Protein expression of Leu-103 mutant isoforms

An amount of *E. coli* lysate (adjusted to give a D_{600} of 0.1 for each isoform) was resuspended in 16 μl of loading buffer and separated by SDS/PAGE. Lane 1, Leu-103-Arg; lane 2, Leu-103-Tyr; lane 3, Leu-103-Asn; lane 4, Leu-103-Met; lane 5, Leu-103-Ile; lane 6, Leu-103-Glu; lane 7, Leu-103-Ala; and lane 8, wild-type, respectively. M, broad-range molecular mass marker.

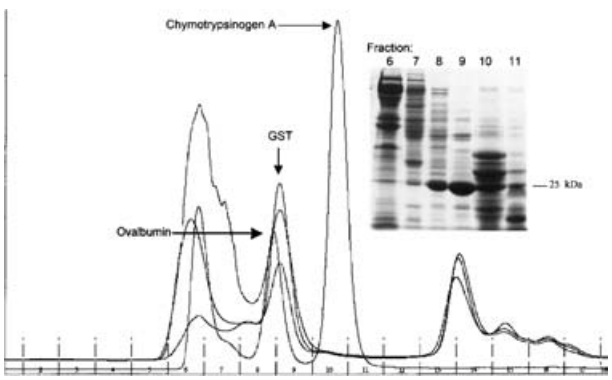

Figure 2 FPLC chromatogram of AdGSTD4-4 (wild-type), Leu-103-Arg, and Leu-103-Tyr

Ovalbumin (43 kDa ± 15 %) and chymotrypsinogen A (25 kDa ± 25 %) were used as molecular mass standard markers for the dimeric and monomeric form, respectively. A polyacrylamide gel (inset) shows relative sizes of fractionated proteins.

observed at a point corresponding to a dimeric molecular mass of 50 kDa (Figure 2). We then concluded that the Leu-103-Arg and Leu-103-Tyr mutants were expressed as homodimers.

Not all recombinant GSTs were effectively purified by the GSTrap column. The expressed enzymes of Leu-103-Glu, Leu-103-Asn, Leu-103-Arg, and Leu-103-Tyr, failed to bind to the GSTrap column but were successfully purified by using an S-hexylglutathione affinity column. Nevertheless, the purification yield of Leu-103-Arg and Leu-103-Tyr was low, approx. 1 % of the wild-type yield (Table 1). Differences in the purification yield for each recombinant protein may have resulted from a reduction in binding affinity to the ligand on the gel matrix of either the GSTrap column or the S-hexylglutathione agarose. However, all the recombinant enzymes retained the ability to bind to the GSH substrate, albeit with different affinities compared with wild-type, as indicated by the K_m values. A point worth noting is that any amino acid substitution, with a non-hydrophobic residue in the 103 position, would lose the binding affinity to the GSTrap, a GSH-based affinity matrix. The X-ray crystal structure of AdGSTD4-4 shows that Leu-103 is not in the active site but is located in helix 4 and is in a hydrophobic environment (Figure 3). It appears that the alteration of this position to a non-hydrophobic residue disrupts the conformation of, and decreases the ability to bind to, the GSH-based affinity matrix. Changes in GST interaction with GSH-based affinity matrices has been reported in studies with an Alpha and a Pi class GSTs involving a single amino acid important in intersubunit interactions [24,25]. This residue (Phe-51 in Alpha and Tyr-50 in Pi class) was involved in a structural lock-and-key motif contributing to the subunit–subunit interface. Although a structural residue, this residue position impacted significantly on enzyme activity such that the mutant proteins displayed a decreased affinity for the GSH affinity matrices. This residue position is located between α helix 2 and β strand 3, and parts of this region contribute to one side of the G-site [25]. In our study, residue 103 is not equivalent to the aromatic residue in Alpha and Pi class GSTs, but is located in the interior of the subunit, also adjacent to the active site and the subunit interface.

Enzyme kinetic properties

The steady-state kinetics followed Michaelis–Menten kinetics for several of the mutants and the kinetic parameters were determined by non-linear regression analysis and compared with the wild-type AdGSTD4-4 (Table 1). Changes of the Leu-103 residue to arginine or tyrosine almost abolished GST activity towards 1-chloro-2,4-dinitrobenzene (CDNB), which precluded the determination of kinetic parameters for these two mutants. However, all other mutant enzymes had a greater maximum rate of reaction or V_{max} (Table 1). The catalytic efficiencies or k_{cat}/K_m of all mutant enzymes towards CDNB were 1.2–3-fold greater than the wild-type enzyme. But the catalytic efficiencies towards GSH of the Leu-103 mutants were 0.02–0.37-fold less than the

Table 1 Yields of purification and kinetic parameters of the seven recombinant mutant GSTs compared with AdGSTD4-4 (wild-type) from *A. dirus*

The data are the means ± S.D. of at least three separate experiments. n.d., not determined.

Clone	Protein yield (mg/l)	V_{max} (μmol/min per mg)	k_{cat} (s^{-1})	CDNB		GSH	
				K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)	K_m (mM)	k_{cat}/K_m ($s^{-1} \cdot mM^{-1}$)
Wild-type	43.2	44.7 ± 2.3	18.60	0.76 ± 0.07	24.5	0.70 ± 0.09	26.6
Leu-103-Ala	8.0	47.6 ± 3.6	19.78	0.62 ± 0.19	31.9	24.0 ± 2.40	0.8
Leu-103-Glu	4.8	76.6 ± 2.7	31.90	0.72 ± 0.07	44.3	3.42 ± 0.27	9.3
Leu-103-Ile	26.6	87.0 ± 1.4	36.21	0.49 ± 0.08	73.9	3.70 ± 0.37	9.8
Leu-103-Met	70.0	72.4 ± 5.2	30.16	0.91 ± 0.14	33.1	6.38 ± 0.55	4.7
Leu-103-Asn	20.0	52.7 ± 3.4	21.94	0.77 ± 0.09	28.5	37.4 ± 2.66	0.6
Leu-103-Tyr	0.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Leu-103-Arg	0.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

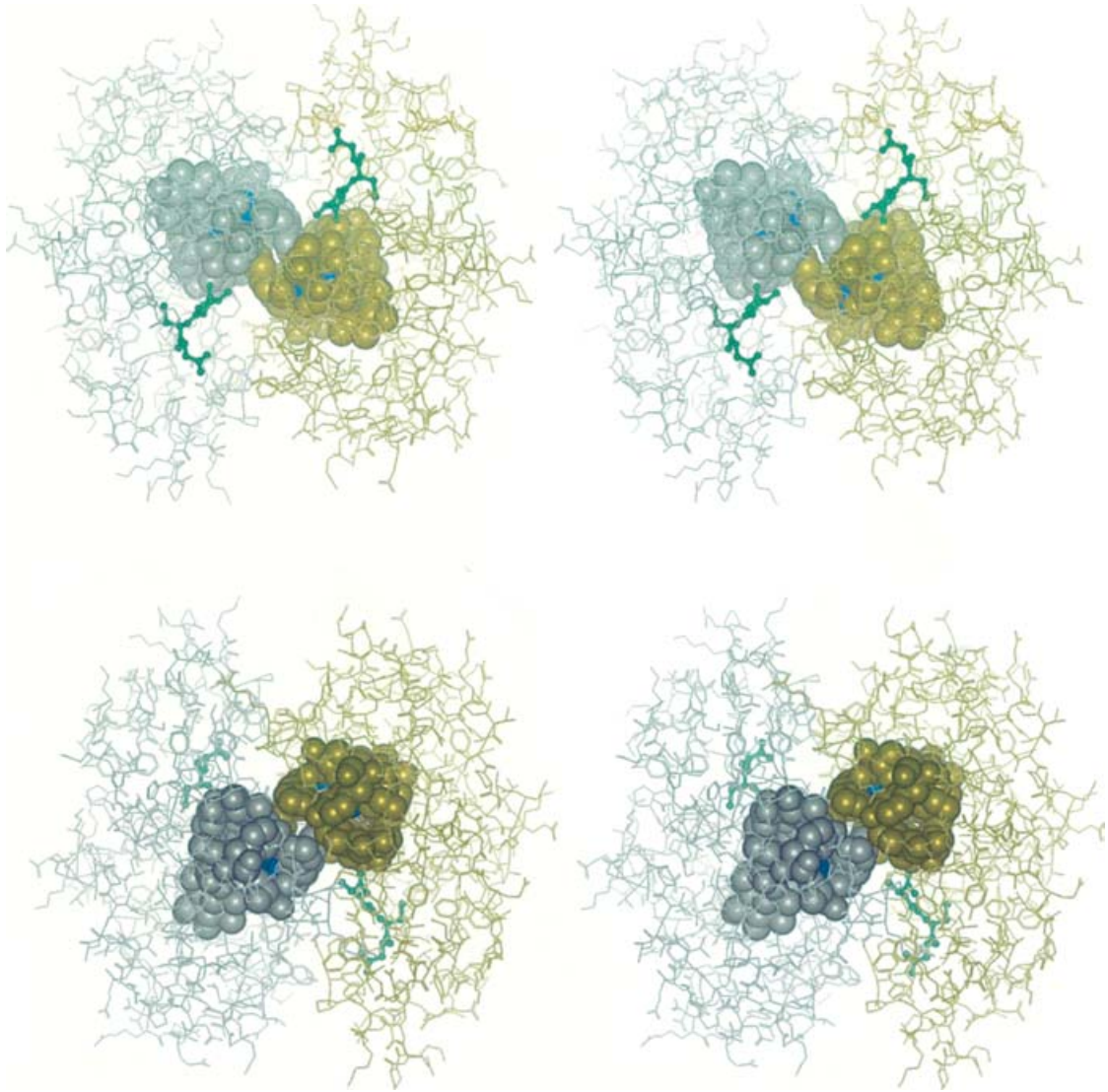

Figure 3 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

The upper panel is looking down on to the active site, with GSH superimposed as a green ball-and-stick figure. The lower panel shows the structure rotated by 180°. The two subunits are shown in grey and gold and L103 is shown in blue. The coordinates for the tertiary structure have been deposited in the Protein Data Bank with the accession number 1JLW [8].

wild-type enzyme. Except for Leu-103-Arg and Leu-103-Tyr, it appears that all other Leu-103 mutants possess a greater K_mGSH (K_m towards GSH substrate), ranging from 3.4–37.4 mM (wild-type, 0.7 mM), indicating a decreased affinity of these mutant enzymes for the GSH substrate. Several of the Leu-103 mutants, most notably Leu-103-Ala and Leu-103-Asn, instead of a hyperbola, yielded a sigmoidal curve-shape for GSH binding. This was also reported for several Tyr-50 mutants in GSTP1-1 which was thought to impact upon part of the G-site [25]. A sigmoidal velocity curve reflects the GSH binding in the first active site, which then facilitates another GSH binding in the second active site by increasing the binding affinity of the vacant binding site [26]. The X-ray crystal structure of AdGSTD4-4 shows that the Leu-103 residue is not in a position to be involved in main-chain or side-chain interactions with GSH in the active site. However, there are 13 residues packed in a sphere around Leu-103 involving 34 H-bonds with contacts extending across the subunits (Figure 4). These 13 residues are Arg-67, Ile-71, Tyr-89, Val-99, His-100, Phe-104, Phe-104B (the other subunit), Asp-106, Val-107, Ile-163, Ala-164, Ser-167 and Ile-168. Six of these residues are in the active site: Arg-67, Phe-104, Phe-104B, Asp-106, Val-107 and Ser-167, and interact with an additional five active site residues: Glu-65, Ala-108, Ala-108B (the other subunit), Tyr-111 and Thr-171. Residue Arg-67, which is a strictly conserved residue through the GST classes, directly interacts with the carboxylic acid of the γ-glutamyl of GSH. The comparative arginine residue, Arg-69 in human alpha class GST, has been shown to be important for GSH binding as well as stabilization of the enzyme conformation [27]. The variation in K_mCDNB may originate from charge distribution effects transmitted between the G-site and H-site. This has previously been suggested for GSTs where the electrostatic field of the active site had been modified or disturbed by residue changes [28–30]. A corollary to changes in the electrostatic field of the active site would be changes in the topology of the active site through residue movement, both through adjustments in packing and conformational flexing differences.

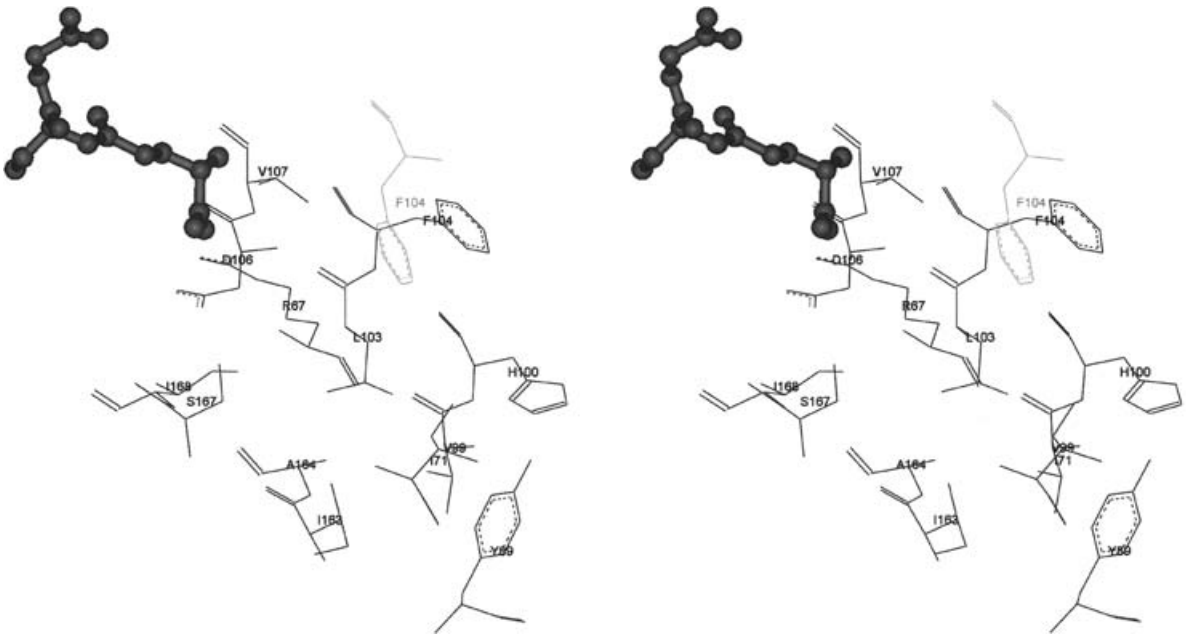

Figure 4 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

GSH is superimposed as a grey ball-and-stick figure. The residues from subunit A are represented in black lines and Phe-104 from subunit B as grey lines. The coordinates for the tertiary structure have been deposited in the Protein Data Bank with the accession number 1JLW [8].

Table 2 Substrate specificities of the seven recombinant mutant GSTs compared with AdGSTD4-4 (wild-type) from *A. dirus*

The substrate concentrations used are in parentheses. DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; PNPB, p-nitrophenethyl bromide; PNBC, p-nitrobenzyl chloride. The data are the means ± S.D. of at least three separate experiments.

	Substrate specificity (μmol/min per mg)				
Clone	CDNB (3 mM)	DCNB (1 mM)	EA (0.2 mM)	PNPB (0.1 mM)	PNBC (0.1 mM)
Wild-type	37.4 ± 2.2	0.016 ± 0.003	0.211 ± 0.019	0.042 ± 0.008	0.060 ± 0.005
Leu-103-Ala	19.7 ± 1.8	0.017 ± 0.009	0.313 ± 0.018	< 0.091	< 0.057
Leu-103-Glu	46.4 ± 0.4	0.032 ± 0.005	0.345 ± 0.050	< 0.078	0.102 ± 0.031
Leu-103-Ile	50.4 ± 4.7	0.046 ± 0.004	0.124 ± 0.020	0.030 ± 0.004	0.058 ± 0.012
Leu-103-Met	38.6 ± 1.9	0.039 ± 0.007	0.182 ± 0.012	0.027 ± 0.002	0.056 ± 0.002
Leu-103-Asn	15.5 ± 0.8	0.009 ± 0.001	< 0.004	< 0.018	0.030 ± 0.004
Leu-103-Tyr	0.66 ± 0.11	< 0.071	< 0.121	< 0.503	< 0.318
Leu-103-Arg	0.36 ± 0.02	< 0.066	< 0.113	< 0.470	< 0.297

Substrate specificities

Substrate specificity determination revealed differences in the specificity or the interaction of the mutant enzymes with several hydrophobic substrates, which indicated that the changed single amino acid residues affected the catalytic specificity of the enzymes (Table 2). It has been shown in human Pi GST (Pi class GST) that there are sensitive structural regions where single residue changes influence inhibitor specificity and decrease enzyme activity [27,31]. Some of these residues are found in the domain 2 α-helices that generate a hydrophobic core. This hydrophobic core is thought to stabilize the subunit tertiary structure. Previously we have shown for AdGSTD1-1 that residues outside the active site can subtly affect the enzyme specificity [9]. In AdGSTD1-1, one of these residues was found on α helix 7 that contributes to the hydrophobic core in domain 2. The differing conformations may bring different residues into proximity or change the proximity/orientation of the residues involved in binding or catalysis, thereby affecting the observed enzyme properties. This has also been shown by mutagenesis studies of human Theta class GSTT2-2 [32].

Enzyme stability

The wild-type enzyme was subjected to a heat-inactivation assay and it was observed that the GST activity began to decrease at 45 °C. This temperature was used to determine half-life stabilities for recombinant enzymes. The half-life corresponds to the time of incubation at which there is 50 % of activity remaining. Most of

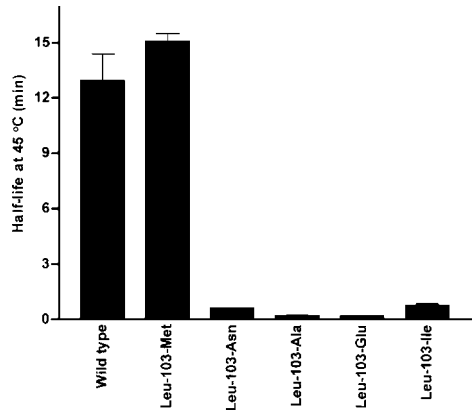

Figure 5 Half-life of recombinant mutant GSTs at 45 °C compared with AdGSTD4-4 (wild-type) from *A. dirus*

The data are the means ± S.D. of at least three separate experiments.

the Leu-103 mutants possess a lower stability, although Leu-103-Met showed a slight increase in stability (Figure 5). It has been shown previously that the mutation of a single amino acid residue can have an impact on stability of the enzyme [24,25]. These studies of Alpha and Pi GSTs characterized a residue at the subunit interface involved in the dimerization of the subunits. The present study characterizes a residue, Leu-103, which is in the interior of the subunit. This position, with its surrounding 13 residues, forms a hydrophobic core completely excluding water molecules in the centre of the subunit. The mutations of residue 103 disturb the packing of these residues, which affects the stability of the structure as well as its enzymic properties.

Conclusion

It has been shown that the structural residue Leu-103 affects the active site through H-bond and van-der-Waal contacts with six active-site residues in the G-site. Changes in this interior core residue appear to disrupt internal packing, affecting active-site residues as well as residues at the subunit–subunit interface. These effects are observed as changes in k_{cat} and K_m for the hydrophobic substrate CDNB as well as changes in K_m for GSH. The Leu-103 effects also influence substrate specificity as well as enzyme stability.

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