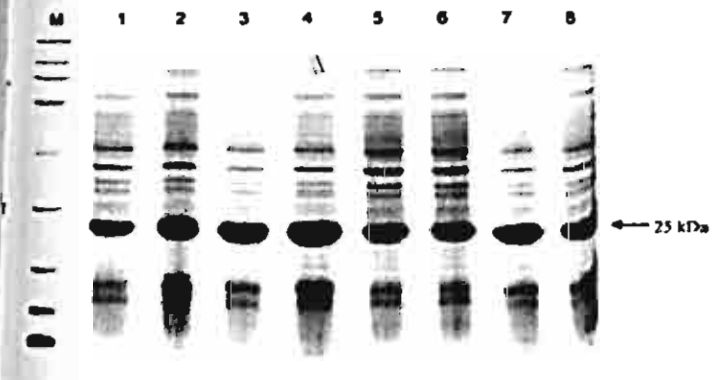

Figure 1 Protein expression of Leu-103 mutant isoforms

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.

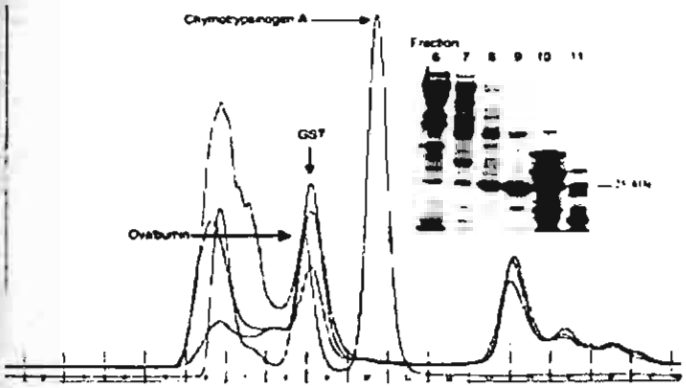

Figure 2 FPLC chromatogram of AdGSTD4-4 (wild-type), Leu-103-Arg, and Leu-103-Tyr

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.

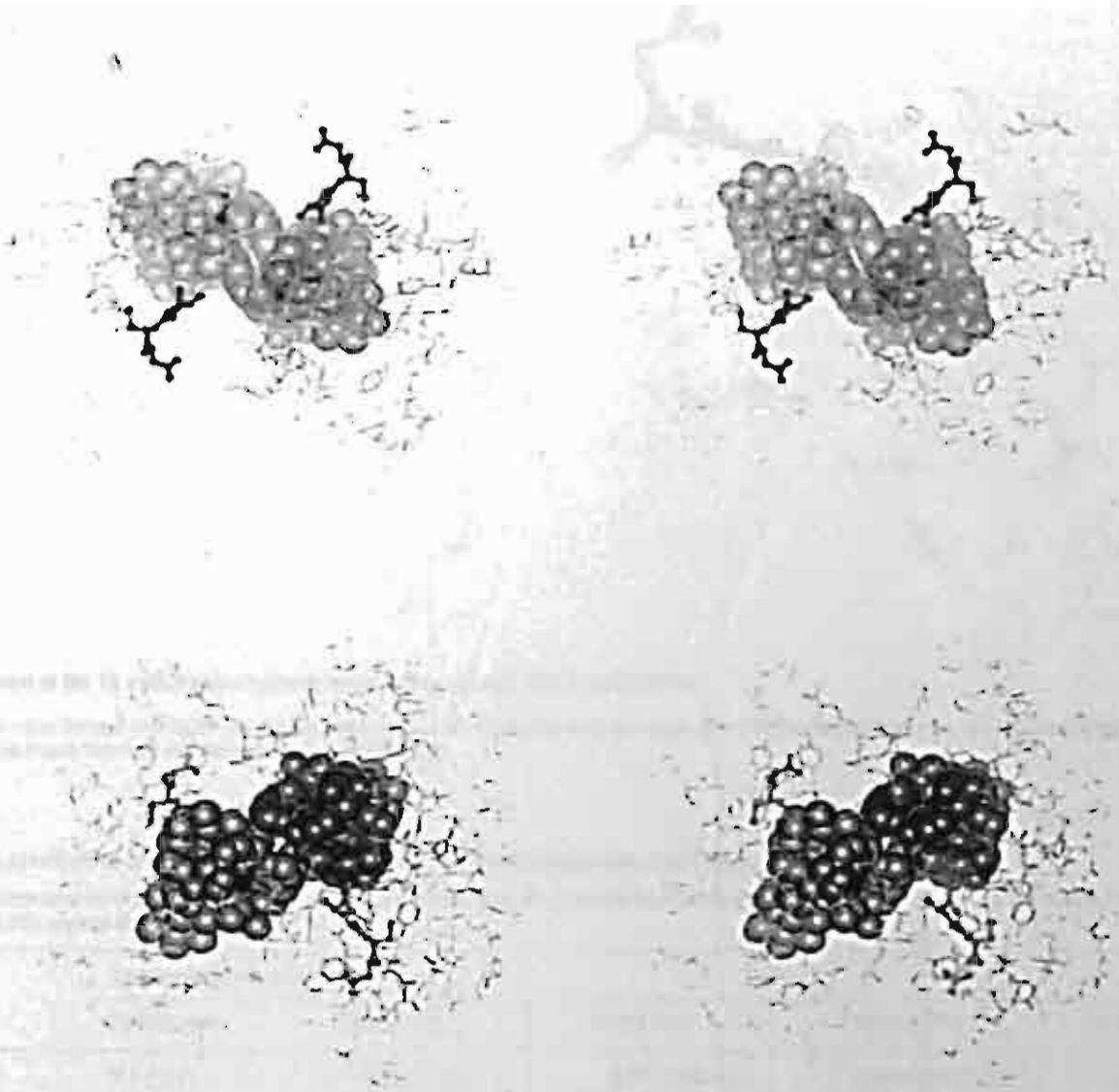

Figure 3 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

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.

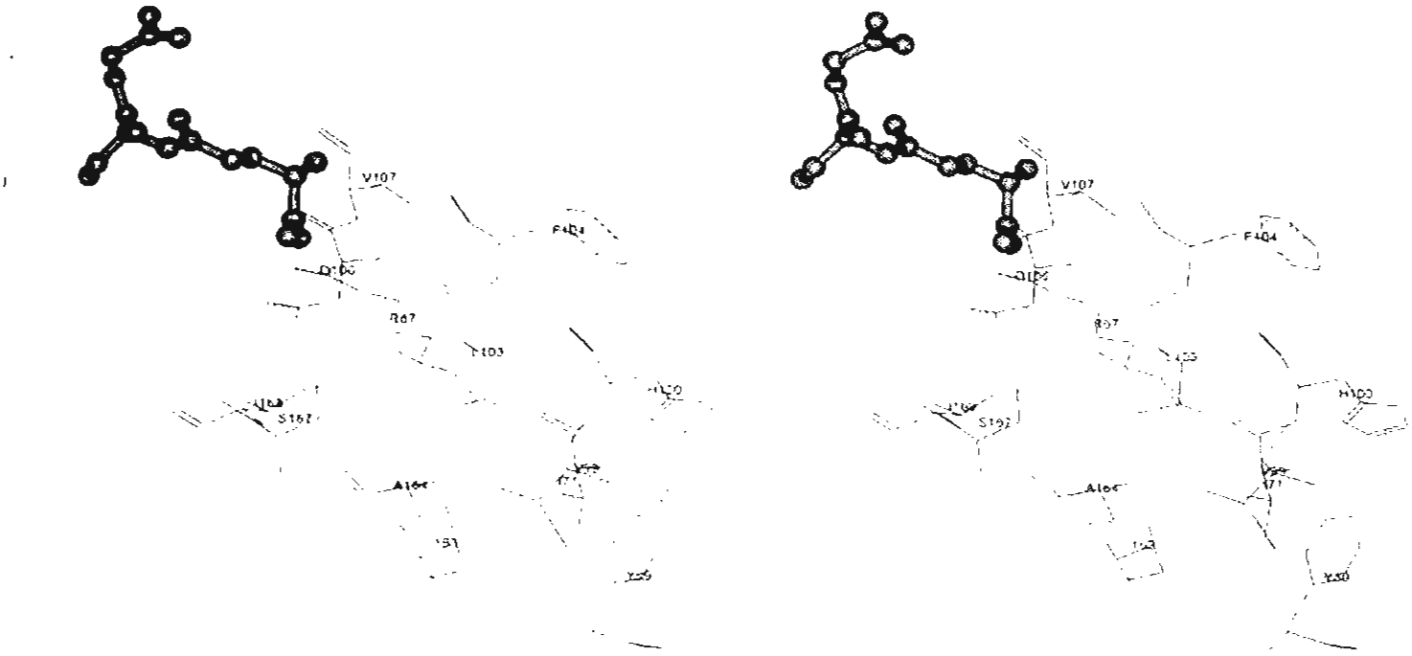

Figure 4 Stereo view of the 13 amino acid residues surrounding residue 103 in AdGSTD4-4

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 co-ordinates 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

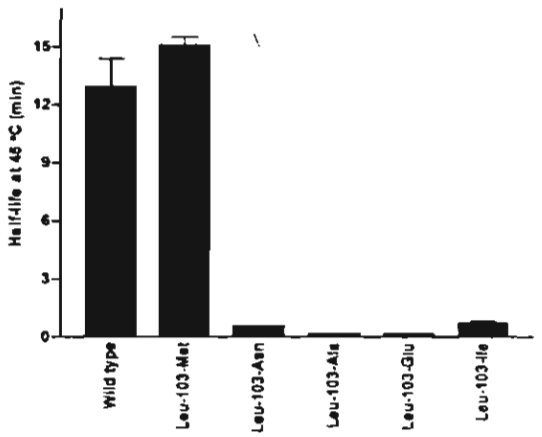

Figure 5 Half-life of recombinant mutant GSTs at 45 °C compared with rdGSTD4-4 (wild-type) from *A. dirus*

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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Non-active site residues Cys69 and Asp150 affected the enzymatic properties of glutathione *S*-transferase AdGSTD3-3

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Abstract

To elucidate how non-active site residues support the catalytic function, five selected residues of AdGSTD3-3 isoenzyme were changed to AdGSTD1-1 residues by means of site-directed mutagenesis. Analysis of the kinetic parameters indicated that Cys69Gln and Asp150Ser showed marked differences in V_{max} and K_m compared with the wild type enzyme. Both residues were characterized further by replacement with several amino acids. Both the Cys69 and Asp150 mutants showed differences with several GST substrates and inhibitors including affecting the interactions with pyrethroid insecticides. Cys69 and Asp150 mutants possessed a decreased half-life relative to the wild type enzyme. The Asp150 mutation appears to affect neighboring residues that support two important structural motifs, the N-capping box and the hydrophobic staple motif. The Cys69 mutants appeared to have subtle conformational changes near the active site residues resulting in different conformations and also directly affecting the active site region. The results show the importance of the cumulative effects of residues remote from the active site and demonstrate that minute changes in tertiary structure play a role in modulating enzyme activity.

Keywords: Site-directed mutagenesis; Enzyme characterization; Protein stability; Molecular modeling

1. Introduction

Glutathione *S*-transferases (GSTs) (E.C 2.5.1.18) are multifunctional enzymes involved in the detoxification and excretion of physiological and xenobiotic substances. The enzymes function by catalyzing the nucleophilic addition of the thiol of reduced glutathione to electrophilic centers in organic compounds (Booth et al., 1961). These proteins have the ability to bind large molecules for storage and transport roles using parts of the substrate binding sites (Oakley et al., 1999). GSTs also have been shown to regulate the stress kinase by acting as Jun-N terminal kinase (JNK) inhibitors (Adler et al., 1999). An additional signaling role shown for these cytosolic enzymes is modulation of ryanodine receptors, which are calcium channels (Dulhunty et al., 2001). Polymorphic GSTs are found in many organisms and organized into many classes e.g. alpha, mu, pi, theta, sigma, delta and kappa based on substrate and inhibitor specificity, immunological property and amino acid sequences (Mannervik and Danielson, 1988; Mannervik et al., 1992; Mannervik, 1985; Armstrong, 1997; Ketterer, 2001).

All of the cytosolic GSTs have the same basic protein fold, which consists of two domains. The N-terminal domain consists of beta sheets and alpha helices. The major function of this domain is to provide the G site, the binding site for glutathione (GSH). The C-terminal domain is an all α-helical domain. It provides structural elements for the recognition of hydrophobic substrates; the H site, and helps to define the substrate selectivity of the enzymes (Board et al., 2000; Ji et al., 1995; Reinemer et al., 1992; Reinemer et al., 1996; Sinning et al., 1993; Wilce et al., 1995). Although dimeric GSTs have a canonical tertiary structure, the subunit interface of the class alpha, mu and pi is characterized by a ball and socket hydrophobic interaction. Whereas, the interface

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Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; EA, ethacrynic acid; AdGSTD3-3, *An. dirus* GST delta 3-3; AdGSTD1-1, *An. dirus* GST delta 1-1

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for the sigma, theta and delta enzymes is flatter, more hydrophilic and lacks the lock-and-key motif (Board et al., 2000; Ji et al., 1995; Reinemer et al., 1992; Reinemer et al., 1996; Sinning et al., 1993; Wilce et al., 1995; Oakley et al., 2001).

At present, several of the G site and H site residues have been studied in mammalian GST classes (Dirr et al., 1994). However, non-active site residues also have effects on the properties of the enzymes through structural effects. Several studies have demonstrated that a single amino acid change outside of the active site could affect the catalytic activity of GSTs. In pi class, the conserved residues at the N-terminus of α-6, an N-capping box motif, has a role in folding and stability. Several mutants at this position showed an increase in k_{cat} and $k_{cat}/K_{m\ CDNB}$ of approximately 1.5-fold (Dragani et al., 1997). In alpha class, Phe 51 has a role in stabilizing the conformation of the dimeric protein through a lock-and-key intersubunit interaction. The mutation of Phe 51 to serine caused a change in binding affinity toward GSH and 1-chloro-2,4-dinitrobenzene (CDNB) substrates (Sayed et al., 2000). In addition, there is an intradomain interaction formed by the side chain of Trp 20 into a hydrophobic pocket in domain 2. The mutation of Trp 20 to alanine reduced the specific activity of GSTA 1-1 (Wallace et al., 2000). Another example of structural residue effects is the mutation of Cys 101 to serine in a pi class GST that increased the binding affinity toward CDNB by inducing a conformational change in α-4 which contains several H-site residues (Park et al., 2001). A study with a delta class GST shows that residue Glu 25 of AdGSTD1-1 effects the hydrophobic core in domain 1, whereas Ala 188 is a part of the hydrophobic core in domain 2 and mutations at these two positions changed the catalytic activity of the enzymes (Ketterman et al., 2001).

Insect GSTs are of particular interest because they may be involved in insecticide resistance. The *adGSTIAS1* gene identified from an *Anopheles dirus* genomic library, expresses alternatively spliced products that yield four mature transcripts (Pongjaroenkit et al., 2001). The four splicing products, adGST1-1, adGST1-2, adGST1-3 and adGST1-4, have been cloned and characterized (Jirajaroenrat et al., 2001; Ketterman et al., 2001). Previously 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 (Chelvanayagam et al., 2001). Where the 'D' refers to the GST delta class and the subunit number remains the same while 3-3, for example, signifies the enzyme is a homodimer. It was found that the AdGSTD3-3 isoenzyme could catalyze CDNB conjugation with a rate of 60 μmol/min/mg whereas the AdGSTD1-1 isoenzyme had a rate of 12 μmol/min/mg. Both isoenzyme subunits are 209 amino acids and share a 77% amino acid identity. From the 26 non-conserved residues in these GSTs, five residues were initially chosen, Cys69, Asp150, His178, Thr186 and Tyr206. These five were selected because of the differences in amino acid properties and the positioning of the side chain of the residues which face into the protein as shown by the tertiary structure of AdGSTD3-3 isoenzyme (Oakley et al., 2001). All of the residues are located outside of the active site and in domain II except Cys69 which is in domain I (Oakley et al., 2001). Therefore this work aimed to study the structure–function relationship of residues outside the active site of an insect GST by identifying important residues in AdGSTD3-3. All mutants were expressed and then characterized using several kinetic parameters, thermal stability and molecular modeling.

2. Materials and methods

2.1. Oligonucleotide-directed mutagenesis

The DNA encoding AdGSTD3-3 in pET3a (Jirajaroenrat et al., 2001) was used as a template in the mutagenesis procedure. The single mutations Cys69Gln, Cys69Asn, Cys69Ala, Asp150Ser, Asp150Tyr, Asp150Ala, His178Asn, Thr186Cys, Tyr206Lys and the double mutations Cys69Gln/Asp150Ser were made with the following oligonucleotides: Cys69Gln: 5′GCG CGC CAT CCA GAC GTA CTT AGC GGA GAA GTA CGG CAA G 3′, Cys69Asn: 5′CTG TGG GAG TCG CGC GCC ATC AAT ACG TAC TTG GCG GAG 3′, Cys69Ala: 5′GAG TCG CGC GCC ATC GCT ACG TAC TTG GCG GAG AAG 3′, Asp150Ser: 5′GCA CAA GTA CGT GGC GGG CTC GAG TCT GAC GAT CGC G 3′, Asp150Tyr: 5′GGG CAC AAG TAC GTA GCG GGC GCG AGT ACG ATC GCG 3′, Asp150Ala: 5′GGG CAC AAG TAC GTA GCG GGC GCG AGT ACG ATC 3′, His178Asn: 5′ GGG CTT CGA GCT CGC GAA GTA CCC GAA TGT GGC GGC GTG GTA 3′, Thr186Cys: 5′ TACGAG CGC TGT CGC AAG GAG GCG CCC GGT GCC GCC AT 3′, Tyr206Lys: 5′ GAG GAG TTC AGG AAG AAA TTC GAA AAG TAA CAT ATG GCT AGC 3′. The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was used in accordance with the manufacturer's instructions. Clones with the required mutation were first identified by restriction digest of the plasmids, and confirmed by full-length sequencing in both directions using a BigDye™ Terminator Cycle Sequencing Kit (Perkin–Elmer).

2.2. Expression and purification of wild type and mutant AdGSTD3-3 isoenzymes

A colony of *E. coli* BL21 (DE3)pLysS which contained a recombinant plasmid was grown at 37 °C until

the OD_{600} was approximately 0.6. IPTG was added to a final concentration of 0.1 mM, then the incubation was prolonged for a further 3 h. The purification of enzymes was performed as follows. The bacterial cells from 200 ml culture were collected by centrifugation at 5000 rpm, 4 °C for 10 min. The pellets were resuspended in 19.2 ml of PBS, pH 7.3 (140 mM NaCl, 27 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 of 50 mM Tris–HCl pH 7.4, 1 mM EDTA), 800 μl of 100 mg/ml lysozyme and 14.4 μl of 1.4 M β-mercaptoethanol were then incubated on ice for 30 min. 200 μl of 1 M DTT was added and the mixture was lysed at 900 psi in a French Pressure cell. The particulate material was removed by centrifugation at 10,000*g* at 4 °C for 20 min. The resulting supernatant was subjected to GSTrap affinity column. All the steps to obtain the purified GSTs were performed at 4 °C. The non-specific binding proteins were removed by washing with PBS buffer. The proteins bound to the column were eluted with 50 mM Tris–HCl (pH 8.0), containing 10 mM reduced–glutathione and 10 mM DTT. The fractions containing recombinant GST were pooled before concentrating by using a centriprep-10 (Amicon) at 2500*g*, 4 °C. Then the bound glutathione was eliminated using HiTrap desalting column (Amersham Pharmacia Biotech) equilibrated with 50 mM phosphate buffer pH 6.5. The concentrated protein was applied to the column and eluted in the same buffer containing 10 mM DTT. The concentration step was repeated until the final volume was 0.5 ml. Glycerol was added to a final concentration of 50% and the purified concentrated GSTs were stored at −20 °C.

2.3. Characterization of the expressed enzymes

The purity of an enzyme preparation was determined by SDS-PAGE using Bio-Rad low range standards as molecular weight markers. K_m and V_{max} for 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) were determined by non-linear regression analysis using Graphpad Prism 2.01 Software. Specific activities toward several GST substrates were determined spectrophotometrically. The GST activities were measured with glutathione and five other substrates; 1-chloro-2,4-dinitrobenzene (CDNB), 1-2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid, 4-nitrophenethyl bromide and 4-nitrobenzyl chloride using the appropriate pH and λ_{max} (Habig et al., 1974). To determine the inhibition kinetics parameter K_i for the GSTs the effect of cumene hydroperoxide, *S*-hexylglutathione and the pyrethroid insecticides, deltamethrin and permethrin, on the CDNB conjugating activity of GSTs was determined in assays at fixed concentration of 10 mM GSH containing varying concentrations of CDNB 0.02–2.5 mM in 0.1 M phosphate pH 6.5. The K_i results were calculated based on double-reciprocal Lineweaver–Burk plots. In the thermal stability assay, the enzyme was incubated 10 min at different temperatures ranging from 30 to 50 °C at a protein concentration of 0.1 mg/ml in 0.1 M potassium phosphate (pH 6.5) containing 5 mM DTT and 1 mM EDTA. The enzyme was heated in sealed eppendorf tubes and the temperature was monitored with a thermometer. The stability time courses were determined by withdrawing suitable aliquots at different time points from the denaturation mixture for assay of remaining activity. Protein concentration was determined using the Bio-Rad protein reagent with bovine serum albumin as the standard protein (Bradford, 1976).

2.4. Molecular modeling

Initial coordinates of AdGSTD3-3 were taken from the X-ray crystal structure of the complex with glutathione (Oakley et al., 2001). Modeling of the tertiary structure was performed using the Insight II software (Biosym/MSI, San Diego, CA). Initial strains in the system were released by a two-step energy minimization, using the steepest descent and conjugate gradient algorithm until the average absolute derivative was 10^{-7}. Then the models of the mutants were generated. The minimization was repeated to obtain the most stable model. To study the effects on tertiary structure of the protein, several parameters were measured e.g. root mean square deviation of the alpha carbon backbone (rms) using swisspdb software, distances across the active site pocket, dihedral angle changes, residue movements, H-bond distances and H-bond angles.

3. Results

3.1. Expression and purification of mutant enzymes

To assess the contribution of the five chosen residues to the differences between AdGSTD3-3 and AdGSTD1-1, five mutants of AdGSTD3-3; Cys69Gln, Asp150Ser, His178Asn, Thr186Cys and Tyr206Lys were generated by PCR with a single amino acid being replaced with the equivalent amino acid in AdGSTD1-1. The locations of the five residues are shown in Fig. 1. Mutants and wild type enzymes were then expressed in *E. coli* and purified by affinity chromatography on immobilized GSH. The purified proteins gave a single band on SDS-polyacrylamide gel electrophoresis with a size of approximately 23 kDa, which corresponds to the calculated molecular weight of the GST subunits.

3.2. Kinetic properties

Comparison of the kinetic parameters of AdGSTD3-3 mutants with the wild type values showed that the residue changes affected enzymatic properties (Table 1). Three of the mutant GSTs, His178Asn, Thr186Cys and

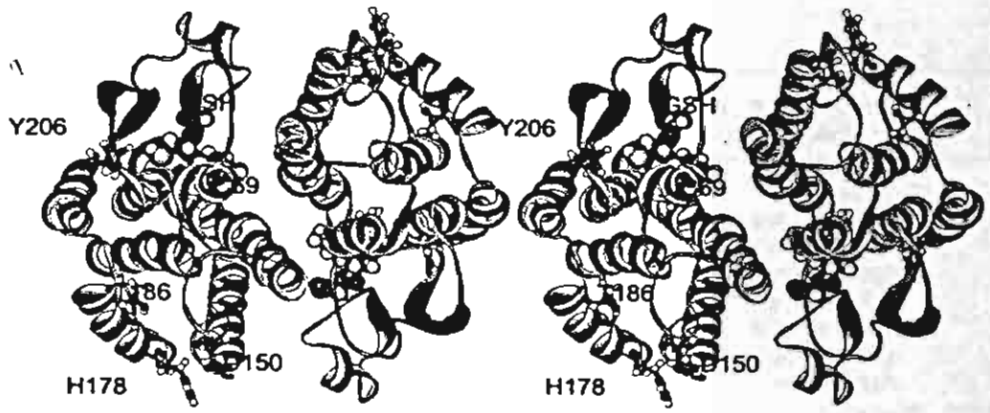

Fig. 1. Stereoview of homodimeric AdGSTD3-3 complexed with glutathione. The five selected residues are labeled and shown in ball-and-stick form.

Table 1
Kinetic constants for the AdGSTD3-3 mutants

Enzyme	V_{max}	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
Wild type AdGSTD3-3	63.6 ± 6.2	25.2	0.29 ± 0.03	86.9	0.13 ± 0.01	194
Wild type AdGSTD1-1*	12.9 ± 0.6	5.03	0.86 ± 0.18	5.86	0.10 ± 0.03	48.4
Cys69Gln	105 ± 7	41.7	0.44 ± 0.03	96.9	0.21 ± 0.03	198
Asp150Ser	70.2 ± 1.0	27.8	0.17 ± 0.02	160	0.07 ± 0.01	391
His178Asn	67.4 ± 2.8	26.7	0.51 ± 0.05	53.6	0.12 ± 0.01	222
Thr186Cys	59.5 ± 3.4	23.6	0.31 ± 0.04	76.8	0.12 ± 0.02	195
Tyr206Lys	63.5 ± 3.3	25.1	0.22 ± 0.02	114	0.25 ± 0.05	100
Cys69Asn	26.7 ± 6.4	9.61	0.53 ± 0.03	18.2	0.08 ± 0.01	117
Cys69Ala	65.5 ± 7.5	25.9	0.33 ± 0.04	79.2	0.11 ± 0.03	235
Asp150Tyr	74.2 ± 6.8	29.5	0.21 ± 0.02	141	0.09 ± 0.01	327
Asp150Ala	80.2 ± 7.0	31.7	0.33 ± 0.02	96.7	0.08 ± 0.02	382
Cys69Gln/Asp150Ser	116 ± 7	46.1	0.40 ± 0.04	117	0.23 ± 0.03	200
Arg96Ala	118 ± 7	46.8	2.75 ± 0.12	17	0.38 ± 0.06	125

The units are: V_{max}, μmole/min/mg; K_m, mM; k_{cat}, s^{-1}, k_{cat}/K_m, mM^{-1} s^{-1}. The data are mean ± standard deviation from at least three independent experiments.

* See Ketterman et al. (2001)).

Tyr206Lys, had the same maximal velocity (V_{max}) as the wild type but the replacement of Cys69 with glutamine caused a significant increase of 65% in the V_{max}. There were approximately 1.7- and 1.8-fold decreases in the K_m value for GSH and CDNB for the mutant Asp150Ser. The k_{cat}/K_m for Asp150Ser shows that it possesses about a 1.8- and 2-fold greater catalytic efficiency for both GSH and CDNB, respectively, compared to the wild type enzyme. In contrast, Tyr206Lys had the lowest efficiency for CDNB and His178Asn had the lowest toward GSH. Only the two positions Cys69 and Asp150 were characterized further. Amino acid substitutions were performed producing Cys69Asn, Cys69Ala, Asp150Tyr and Asp150Ala. In addition, the double mutant Cys69Gln/Asp150Ser, was also generated to examine the possible combined effect of both residues (Table 1). It was found that the alteration of Cys69 with asparagine significantly decreased the k_{cat} 2.6-fold compared to the wild type AdGSTD3-3. However, there was no significant change observed in the kinetic parameters of Cys69Ala. The double mutant enzyme Cys69Gln/Asp150Ser possessed kinetic constants very similar to those observed for the single mutant Cys69Gln. Kinetic constants for the single mutant Cys69Gln and Cys69Asn strongly suggest that the amino acid at position 69 has a major structural influence on the catalytic activity of the enzymes. The Asp150Tyr mutant showed a slightly lower $K_{m\ GSH}$ than the wild type AdGSTD3-3 whereas Asp150Ala had a greater value. However both mutants displayed a significantly lower K_m for CDNB, as did the Asp150Ser mutant.

To establish whether the mutant and wild type enzymes have similar or distinct catalytic specificity, their activities toward a panel of established GST substrates; (1-chloro-2,4-dinitrobenzene (CDNB), 1-2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA),

Table 2
Specific activity of the AdGSTD3-3 mutants towards five different substrates

Enzyme	CDNB (1 mM)	DCNB (1 mM)	4-Nitrobenzyl chloride (0.1 mM)	4-Nitrophenethyl bromide (0.1 mM)	Ethacrynic acid (0.2 mM)
Wild type AdGSTD3-3	57.2 ± 3.2	0.248 ± 0.003	0.135 ± 0.013	0.019 ± 0.007	0.087 ± 0.010
Cys69Gln	95.6 ± 2.0	0.249 ± 005	0.165 ± 0.013	0.070 ± 0.008	0.126 ± 0.016
Cys69Ala	50.2 ± 0.9	0.205 ± 0.003	0.196 ± 0.025	0.057 ± 0.003	0.109 ± 0.022
Cys69Asn	32.0 ± 0.6	0.252 ± 0.007	0.165 ± 0.024	0.030 ± 0.015	0.064 ± 0.006
Asp150Ser	64.7 ± 5.5	0.249 ± 0.005	0.142 ± 0.009	0.070 ± 0.008	0.126 ± 0.016
Asp150Ala	68.9 ± 3.8	0.284 ± 0.009	0.111 ± 0.004	0.021 ± 0.004	0.050 ± 0.017
Asp150Tyr	63.9 ± 3.7	0.289 ± 0.003	0.173 ± 0.020	0.067 ± 0.013	0.100 ± 0.016
Cys69Gln/Asp150Ser	97.7 ± 7.7	0.315 ± 0.002	0.209 ± 0.006	0.069 ± 0.017	0.100 ± 0.009

The units are μmole/min/mg of protein. The data are mean ± standard deviation from at least three independent experiments.

4-nitrophenethyl bromide and 4-nitrobenzyl chloride) were determined (Table 2). The Cys69 mutants showed differences with several of the GST substrates especially CDNB and EA. The Asp150 mutants displayed several differences in the conjugating activity towards the tested substrates most notably with 4-nitrophenethyl bromide and EA. The double mutant enzyme had a significant increase in the DCNB, 4-nitrobenzyl chloride and 4-nitrophenethyl bromide activity compared to the wild type AdGSTD3-3. It also possessed greater activity towards DCNB and 4-nitrobenzyl chloride than the respective single residue mutant enzymes. An inhibition kinetics parameter, K_i, for adGSTD3-3 mutants was determined using four different compounds, cumene hydroperoxide, deltamethrin, permethrin and *S*-hexylglutathione (Table 3). The K_i ranges of the GSTs varied from 3-fold for deltamethrin up to 80-fold for *S*-hexylglutathione. All mutations increased the affinity of interaction, relative to the wild type enzyme, up to 30-fold with CuOOH but decreased the affinity up to 5-fold for interaction with permethrin. Of special interest was the Asp150Ala mutant which displayed the greatest affinity, lowest K_i value, for deltamethrin but the lowest affinity, greatest K_i value, for permethrin demonstrating conformational differences in the adGSTD3-3 are involved in governing the binding for the pyrethroid insecticides.

The stability experiment was performed at different temperatures and demonstrated that the wild type and most of the mutant enzymes were stable up to 40 °C. The half-life of the enzymes were calculated based on the relationship between the log percentage of original activity incubated at 4 °C and the activity at different temperatures of preincubation. The half-life of the wild type and Cys69Gln at 40 °C was approximately 31.9 and 40 min whereas both Cys69Ala and Cys69Asn possessed a shorter half-life, of 28.8 and 1 min(s), respectively. At 45 °C, Cys69Asn lost all activity, however the wild type, Cys69Gln and Cys69Ala retained their activities each with a half-life of approximately 6.2, 1.8 and 1.5 min, respectively. The results suggest that the amino acid at position 69 affects the core residues in domain 1, the mutational changes thereby disrupting the enzyme stability. The Asp150 mutants and the Cys69Gln/Asp150Ser double mutant were relatively stable to incubation at temperatures up to 40 °C. The exception was the Asp150Tyr mutant, which lost all activity at 40 °C. At 45 °C the mutants Cys69Gln/Asp150Ser, Asp150Ser and Asp150Ala showed a decrease in half-life to 1.9, 1.6 and

Table 3
Determination of K_i for AdGSTD3 mutants

Enzymes	K_i (μM)			
	CuOOH	Deltamethrin	Permethrin	*S*-Hexyl
adGSTD3-3	34,900	48.1	31.3	3.1
Cys69Gln	1300	63.7	75.2	3.8
Cys69Ala	23,300	50.1	47.0	2.9
Cys69Asn	1200	61.8	51.0	3.0
Asp150Ser	13,100	56.0	39.0	97
Asp150Ala	8500	21.2	166	4.1
Asp150Tyr	8300	40.1	46.1	4.4
Cys69Gln/Asp150Ser	23,000	59.7	128	231

CuOOH is cumene hydroperoxide, and *S*-hexyl is *S*-hexylglutathione.

0.94 min, respectively. Therefore the amino acid at position 150 also appeared to influence the enzyme structure as demonstrated by the stability effects.

3.3. Molecular modeling

After the residues of the wild type were replaced and the energy of the system was minimized to an average absolute derivative of 10^{-7} then the reliability of the model was tested by changing a mutant residue back to the wild type. The energy of the system was re-minimized to an average absolute derivative of 10^{-7}. Two parameters; dihedral angle and residue movement of the two minimized wild type structures, were compared and only very small changes were observed; 0.93° and 0.3 Å, respectively indicating a relatively good reproducibility of the model.

The analysis of the three dimensional models of the Cys69Gln, Cys69Ala and Cys69Asn indicated that the root mean square deviation of the alpha carbon backbone (rms) ranged from 0.01 to 0.74 Å suggesting that the alteration of Cys69 caused no effects on the main chain of the protein. Another parameter measured was the dihedral angle. All the Cys69 mutants showed dihedral angle changes of approximately 0.02–2.03° in helix 1 to helix 8 suggesting that there was no significant change in conformation. The distances across the active site were measured by selecting 10 pairs of residues that line the active site. The changes in distance were less than 1 Å therefore the alteration of the residues did not appear to change the conformation of the active site. All the H-bond distances and H-bond angles were measured within one GST subunit. The range of the H-bond distance was 1.6–2.6 Å whereas the range of the H-bond angle was 110–180°. All of the Cys69 models showed no differences in H-bond distances but they demonstrated greater changes in the main chain H-bonds which were 18.27°, 8.47°, 7.12° in Cys69Gln, Cys69Ala and Cys69Asn, respectively. The residue movements were measured from the alpha carbon of the residue 69 to all the neighboring residues within 25 Å range of the dimeric GST model. All the Cys69 models showed small movements in the atoms ranging from 0.3 to 1.67 Å.

In the Asp150 mutant models including the double mutant Cys69Gln/Asp150Ser, the rms of the α carbon backbone, the active site distances and the H-bond distances were in the same ranges as the Cys69 mutants. However, the remaining parameters showed greater changes compared to the wild type model. The dihedral angle of helix 7 in the Asp150Tyr, Asp150Ser and Cys69Gln/Asp150Ser models was changed approximately 5°. In addition, the dihedral angle of Asp150Ala was changed 34° in helix 7 and 7–8° in helix 5 and 6. All the results strongly suggest that there are conformational changes in the Asp150 mutants in domain 2. The residue movements of Asp150Tyr and Asp150Ser mutants revealed maximum changes in the distances measured to His144, located in the loop before helix 6, of approximately 2.25 and 1.48 Å. Whereas the Asp150Ala mutant showed a maximum change of 2.65 Å measured to Gln189 which is located in helix 7. These results provide further evidence of subtle conformational changes of the Asp150 mutants. The maximum movement of Cys69Gln/Asp150Ser was 1.78 Å when measured from the α carbon of residue 150 to Thr18 and 1.29 Å when measured from the α carbon of residue 69 in one subunit to residue 69 in the other subunit. The maximum changes in H-bond angles were also found in domain 2 of Asp150 mutants. In each model the maximum changes ranged from 4.5 to 18.7° and the involved residues, Leu141, Gly143, Lys145, Arg185 and Glu189, were located in similar positions in helix 7 and the loop before helix 6. However, the Cys69Gln/Asp150 mutant demonstrated a maximum change in the main chain H-bond angle in domain 1 that forms between the NH of Thr18 and the oxygen from Ala14 of approximately 18.54° which was similar to Cys69Gln.

4. Discussion

Characterization of the mutant enzymes of the first five residues initially chosen demonstrated that single residue changes affected the enzymatic property. The mutations at position 69 and 150 appeared to affect sensitive regions of the tertiary structure resulting in changes in V_{max} and substrate specificity of the enzyme as shown in Tables 1 and 2. Additional five mutations of the two residues showed differences in substrate specificity, percent inhibition and thermal stability when compared to the wild type AdGSTD3-3.

To aid in interpretation of the observed kinetic data, six modeling parameters were explored using molecular modeling. It was found that three parameters; root mean square deviation of the alpha carbon backbone, the configuration of the active site, and the hydrogen bond distance did not change significantly. However, subtle conformational changes can be observed from the remaining three parameters; the dihedral angles of the helices, residue movements and hydrogen bond angles suggesting that the change in the kinetic data occurs due to differing conformational changes of the enzymes.

The modeling data suggests that the changes in the Cys69 mutant kinetics occur from subtle conformational shifts. Among the six parameters observed, only H-bond angles showed the greatest changes. Based on the models of Cys69Asp, Cys69Ala and Cys69Gln, the NH group from the main chain of Cys69 formed an H-bond with the main chain carboxyl group of Ser65, which is an active site residue directly interacting with GSH. When compared to the wild type, those H-bond angles were changed approximately 5–8°. A second change is

between residue Thr18 and Ala14. The NH group from Thr18 forms an H-bond with the carboxyl group of Ala14. In the Cys69Gln and Cys69Gln/Asp150Ser models, these angles are changed approximately 18°. Both locations are near the active site residues 64–66 that directly interact with GSH. These changes suggest that atom movements occur that yield different conformations and directly affect the active site region. In addition, the residue changes also affect the packing of the hydrophobic core in domain 1 which is formed by the residues in helix 1 and helix 3 (Dirr et al., 1994). Cys69 is located in helix 3 and functions in structural stabilization. All the Cys69 mutants possessed a much shorter half-life at 45 °C. The mutation at residue 69 would affect the neighboring residues that interact with Ile68 such as Ala67 and Tyr71. Ile68 appears equivalent to an invariant isoleucine found in alpha, mu and pi class GST. Mutation of this isoleucine was shown to disrupt the hydrophobic core and inactivate the enzyme (Manoharan et al., 1992).

The effects of the Asp150 residue on the active site can be proposed to occur through two pathways based on the results from the models. The first pathway is an intra-subunit route. Asp150 forms an ionic interaction with His144, which showed maximum movement in the models. This interaction may stabilize the loop of residues 142–155 before helix 6. There are two conserved hydrophobic residues within the loop, Val147 and Ala148 (which are Ala147 and Ala148 in AdGSTD1-1; Val151 and Ala152 in AdGSTD2-2; Val155 and Ala156 in AdGSTD4-4; Val147 and Ala148 in *L. cuprina*; Ala148 and Ala149 in *D. melanogaster*). These two hydrophobic residues have atom–atom interactions with neighboring residue Arg96 in helix 4. The varying interactions in the different mutants would generate positional differences in Arg96 that would then affect interactions with Arg66, which is an active site residue. The other possible pathway is between subunits of the protein. The changes in residue interactions affecting Arg96 would also effect the interaction with Trp63 in the other GST subunit. The positional changes in Trp63 would then influence the interaction with Gln49, which is an active site residue. There is evidence of signaling across the GST subunits through an interaction of Arg69 and Gln100 with the active site residue Arg15 in alpha class (Xiao et al., 1999).

To test the hypotheses the mutant Arg96Ala, a pivotal residue shared by both proposed pathways, was generated. It showed dramatic effects on the enzyme affinities and stability. The change in k_{cat} for Arg96Ala was only increased approximately 2-fold compared to the wild type AdGSTD3-3 (Table 1). However, the K_m for CDNB increased 3-fold and the K_m for GSH increased approximately 10-fold. In addition, Arg96Ala possessed a half-life 30-fold greater, 187 min compared to 6.2 min for the wild type. Therefore Arg96Ala appeared to be involved with both catalysis and structural support. However, the major residues involved in affecting Arg96 via the two proposed pathways remain to be elucidated.

The AdGSTD Asp150 mutation also would affect neighboring residues that generate two important motifs, the N-capping box and the hydrophobic staple motif. These motifs are located in the loop before helix 6 and play a major role in the folding and stability of GSTs. The N-capping box motif consists of Thr153 and Asp156 that H-bond to each other. In addition, Val147 also forms an H-bond with Asp156. Mutations at these positions in pi class destabilized the enzyme structure at higher temperatures by disrupting all the hydrogen bonding network (Dragani et al., 1997; Rossjohn et al., 2000). Based on the structure of the wild type AdGSTD3-3, Tyr146, Ala148 and Gly149 are analogous residues with similar H-bond interactions that generate this motif. Several atom movements observed from the Asp150 mutant models might lead to the loss of these interactions that would result in more disordering of the loop and destabilization of helix 6. The hydrophobic staple motif in AdGSTD3-3 consists of Leu152 and Leu157 located adjacent to the N-capping box motif. Several residues in the loop e.g. Thr153, Ile154 and Ala155 contact Leu152 and Leu157 both of which directly interact with many residues which are important structural elements influencing the active site residues in helix 1. A single mutation in either of Leu157 or Leu152 at the equivalent residues in alpha and pi class increased the k_{cat} and k_{cat}/K_m values for CDNB as well as destabilizing the enzymes (Cocco et al., 2001; Stenberg et al., 2000). Our Asp150 mutants e.g. Asp150Ser also showed a similar result suggesting that the residue interactions induce conformational changes in this region that destabilize helix 6 and affect the active site residues in helix 1 that result in changes in both the stability property of the protein and kinetic parameters. Further support for tertiary structure changes is given by the modeling data. The Asp150 mutants showed a large change in dihedral angles of helices 5–7 that would affect the packing of the hydrophobic core in domain 2. When the loop becomes less flexible, the residues on the loop would be packed in the hydrophobic core with less movement thereby yielding more stability and giving the wild type enzyme a longer half-life at 45 °C.

In our study, when the residues from isoenzyme AdGSTD3-3 were replaced with residues from isoenzyme AdGSTD1-1, the GST mutants Cys69Gln and Asp150Ser did not show any kinetic properties of isoenzyme AdGSTD1-1 as expected (Table 1), demonstrating that contributions from other residues are also needed. A mutation study in alpha class GST was performed to increase the activity of rat GST A1-1 toward 4-hydroxyalkenal similar to isoenzyme rGSTA4-4 (Björnestedt et al., 1995). It was shown that a substitution of Ala12Gly of GST A1-1 cannot increase the alkenal activity. A

chimeric rGSTA4-4 was generated by replacing the first 25 residues in the N-terminus with A1-1 residues resulting in a mutant enzyme with low alkenal activity. Then it was shown that the Ala12Gly point mutation of the chimeric enyzyme can confer high alkenal activity demonstrating that the non-active site residues are essential. In addition, human GST A2-2 was also improved in steroid isomerase activity by replacing five amino acids in the H-site with the residues of GST A3-3, the most efficient catalyst of double-bond isomerization (Pettersson et al., 2002; Johansson and Mannervik, 2001; Pettersson et al., 2002). These mutations resulted in a 3500-fold increase in catalytic efficiency with Δ^5-androstene-3, 17-dione which was less than the parental A3-3 by approximately 1500-fold, suggesting that the lower activity is from an indirect role of non-active site residues. An additional study succeeded in redesigning the substrate-selectivity of GST A1-1 to yield a high catalytic efficiency with toxic alkenal products of lipid peroxidation (Nilsson et al., 2000). The improved enzyme was generated by replacing four important active site residues and the C-terminus part containing several non-active site residues with the residues from GST A4-4. The mutant had a 300-fold increase in catalytic efficiency with nonenal compared to the original A1-1 and was 3.1-fold more efficient than the GST A4-4 that normally possessed the highest alkenal activity. However, the mutant still showed approximately 3-fold lower catalytic efficiency toward HNE when compared to GST A4-4 suggesting a role for other non-active site residues in influencing the catalytic residues in their required orientations.

Both amino acids Cys69 and Asp150 are non-active site residues, but their replacement appears to yield a large number of small changes in the enzyme that leads to different flexing or conformational changes thereby changing the enzymatic properties. This finding was also found in enzymes other than GSTs. For example, aspartate aminotransferase was mutated to a valine aminotransferase (Oue et al., 1999). This enzyme with 17 mutations demonstrated a 2.1×10^6-fold increase in k_{cat}/K_m for valine, a non-native substrate. However, only one of the mutated residues directly interacted with the substrate. The crystal structure demonstrated that the mutations affected the enzyme structure by changing the subunit interface including shifting of the enzyme domain that enclosed the substrate.

In conclusion, the influence of the non-active site residues in affecting the positioning of the catalytic residues depends on the specific amino acids involved including the surrounding milieu of amino acids. Therefore the results suggest the importance of the cumulative effects of residues remote from the active site and demonstrate that minute changes in tertiary structure play a role in modulating enzyme activity.

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MULTIPLE ROLES OF GLUTATHIONE BINDING-SITE RESIDUES OF GLUTATHIONE S-TRANSFERASE

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Abstract: This study was designed to characterize residues in the glutathione binding site of AdGSTD4-4 from the mosquito malaria vector *Anopheles dirus*. The data revealed that Leu33, His38 and His50 each play a role in enzyme catalysis and glutathione binding. The mutants of these three residues also displayed differences in hydrophobic substrate specificity, suggesting that changes in the active site conformation occurred. Differences in conformations was also suggested by protein stability changes. These results indicate that residues in the glutathione binding site are not only important in the catalytic function but also play a role in the structural integrity of the enzyme.

Keywords: active site, *Anopheles dirus*, catalysis, glutathione transferase, mosquito, structure.

INTRODUCTION

Glutathione S-transferases (GSTs: E.C. 2.5.1.18) are intracellular proteins which are widely distributed in nature and found in most aerobic eukaryotes and prokaryotes. They are involved in xenobiotic metabolism as well as protection against peroxide damage [1, 2]. GSTs catalyze the S-conjugation between the thiol group of the tripeptide glutathione (GSH, γ-glutamyl-L-cysteinyl-glycine) and a large number of electrophilic moieties in the hydrophobic substrates [2-4]. A simplified enzyme catalysis scheme for GSTs first involves GSH substrate binding to the active site, then GSH ionization to form a nucleophilic thiolate anion (GS^-), followed by substrate conjugation, product formation and finally product release [5-7]. Once the GSH thiolate anion (GS^-) is formed in the binding site, it is capable of reacting spontaneously by nucleophilic attack with electrophilic compounds in close proximity. The conjugation increases the solubility of the target molecule thus facilitating the excretion of the molecule from the organism.

All cytosolic GSTs consist of two domains [5, 8-10]. The N-terminal domain constitutes roughly one-third of the protein and adopts a βαβαββα topology which contributes most of the contacts to the GSH substrate and is referred to as the GSH binding site (G-site). The C-terminal domain is all α-helical, is two-thirds of the protein and provides some of the contacts to the hydrophobic binding site (H-site) that lies adjacent to the GSH binding site. The H-site shows the greatest variability across GST classes and defines the substrate selectivities of the various isoenzymes [5, 8, 10]. The functional implication of the dimeric quaternary structure allows for the construction of a fully functional active site, part of which is situated near the subunit interface and is also a significant source of stabilization for the tertiary structures of the individual subunits.

There is evidence for at least five classes of insect GSTs based on their amino acid sequence homology, substrate specificity, physical properties and immunological properties. The insect class I GSTs or delta class are of interest because of their potential roles in insecticide resistance. The resistance to pesticides in insects has been shown to correlate with elevated levels of GST activity [2,11].

The *Anopheles dirus* mosquito *adgst1AS1* gene, obtained from a genomic library, is spliced to yield four mature GST transcripts; AdGSTD1, AdGSTD2, AdGSTD3 and AdGSTD4 [12]. This study focuses on AdGSTD4 only. AdGSTD4 has very high heterologous expression levels in *E. coli*. The high activity and catalytic efficiency toward GSH and CDNB substrates and the available crystal structure make this protein ideal for structure-function relationship studies [13,14].

This project aims to study the structure-function relationship of AdGSTD4 by characterizing critical residues that are in the active site. Leu33, His38 and His50 were investigated in this study. These residues are in the glutathione binding site (G-site) and His38 and His50 interact directly with the carboxylic group of glycine in GSH. These residues are conserved across insect delta class GSTs which suggests they play an important role. The selected residues were mutated and characterized for enzymatic properties as well as physical properties and compared to the AdGSTD4 wild type enzyme. The mutations showed effects not only in the kinetic properties and substrate specificity but also in the stabilities of the enzymes.

MATERIALS AND METHODS

Site Directed Mutagenesis

The mutants were generated using Stratagene's QuickChange Site directed mutagenesis protocol. The selected residues Leu33, His38 and His50 are in the G site. His38 and His50 directly interact with GSH. The mutagenic primers used in this experiment have been designed according to the 5' and 3' sequence of the AdGSTD4 wild type gene (Genbank accession number AF273040). The oligonucleotide primers, each complementary to opposite strands of the vector, were extended during temperature cycling by means of *Pfu* DNA polymerase, which replicates both plasmid strands with high fidelity. Each mutant was randomly screened by restriction digestion analysis. Mutant plasmids could be distinguished from the template by digestion with the restriction enzyme corresponding to the restriction recognition site introduced by the mutagenic primers. Then full length DNA sequencing in both directions was performed to confirm the mutant clones.

Protein Expression and Purification

After transformation of the mutant plasmids into *E. coli* BL21(DE3)pLysS, protein expression was performed. All the AdGSTD4 mutants clones and wild-type were expressed in 200 ml LB broth (containing 100 μg/ml ampicillin and 34 μg/ml chloramphenicol) and induced with 0.1 M IPTG for 3 hours at 37 °C. The pellets were collected and kept at -20°C until used. The expression levels of the protein were determined by SDS-PAGE. The recombinant AdGSTD4 mutants and wild type were purified by using either glutathione affinity chromatography (GSTrap) or by using cation exchange followed by hydrophobic interaction chromatography. The GSTrap was used according to manufacturers instructions (Amersham Biosciences). The cation exchanger chromatography employed a SP-XL column equilibrated with Buffer A which was 20 mM Phosphate buffer pH 7 containing 10 mM dithiothreitol (DTT). After the lysate was applied to the column, the column was washed with buffer A. Then the protein was eluted with a linear gradient from 80-500 mM NaCl in buffer A. The major amount of GST enzyme eluted in buffer A containing 250 mM NaCl. The eluted GST fractions were pooled and loaded to a phenyl Sepharose column which was equilibrated with buffer A containing with 2 M NaCl. Washing steps were performed by using stepwise decreases in salt concentration in buffer A. The GST enzyme was eluted from the phenyl Sepharose column by using Buffer A containing 20% ethylene glycol and 10 mM DTT. The purified enzymes were stored in 50% glycerol at -20 °C until used. The concentrations of the proteins were determined by Bio-Rad protein reagent (Bio-Rad) and the purity of the proteins was analyzed by SDS-PAGE.

GST Activity Determination

The GST activity was determined for all the AdGSTD4 mutants and compared with the wild type protein. The standard GST assay was performed in 0.1 M potassium phosphate buffer pH 6.5 in the presence of 3 mM CDNB and 10 mM GSH [13]. The rate of conjugation between GSH and CDNB was monitored by continuously measuring the change in absorbance at 340 nm for 1 minute using a SpectraMax 250 at 25-27 °C. The extinction coefficient of 9.6 $mM^{-1}cm^{-1}$ was used to convert the absorbance to moles [15].

Kinetic Parameters Determination

The kinetic experiments were performed as previously described [13]. In brief, CDNB was chosen as electrophilic substrate for determination of V_{max}, K_m, k_{cat} and k_{cat}/K_m values. The kinetic parameters were determined by varying the CDNB concentration (0.031-3.0 mM) while GSH was held constant at a saturating concentration and by varying the GSH concentrations (0.25-20 mM) at a saturating concentration of CDNB. The initial rate of the enzymatic reaction was measured spectrophotometrically as described for the GST activity assay determination. The steady state kinetics followed Michaelis-Menten kinetics except where stated. The maximal velocity (V_{max}) and the Michaelis constant (K_m) were determined by non-linear regression software analysis. The catalytic constant (k_{cat}) and the catalytic efficiency (k_{cat}/K_m) were calculated on an active-site basis using the subunit molecular mass of each enzyme. The kinetic parameters are the mean ± standard deviation for at least three independent experiments.

Substrate Specificities Determination

The specific activities toward several GST substrates were determined by spectrophotometer, as previously described [13]. All measurements were performed at 25-27 °C in 0.1 M potassium phosphate buffer pH 6.5 or pH 7.5. The GST activities were measured with glutathione and five hydrophobic substrates; 1-Chloro-2,4-dinitrobenzene (CDNB), 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), p-nitrophenethyl bromide (PNPBr) and p-nitrobenzyl chloride (PNBC). Specific activities were calculated according to the molar extinction coefficient (ε) for each substrate. The extinction coefficients were used to convert the absorbance to moles are of 9.6 $mM^{-1}cm^{-1}$, 8.5 $mM^{-1}cm^{-1}$, 5.0 $mM^{-1}cm^{-1}$, 1.2 $mM^{-1}cm^{-1}$ and 1.9 $mM^{-1}cm^{-1}$, respectively [15].

Half-Life Determination

The enzymes 0.1 mg/ml in 0.1 M phosphate buffer pH 6.5 containing 1 mM EDTA and 5 mM DTT were incubated at 45°C for various times and then the activity was measured in the standard GST assay. The data were plotted as log percentage of remaining activity versus pre-incubation time. The half-life of the enzyme at 45°C was calculated from the slope of the plot using the equations: Slope = k/2.3, $k = 0.693/t_{1/2}$.

RESULTS AND DISCUSSIONS

Kinetic Determination

As shown in Figure 1, the three residues Leu33, His38 and His50 are in the G-site [14]. However, the steady state kinetics of these mutants revealed that the role of these residues and their involvement in the GSH binding and enzyme catalysis varies (Table 1). The K_m toward CDNB of Leu33, His38, and His50 mutants are comparable to the wild type. This clearly demonstrates the amino acid changes at these positions in the GSH binding site did not affect the affinity of the enzyme to bind with this hydrophobic substrate. Leu33 mutants showed changes in both catalytic rate and GSH binding, although this residue does not interact directly with GSH. The data suggests that the mutations at this position affect the neighboring residues that influence GSH orientation in the active site.

The amino acid changes at position His50 drastically decreased V_{max} and k_{cat}, demonstrating this is a critical residue that functions in enzyme catalysis as well as in GSH binding. His38 mutants also exhibit differences in V_{max} or k_{cat} and K_m toward GSH. Notably the affinity toward GSH is very affected by the His38 mutations. The His38E enzyme steady-state kinetics did not obey the usual hyperbolic rate equation. The data fitted well to a rate equation expressing positive cooperativity between the two GSH binding sites. This means that during GSH binding, the glutamic acid in this position induces an active site conformational change which increases the GSH binding affinity in the second active site. Sigmoidal velocity curves have been reported previously for residues that impact upon part of the G-site [16-18].

These data suggest that His50 contributes more to enzyme catalysis than His38A although both His38 and His50 interact with the glycine of GSH. However the ionic interaction of His38 is implicated to be more important in the GSH binding compared to the H-bonding of His50.

The mutations at Leu33, His38 and His50 abolished the functional groups that either directly bind to GSH or to the adjacent residues. These residues influence the GSH interaction via hydrogen

bonding or assist in proper positioning of GSH in the active site during association. In addition the active site residues are well packed and mutations would change the topography of the active site yielding differences in the orientation of the residues thereby causing effects in substrate binding and enzyme catalysis.

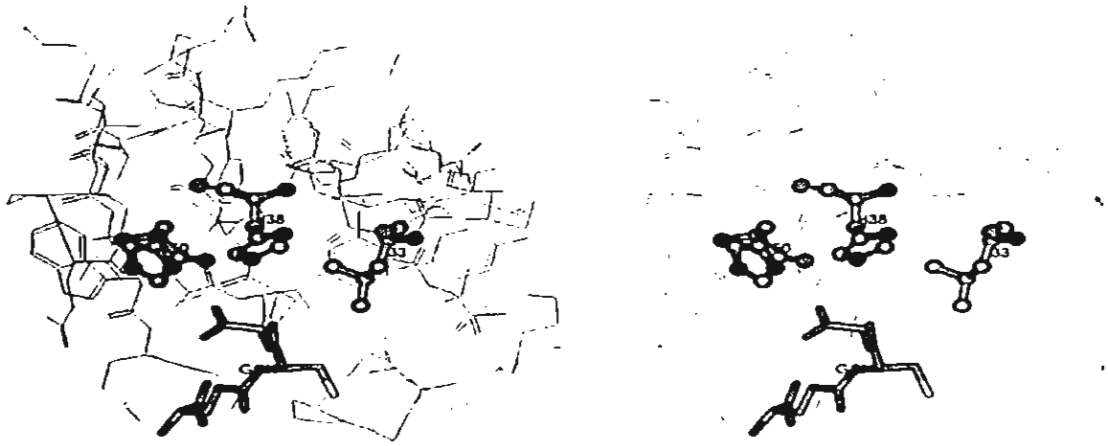

Figure 1. Stereoview of Leu33, His38 and His50 in the glutathione binding site. The coordinates for the tertiary structure are available from the Protein Data Bank with the accession number 1JLW.

Table 1. Kinetic parameters of the adGSTD4 wild type and mutant enzymes.

CLONE			CDNB		GSH	
	Vmax	kcat	Km	kcat/Km	Km	kcat/Km
	(μmol/min/mg)	(S^{-1})	(mM)	($sec^{-1}mM^{-1}$)	(mM)	($sec^{-1}mM^{-1}$)
Wild type	37.4 ± 1.4	15.60	0.74 ± 0.05	21.14	0.54 ± 0.07	28.96
L33A	23.5 ± 0.5	9.70	1.17 ± 0.05	8.30	8.20 ± 0.43	1.18
L33Y	0.32 ± 0.01	0.14	0.81 ± 0.12	0.17	1.10 ± 0.12	0.13
L33F	1.55 ± 0.03	0.65	1.30 ± 0.06	0.50	2.42 ± 0.22	0.27
H38A	50.0 ± 0.90	20.80	1.25 ± 0.12	16.64	15.8 ± 1.19	1.32
H38E	14.91 ± 0.44	6.23	0.92 ± 0.11	6.76	-*	-*
H38F	9.71 ± 0.43	4.06	0.98 ± 0.09	4.14	35.26 ± 0.86	0.12
H50A	8.3 ± 0.3	3.50	1.10 ± 0.15	3.18	10.9 ± 1.00	0.32
H50E	0.21 ± 0.01	0.09	0.80 ± 0.04	0.11	12.34 ± 0.36	0.01
H50Y	0.87 ± 0.02	0.36	0.83 ± 0.06	0.43	12.57 ± 0.73	0.03

*-Km cannot be determined, as the steady-state kinetics of this mutant does not obey the usual hyperbolic rate equation. $[S]_{0.5} = 7.74 \pm 0.12$ mM

Substrate Specificity

The substrate specificity data shows all three residue positions have influences on specificity (Table 2). The results show that the changes in the Leu33, His38 and His50 residues, which are in the GSH binding site, affected the ability of the enzyme to interact with a hydrophobic substrate. GSTs bind to GSH by an induced-fit mechanism [14, 19], therefore changing one amino acid in the active site may affect the topography of the enzyme binding pocket and the nearby residues that interact with both substrates.

Table 2. Substrate specificity determination using several GST substrates.

CLONE	Substrate Specificity (μmol/min/mg)				
	CDNB	DCNB	EA	PNPBr	PNBC
	(3 mM)	(1 mM)	(0.2 mM)	(0.1 mM)	(0.1 mM)
Wild-type	32.1 ± 1.2	0.035 ± 0.006	0.286 ± 0.062	0.074 ± 0.012	0.064 ± 0.002
L33A	18.3 ± 0.3	0.031 ± 0.015	0.332 ± 0.061	0.019 ± 0.004	0.024 ± 0.004
L33Y	0.253 ± 0.001	< 0.002	0.092 ± 0.006	< 0.006	< 0.007
L33F	1.023 ± 0.018	< 0.002	0.059 ± 0.007	< 0.006	< 0.007
H38A	21.7 ± 0.2	0.037 ± 0.001	0.146 ± 0.016	0.040 ± 0.004	0.013 ± 0.002
H38E	11.21 ± 0.24	< 0.001	0.191 ± 0.026	< 0.008	< 0.005
H38F	3.76 ± 0.09	< 0.001	0.230 ± 0.019	< 0.005	< 0.003
H50A	3.8 ± 0.1	0.015 ± 0.007	0.146 ± 0.050	0.012 ± 0.002	0.016 ± 0.007
H50E	0.149 ± 0.004	< 0.001	0.026 ± 0.004	< 0.005	< 0.005
H50Y	0.466 ± 0.011	< 0.001	0.064 ± 0.008	< 0.008	< 0.005

The active site pocket of GSTs are composed of a GSH binding site and a hydrophobic substrate binding site. Although the residues in the present study are in the GSH binding site, there are differences in the substrate specificity for several compounds. It has been suggested that mutations of active site amino acids affects the whole electrostatic field in the active site. The electrostatic field in the active site involves many amino acids that influence atom movement, active site packing, charge distribution, GSH ionization, and GSH orientation to a suitable position for conjugation with the electrophilic GST substrates [20-22]. If the electrostatic field is affected, it will affect the charge distribution and the topography of the active site thereby altering the substrate conjugation ability of the enzyme.

Half-Life Determination

The half-life of the enzymes at 45°C compared to the AdGSTD4 wild-type showed remarkable differences in the Leu33 mutants (Table 3). However the His38 and His50 mutant enzymes displayed only small increases in stability suggesting a minimal role in structural maintenance. The crystal structure shows Leu33 and its neighboring residues Leu6, Thr31 and Ile52 form part of the wall in the GSH binding

site. Therefore the Leu33 residue is involved in the packing of the active site which also impacts upon the tertiary structure of the whole protein.

Table 3. Half life determination at 45 °C.

CLONE	Half-life at 45 °C (Min)
Wild-type	15.32 ± 0.31
L33A	45.29 ± 1.56
L33Y	71.65 ± 1.71
L33F	211.73 ± 17.90
H38A	15.33 ± 0.88
H38E	40.17 ± 1.26
H38F	19.33 ± 0.59
H50A	25.81 ± 1.99
H50E	15.31 ± 0.54
H50F	20.79 ± 0.34

CONCLUSIONS

The present study investigated three residue positions in the glutathione binding site in proximity to interact with the glycine moiety of the glutathione substrate. Each of these residues appeared to have unique and multiple roles in the enzyme including contributions to substrate binding, the multiple steps of catalysis as well as influencing substrate specificity. Some of these functions appear to originate or are modified by the changes in the topography of the active site, for example Leu33 did not appear to directly interact with glutathione but was involved with formation of the active site wall. The changes in the topography or conformation of the active site also appeared capable of exerting major influences on tertiary structural organization of the whole GST protein such that a 13-fold increase in enzyme stability was observed for one Leu33 mutant. These results demonstrate that residues in the active site are not only important in the catalytic function but also may play a role in the structural integrity of the enzyme.

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A NON-ACTIVE SITE RESIDUE, CYSTEINE 69, OF GLUTATHIONE S-TRANSFERASE ADGSTD3-3 HAS A ROLE IN STABILITY AND CATALYTIC FUNCTION.

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ABSTRACT: The Cys69 residue of an *Anopheles dirus* glutathione S-transferase isoform (adGSTD3-3), was characterized to elucidate its contribution in both catalysis and structural support. Nine mutants were generated at this position by replacing the residue with polar, non-polar and charged residues. The polar residues changed the V_m of the enzymes. With non-polar residues, the enzymes were unable to fold and were expressed in the insoluble inclusion form. With charged residues, the soluble enzyme yields were only 3% of the wild type protein. Molecular dynamics simulation also was performed to understand the changes in the enzyme structure. These findings are additional evidence of the importance of structural residues that affect the enzymatic properties such as V_m, K_m and enzyme specificity.

Keywords: Glutathione transferase, *Anopheles dirus*, mosquito, structure, mutagenesis, catalysis.

INTRODUCTION

Glutathione S-transferases (GSTs; EC. 2.5.1.18) are dimeric multifunctional enzymes that have a major role in detoxication of physiological substances as well as xenobiotic compounds such as nitric oxide, drugs, herbicides and insecticides [1, 2]. The enzymes function by catalyzing the nucleophilic addition of the thiol group of reduced glutathione to electrophilic centers in organic compounds [3]. They also have a ligand-binding function using either the hydrophobic substrate-binding site or the cleft located along the dimer interface [4-6]. Recently identified functions of GSTs include roles in signal transduction through inhibition of Jun-N terminal kinase (JNK) and Apoptosis signal-regulating kinase 1 (ASK1) [7, 8]. GSTs have been grouped into distinct classes on the basis of sequence and structural homology, immunochemistry, and substrate specificity. There are at least 12 classes of cytosolic GST; alpha, mu, pi, sigma, theta, kappa, omega, zeta, beta, phi, tau and delta [9, 10].

All GST molecules are composed of two subunits, each containing two domains, an N-terminal and a C-terminal domain that provide two functional active sites per native dimer. Each active site can be divided into subsites that bind the glutathione (the G-site) and the hydrophobic electrophilic substrate (the H-site). The G-site is formed by the N-terminal domain, which is structurally related to thioredoxin. The H-site is formed by the C-terminal region. The G-site is well conserved but the H-site is highly variable and contributes to the varying substrate specificities of the different isoenzymes [11-16]. The interaction at the subunit interface of GSTs in sigma, theta and delta classes are predominantly hydrophilic compared with the more hydrophobic nature of the interfaces of the alpha, mu and pi class GSTs [17-23].

Based on many GST tertiary structures, there are only a few cysteine residues found in each subunit. In pi class, there are four cysteine residues at position 14, 47, 101 and 169 from the amino terminus. Although it is not a predominant amino acid it was shown to have an important role in both catalysis and structural support contributed by disulfide bonds. The intrasubunit disulfide bond is formed by the interaction of Cys47 and Cys101 whereas the intersubunit disulfide bond is formed between Cys47 residues in different subunits [24]. The carboxymethylation of cysteine at position 47 increased the K_m for glutathione by three orders of magnitude [25]. In addition, site-directed mutagenesis of four cysteine residues in pi class revealed that Cys14 was involved in the catalysis by stabilizing the structure of an active site loop. A Cys47Ser mutation decreased the binding affinity toward glutathione. In contrast the substitution of Cys101Ser increased the binding affinity of CDNB substrate by changing the conformation of helix 4. The Cys169 residue appeared to have an important role for maintaining a stable conformation of the enzyme [26].

The insect delta class GSTs are thought to have a role in insecticide resistance. Thus understanding structure-function relationships of amino acids in insect GSTs is necessary to identify important residues that affect the enzymatic properties. The adGSTD3-3 isoform, one of four splicing products of adGST1AS1 gene [27], contains three cysteine residues per subunit, Cys12, Cys69 and Cys106. Cys12 and 106 are conserved among all four isoforms whereas Cys69 is found only in adGSTD3-3. The Cys69 residue, located at the N-terminus of helix 3 in domain I, was one out of five residues chosen in a previous study. The replacement of Cys69 with glutamine, the residue of the adGSTD1-1 isoform, significantly increased the V_m of the enzyme approximately 65%. Therefore this work aimed to continue the characterization of Cys69 by replacing the residue with different amino acids.

Methods

Site-directed mutagenesis

The plasmid pET3a containing adGSTD3-3 coding sequence has previously been constructed [28]. The construct was used as a DNA template in a PCR reaction according to Quick Change™ site-directed mutagenesis described by Stratagene. The following oligonucleotides C69Q-f: 5'GCGCGCCATCCA GACGTACTTAGCGGAGAAGTACGGCAAG3', C69E-f:5'GAGTCGCGCGCCATCGAGACGTACCTGG CGGAGAAGTAC3', C69F-f: 5'GAGTCGCGCGCCATCTTCACGTACCTGGCGGAGAAGTAC 3', C69K-f: 5'GAGTCGCGCGCCATCAAAACGTACCTGGCGGAGAAGTAC 3', C69W-f: 5'GAGTCGCGCGCCAT

CTGGACGTACCTGGCGGAGAAGTAC 3', C69Y-f: 5' GAGTCGCGCGCCATCTATACGTACTTGGCG GAGAAG 3', C69P-f: 5'CTGTGGGAGTCGCGCGCCATCCCTACGTACTTGGCGGAG 3', C69A-f: 5' GA GTCGCGCGCCATCGCTACGTACTTGGCGGAGAAG 3', C69N-f: 5' CTGTGGGAGTCGCGCGCCATC AATACGTACTTGGCGGAG 3' were used as mutagenic primers for the Cys69 mutants. The changed nuclotide or amino acid residues are shown in bold type and the additional recognition site for restriction endonuclease are underlined. The recombinant plasmids were randomly screened by restriction analysis then the nucleotide sequences of the plasmids carrying the mutations were verified by full-length sequencing in both directions using a Bigdye™ Terminator Cycle Sequencing Kit (Perkin Elmer).

Protein expression and purification

The protein expression and purification were carried out as previously described [29]. After affinity purification, the wild type and Cys69 mutant enzymes were homogeneous as judged by SDS-PAGE. The protein concentration was determined by the method of [30] using Bradford protein reagent with bovine serum albumin as the standard protein.

Kinetic studies

The GST activity assays were performed as previously described [31]. Thermal stability was measured as a function of time. The different Cys69 mutants were incubated (0.1 mg/ml in 0.1 M potassium phosphate pH 6.5 containing 5 mM DTT and 1 mM EDTA) at 45 °C and aliquots were assayed for activity in the standard system at different time-points. Half-lives for the enzymes represent the time of incubation when there is 50% residual activity and were calculated using the equations: Slope = k/2.3, $k = 0.693/t_{1/2}$. The slope was obtained from the linear plot between log percentage of original activity and the time point of preincubation.

Molecular dynamics simulation

Several one-nanosecond molecular dynamics trajectories of adGSTD3-3 in complex with glutathione was generated. Gromac 3.0 (http://www.gromacs.org) with Gromacs force fields was used throughout the study [32, 33]. The same adGSTD3-3 coordinates were used as the initial structure for the simulation. Residue Cys69 was replaced with glutamine and asparagine residues using Deepview Swiss-PdbViewer software (http://www.expasy.org/spdbv/)[34]. The file was then converted into (.GRO) format. The protein was immersed in a cubic box full of water molecules. Na^+ ions sufficient to neutralize the systems were added by replacing some of the water molecules to minimize the protein-ion electrostatic interaction. A steepest descent energy minimization was performed followed by positional restraint to fix the protein structure and allow interaction with water molecules. The simulation was carried out for 1 ns. The crystal structure and simulated structures were visualized either by DS ViewerPro 5.0 (Accelrys Inc) or Deepview Swiss-PdbViewer v3.5b4.

Figure 1. The stereoview of domain 1 of adGSTD3-3 isoform crystallized with glutathione. Three active site residues in helix 3; Glu64, Ser65 and Arg66, glutathione and Cys69 residue are shown using ball and stick representation. This figure was created with DS ViewerPro 5.0.

RESULTS

Expression and purification

To understand the role of Cys69 in both catalysis and structure, nine mutants at this position were generated using PCR based site-directed mutagenesis. The Cys was replaced with amino acids of different properties; polar, non-polar and charged. The location of Cys69 is shown in Figure 1. Cys69Gln, Cys69Ala and Cys69Asn mutants were expressed in *E. coli* at 37°C with a band size of approximately 23 kDa, but the yields of soluble protein varied for the different mutant enzymes. The wild type enzyme and mutant Cys69Gln yielded approximately 30-40% of total soluble protein from the bacterial lysate. For the Cys69Ala and Cys69Asn mutants this value was 17% and 10% respectively. There were six Cys69 mutants, Cys69Pro, Cys69Phe, Cys69Lys, Cys69Glu, Cys69Trp and Cys69Tyr that expressed GST in an inclusion form. Therefore the expression temperature was lowered to 25°C or 18°C. At 25°C, only Cys69Glu was soluble. However, the GST was expressed at a relatively low level approximately 3% of the total protein in the lysate. The majority of the protein expressed as inclusion body. Cys69Phe and Cys69Lys had detectable activity in the lysate however the protein concentration obtained after the purification was approximately 0.02-0.03 mg/ml. The specific activity of both mutants was therefore calculated to be 12.9 and 1.3 μmol/min/mg. The actual activity is less since the SDS-PAGE showed contaminating bands indicating that the enzymes were not absolutely pure. The remaining Cys69 mutants, Cys69Pro, Cys69Trp and Cys69Tyr, were all expressed as inclusion bodies at both 25°C and 18°C.

Table 1. Michaelis-Menten parameters for adGSTD3-3 and Cys69 mutants.

Enzyme	V_m	k_{cat}	GSH		CDNB	
			K_m	k_{cat}/K_m	K_m	k_{cat}/K_m
AdGSTD3-3	63.6 ± 6.2	25.2	0.29 ± 0.03	86.9	0.13 ± 0.01	194
Cys69Gln	105 ± 7	41.7	0.44 ± 0.03	96.9	0.21 ± 0.03	198
Cys69Asn	26.7 ± 6.4	9.61	0.53 ± 0.03	18.2	0.08 ± 0.01	117
Cys69Ala	65.5 ± 7.5	25.9	0.33 ± 0.04	79.2	0.11 ± 0.03	235
Cys69Glu	57.1 ± 1.6	22.6	0.53 ± 0.03	42.6	0.13 ± 0.01	173

All measurements were performed at 25-27°C as described under Methods. The k_{cat}/K_m values were calculated from the initial linear plot of the saturation curve at low concentrations of the varied substrates.

Table 2. Specific activity of the Cys69 mutants towards five different substrates.

Enzyme	CDNB (1 mM)	DCNB (1 mM)	4-nitrobenzyl chloride (0.1 mM)	4-nitrophen-ethyl bromide (0.1 mM)	Ethacrynic Acid (0.2mM)
AdGSTD3-3	66.6 ± 1.8	0.326 ± 0.007	0.099 ± 0.006	0.003 ± 0.002	0.093 ± 0.005
Cys69Gln	94.6 ± 3.2	0.259 ± 0.014	0.132 ± 0.010	0.018 ± 0.002	0.143 ± 0.008
Cys69Asn	40.9 ± 2.0	0.350 ± 0.007	0.161 ± 0.020	0.029 ± 0.006	0.146 ± 0.019
Cys69Ala	54.2 ± 3.5	0.217 ± 0.002	0.131 ± 0.014	0.030 ± 0.009	0.163 ± 0.001
Cys69Glu	63.6 ± 3.8	0.234 ± 0.016	0.184 ± 0.019	0.028 ± 0.009	0.345 ± 0.056

The units are μmole/min/mg of protein. The data are mean ± standard deviation from at least 3 independent experiments.

Catalytic properties

The catalytic properties of adGSTD3-3 and Cys69 mutants are summarized in Table 1. It is shown that the residue in position 69 has a strong influence on kinetic parameters investigated. The effect is greatest for the Cys69Gln and Cys69Asn mutants. Cys69Gln possessed a V_m more than 1.6-fold of the wild type whereas Cys69Asn is approximately 2.4-fold less than the wild type. The change of Cys69 with asparagine significantly decreased the k_{cat}/K_m^{GSH} 4.8-fold compared to the wild type adGSTD3-3. The effect results from a combination of reduced k_{cat} and increased K_m^{GSH} values. For the Cys69Glu mutant on the other hand, a significantly increased K_m^{GSH} is the cause for the lower catalytic efficiency. Although the replacement of Cys69 with asparagine increased the binding affinity toward CDNB substrate the k_{cat}/K_m^{CDNB} is significantly lower, approximately 1.6 fold due to the low k_{cat} value. Kinetic constants for the Cys69 mutants strongly suggest that the amino acid at position 69 has a major structural influence on the catalytic activity of the enzyme. The specific activities of the purified enzymes were determined with five different substrates (Table 2). Replacing the cysteine in position 69 by alanine or asparagine reduced the specific activity with CDNB approximately 1.2-1.6-fold whereas the replacement by glutamine significantly increased the activity about 1.4-fold. Specific activities determined with DCNB, a mu class substrate, were lower for the mutant Cys69Gln, Cys69Ala and Cys69Glu approximately 1.2-1.5-fold compared to the wild type. Greater specific activities toward 4-nitrobenzyl chloride, 4-nitrophenethyl bromide and ethacrynic acid were observed with all the Cys69 mutants. Cys69Glu showed the highest conjugating activity towards 4-nitrobenzyl chloride and ethacrynic acid compared to the other Cys69 mutants or the wild type enzymes. An inhibition study of CDNB activity was performed using several

different compounds that represent hydrophobic substrates or glutathione analogs including pyrethroid insecticides (Table 3). The inhibition of Cys69Gln with 4-nitrobenzyl chloride and 4-nitrophenethyl bromide was less than for the wild type, approximately 1.3 and 1.5 fold respectively. With the pyrethroid insecticide deltamethrin, Cys69Gln and Cys69Ala had approximately 1.5 fold more inhibition than the wild type. Cys69Asn demonstrated the lowest inhibition toward cumene hydroperoxide and ethacrynic acid compared to the wild type and the other mutants. Based on the results obtained from Tables 2 and 3, it is suggested that the replacement of amino acid 69 in domain I affected the binding of both glutathione and hydrophobic substrates since the enzymes possessed different conjugating activities and inhibition characteristics.

Table 3. Percent inhibition study of adGSTD3-3.

Inhibitor	Wild type	Cys69Gln	Cys69Asn	Cys69Ala	Cys69Glu
4-NBC 1mM	49 6 ± 4 9	37 4 ± 1 6	55 0 ± 4 9	55 2 ± 2 1	46 4 ± 1 9
CuHOOH 2 5 mM	26 9 ± 2 5	25 7 ± 0 2	17 5 ± 0 9	26 6 ± 4 5	27 8 ± 1 4
DCNB 1 mM	46 0 ± 3 1	49 9 ± 4 2	46 6 ± 4 0	51 6 ± 3 6	43 1 ± 2 0
4-NPB 0.1 mM	27 8 ± 3 1	18 2 ± 2 1	36 5 ± 0 6	33 4 ± 1 9	26 2 ± 3 7
Deltamethrin 0 01 mM	47 7 ± 5 9	70 0 ± 4 2	44 4 ± 2 4	68 3 ± 1 5	46 7 ± 1 1
Permethrin 0 01 mM	38 9 ± 6 7	59 8 ± 2 7	32 4 ± 2 6	52 8 ± 7 9	39 1 ± 5 9
EA 0 1 mM	96 0 ± 1 2	96 6 ± 1 8	96 9 ± 0 8	96 0 ± 2 0	95 5 ± 0 5
EA 0 01 mM	74 6 ± 1 9	77 1 ± 1 1	74 5 ± 1 6	79 4 ± 4 2	73 7 ± 3 2
EA 0 001 mM	27 6 ± 2 1	25 0 ± 7 9	17 8 ± 3 7	37 0 ± 4 8	41 0 ± 1 5
S-hexyl 0.1 mM	63 5 ± 2 9	71 7 ± 1 4	58 7 ± 3 7	65 1 ± 2 9	54 1 ± 2 9
S-hexyl 0 01 mM	20 3 ± 3 6	24 1 ± 4 5	15 2 ± 2 5	13 1 ± 2 4	33 4 ± 4 9

The data are means ± standard deviation from at least three separate experiments. The tested compounds are 4-NBC, 4-nitrobenzyl chloride, CuHOOH, Cumene hydroperoxide, DCNB, 1,2-dichloro-4-nitrobenzene, 4-NPB, 4-nitrophenethyl bromide, deltamethrin, permethrin, EA, ethacrynic acid, S-hexyl, S-hexylglutathione. The activity was measured using the standard GST assay

Stability assay

The nature of the side-chain in position 69 is of importance for thermal stability as shown in Table 4. Removal of the sulfhydryl group of Cys69 gives mutant Cys69Ala a significantly less stable enzyme with a 2.5-fold decrease in half-life. The replacement of cysteine with a negatively charged amino acid, Cys69Glu, reduces the thermal stability 3.8-fold. The replacement of the sulfhydryl group with a larger polar amino acid, Cys69Gln, reduces the stability 1.4-fold. On the other hand, the replacement with a smaller hydrophilic R group Asn, one methylene group different, significantly decreased the stability of the enzyme approximately 5.4 fold. The 69 residue is also involved in the packing of the hydrophobic core in domain I since the replacement of Cys69 with Phe, Trp, Pro, Lys and Tyr which consist of larger side chains compared to Gln, caused the protein to be expressed in an inclusion form. Therefore this residue position appeared to have a role in both folding and stability of adGSTD3-3.

Molecular dynamics simulation

To explain the changes in enzymatic activity including the stability of the Cys69 mutants, models of Cys69Gln and Cys69Asn were generated and dynamics simulations were conducted for 1000

Table 4. Thermal stability of GST mutants.

Enzymes	Half-life (minutes)
Wild type	3.8 ± 0.03
Cys69Gln	2.8 ± 0.16
Cys69Asn	0.7 ± 0.08
Cys69Ala	1.5 ± 0.25
Cys69Glu	1.0 ± 0.06

Half-lives represent the time of incubation after which 50% activity remains measured in the standard assay system, the conjugation between CDNB (1 mM) and GSH (10 mM).

Figure 2. Comparison of the wild type adGSTD3-3 (blue) and Cys69Asn (yellow) conformations obtained from the molecular dynamics trajectories. Shown is the superimposition using the time point that demonstrated maximum changes in the center of gravity at 1000 ps. Both 69 residues are shown in stick form, Cys69 (red) and Asn69 (green).

picoseconds (ps). The variations in the system were also determined by performing four simulations of the wild type for 200 ps each using the structure obtained from 1000 ps as the starting coordinates and four different random seed numbers in the system. The parameters were analyzed during the 500-1000 ps duration. During this period the structure reaches a conformational fluctuation steady state, with similar values to the average values of the wild type models obtained from 200 ps, e.g. hydrogen bonds, radius of gyration, RMSD and dihedral angle. In addition, Root Mean Square Fluctuation (RMSF) was also computed for each residue to illustrate the fluctuation pattern within the protein. The RMSF pattern showed that residues 24-41, 77-87, 119-124, 142-155 and 190-193 are the flexible regions of the protein. These residues are in the loops between helices except 24-41, which also includes β2. The helix 2 in pi class was shown to undergo the most prominent conformational changes in a dynamics simulation [35]. No appreciable change of the monitored structural parameters takes place relative to the structure of the wild type at 1000 ps, except for the location of Cys69 relative to the Center of gravity (Cg) that indicates a different conformation. A snapshot of that time point in both mutants was generated and superimposed on the wild type. It was found that helix 3 was shifted in both Cys69Asn and Cys69Gln approximately 1.4 and 1.7 Å, measured between C-alpha of residue 69 in wild type and mutant (Figure 2). The most relevant conformational changes are observed in the structure comprising residues 32-41, the loop between β2 and α2 (maximum deviation 4 Å) and residues 77-87, the loop before helix 4 (maximum deviation 5 Å) that correlates with the RMSF parameters.

DISCUSSION

The Cys69 position appears to have an impact on a sensitive structural region of the adGSTD3-3 isoform by changing both catalysis and stability of the enzyme. The tertiary structure of adGSTD3-3 shows the Cys69 residue relatively distant from the other two cysteine residues. Therefore, it is unlikely that the residues could form disulfide bonds to each other to stabilize the structure. It was found that the Cys69

mutants that possessed larger side chains such as Trp, Tyr and Phe were unable to fold into the appropriate structures and were expressed in the inclusion form, indicating severe structural perturbations. Although it had no effect on the kinetic parameters, the smaller side chain alanine reduced the enzyme half-life more than 50%. In addition, when residue 69 was replaced with either a negatively charged amino acid (Glu) or a positively charged residue (Lys), both mutants were expressed as inclusions at 37°C but only the Cys69Glu could express as soluble protein at a lower temperature. However, the soluble GST form obtained was only 3% because the majority of the expression was still in an inclusion form. This was also found in two temperature-sensitive mutants of GST pi, Asp153Ala and Ser150Ala/Asp153Ala mutants [36]. The expression of both mutants depended on the temperature of host cell growth. The yield of the enzymes was lower and clearly decreased with increasing temperatures. All these results strongly suggest that residue 69 is involved in the enzyme folding as well as stability since all Cys69 mutants demonstrated dramatically decreased half-lives. However the relationship between kinetic parameters including the thermal stability of the enzyme and the property of the amino acid at position 69 remains unclear.

The adGST Cys69 mutation also may affect residues that maintain an intersubunit salt bridge of the protein. The crystal structure shows Glu74 in helix 3 in domain 1, forms an intersubunit salt bridge with Arg90 in helix 4 in domain 2 of the neighboring subunit, to stabilize the structure. The distances of both residues measured in Cys69Gln and Cys69Asn were 5.43 and 4.86 Å therefore the replacement of Cys69 residue would cause both mutants to lose their intersubunit salt bridges. A salt bridge interaction was also found to stabilize GST structure in a rat mu class GST [37]. Equilibrium folding studies of insect GSTs will increase our understanding of the roles played by individual domains in maintaining the stability of GST dimers and their monomers. GST folding is thought to originate by the formation of three dimensional structural formations (nucleation) occurring independently in the individual domains. Based on the structure, domain 1 (approximately residues 1-80) adopts a topology similar to that of the thioredoxin fold consisting of four β-sheets with three flanking α-helices. The folding in this domain is initiated by interaction between β2 and β4 regions [38]. Domain 2 (approximately residues 81-207) consists of two important motifs, an N-capping box and a hydrophobic staple motif, located at the beginning of helix 6. The N-capping box has an important role in folding and stability in GSTs by determining the direction of the forming helix [36]. The latter motif is required in the cooperativity between α6 helix in domain 2 and α1 in domain 1 [39, 40]. Both motifs have been proposed as nucleation sites of folding. Moreover, helix 3 was also found to have a critical role in maintaining the correct folding of domain 2 [41, 42].

The interaction at the subunit interface also has a major effect on the folding in GST. It also may have a role in determining GST stability. Based on GST tertiary structures, the equilibrium unfolding of a homodimeric delta class GST should be most closely related to the sigma and theta class since all three structures have hydrophilic interactions at the subunit interface. A four-state pathway for a sigma class GST was proposed with triphasic unfolding transition [43]. The first transition is protein concentration independent and involves a change in the subunit tertiary structure producing a partially active dimeric intermediate ($N_2 \leftrightarrow I_2$). The second step is protein concentration dependent with I_2 dissociation into two

monomeric intermediates. The final step of unfolding transition is protein concentration independent and involves the complete unfolding to the monomeric state. Although having a lock-and-key intersubunit interaction, the unfolding of alpha and pi class GSTs are two-state pathways [44, 45] but the equilibrium unfolding/refolding of class mu proceeds via a three-state process [46].

The replacement of Cys69 significantly affected the kinetic parameters especially the V_m, K_m^{GSH} and k_{cat}/K_m for GSH (Table 1) suggesting that the conformation of the G-site was altered. Cys69 is located at the N-terminus of α3-helix in domain 1 adjacent to residues Glu64, Ser65 and Arg66, active site residues that directly interact with the glutamate residue of glutathione. Gln64 in GSTPi, the equivalent residue of our Glu64, was shown after mutation to alanine to significantly decrease the yield of protein expression and glutathione-binding affinity including the specific activity [47, 48]. Static models also provided a clue of subtle conformational changes of adGSTD3-3 by detecting the atom movement between residues 69 and 65. The movement may also induce the positional change of Arg66 since Ser65 forms an H-bond with Arg66, which forms a salt bridge with Asp100 and appears to stabilize the intra-subunit domains of the protein. Based on the superimposition of wild type, Cys69Gln and Cys69Asn obtained from dynamics simulation, the most conspicuous conformational changes are observed in helix 3. The whole helices of both mutant models were shifted to a greater extent than the wild type structure at the C-terminal region. Other regions such as residues 32-41 in the loop before helix 2, and residues 77-87 in the loop before helix 4 also demonstrated relevant structural rearrangement with maximum deviations of 4-5 Å. These also provided additional evidence of conformational changes. The effect of the structural changes may cause a major change of the catalytic property of adGSTD3-3 since some regions e.g. the loop before helix 2 contains several residues in the active site region of adGSTD3-3. Indeed, this has been shown in other GSTs to control the movement of helix 2 that participates in the formation of active site and modulates the G-site affinity for glutathione [49].

In conclusion, these results strongly suggest that residue 69 is involved in the initial enzyme folding as well as stability since all Cys69 mutants have dramatically decreased half-lives and several mutants could only be expressed as insoluble inclusion bodies. These findings are additional evidence of important structural residues that affect the enzymatic properties such as V_m, K_m and enzyme specificity.

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Expression and characterization of a novel class of glutathione S-transferase from *Anopheles dirus*

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Abstract

A new *Anopheles dirus* glutathione S-transferase (GST) has been obtained and named adGST4-1. Both genomic DNA and cDNA for heterologous expression were acquired. The genomic sequence was 3188 bp and consisted of the GST gene as well as flanking sequence. The flanking sequence was analyzed for possible regulatory elements that would control gene expression. In *Drosophila* several of these elements have been shown to be involved in development and cell differentiation. The deduced amino acid sequence has low identity compared with the four alternatively spliced enzymes, adGST1-1 to 1-4, from another *An. dirus* GST gene *adgstIAS1*. The percent identities are 30–40% and 11–12% comparing adGST4-1 to insect GSTs from Delta and Sigma classes, respectively. Enzyme characterization of adGST4-1 shows it to be distinct from the other *An. dirus* GSTs because of low enzyme activity for customary GST substrates including 1-chloro-2, 4-dinitrobenzene (CDNB). However, this enzyme has a greater affinity of interaction with pyrethroids compared to the other *An. dirus* GSTs. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: Glutathione transferase; Anopheles dirus; Gene regulation; Promoter

1. Introduction

The glutathione S-transferases (GST) are a super-family of dimeric enzymes which currently has at least 25 possible families of GST-like proteins (Snyder and Maddison, 1997). Based on their sequences, the mammalian GSTs can be divided into seven distinct classes termed Alpha, Mu, Pi, Sigma, Theta, Zeta and Omega (Hayes and Pulford, 1995; Board et al., 1997, 2000). The insect GSTs can be grouped into two distinct classes termed Class I or Delta class and Class II or Sigma class (Toung et al., 1990 Fournier et al., 1992). Generally amino acid sequence identity within a class is 50% or greater, while inter-class identity is less than 30% (Mannervik et al., 1992). Therefore, the GST classes span multiple species with enzymes from the same class but from different species being more similar to each other than enzymes from different classes from within a single species. Currently Delta class has only been reported in insects although Sigma class GSTs have also been reported from cephalopods (Ji et al., 1995; Board et al., 1997).

Previously we have reported *adgstIAS1* which is an *Anopheles dirus* alternatively spliced GST gene (Pongjaroenkit et al., 2001). This gene encoded four Delta class GST enzymes, adGST1-1, 1-2, 1-3, and 1-4, that we had heterologously expressed and characterized (Jirajaroenrat et al., 2001 Ketterman et al., 2001). Although splice products from the same gene, the four enzymes possessed distinct enzyme kinetic properties for substrates and inhibitors including insecticides. Several allelic variants for one of the spliced products, adGST1-1, each had single amino acid changes outside the active site that significantly affected kinetic properties of the enzymes (Ketterman et al., 2001). Molecular modeling showed that the single residue change appeared to modulate the conformations attainable by the different variants.

In this report, we describe a novel *An. dirus* GST gene including putative regulatory elements. The gene encodes a protein from a new class of insect GST that we heterologously expressed and characterized. This enzyme appears to possess little activity for customary GST substrates and may be regulated by several

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elements that have been shown in *Drosophila* to be involved in development and cell differentiation.

2. Materials and methods

2.1. An. dirus genomic DNA sequencing

The recombinant bacteriophage, derived from an *An. dirus* genomic library (Pongjaroenkit et al., 2001) was partially digested with *Sal*I and *Xho*I. The DNA hybridization was performed by using the Digoxigenin (DIG)-labeled 5′ part of *adgst1-1*, the first 200 bp of the coding sequence, as the probe. The probe preparation and hybridization procedure were described previously (Pongjaroenkit et al., 2001). Positive signal DNA were subcloned into pBluescript II SK(+). The contiguous sequence of 3188 bp was obtained by automated sequencer (ABI PRISM™ 377, Perkin–Elmer) and assembled using BioEdit software.

2.2. RT-PCR and cDNA sequencing

Total RNA of fourth instar larvae of *An. dirus* was extracted by TRIzol™ Reagent. This RNA was used as the template to synthesize first strand cDNA. PCR was performed by using primers 5′CCGAGAGCATATGGATTACTACTACAGCCTC3′ and 5′CCGAGAGCATATGTCACTTTC-GGCTCGCGAC3′ and 300–500 ng of cDNA as template. Optimal PCR conditions (40 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 1 min) were carried out in a Perkin–Elmer thermocycler to generate the coding sequence of adGST4-1. A single product of 630 bp was obtained and subcloned into pET3a (Novagen) by the *Nde*I restriction site contained in the primers, underlined above. The DNA sequencing was performed in both directions several times. The expression of the recombinant proteins was performed in *E. coli* BL21(DE3)pLysS.

2.3. Preparation of recombinant protein

The protein expression was performed as previously described (Ketterman et al., 2001). The soluble target protein was purified by HiTrap affinity columns (glutathione ligand coupled via a 10-carbon linker arm) as described in the user's instructions (Amersham Pharmacia Biotech). The bound proteins were eluted with 10 mM reduced-glutathione. The fractions containing active enzymes were concentrated using centriprep-10 ultrafiltration units (Amicon) by centrifugation at 2500*g* for 3 h, at 4°C and passed through Hitrap desalting columns (Amersham Pharmacia Biotech) to remove the glutathione. The enzymes were stored in 50 mM phosphate buffer (pH 6.5), 10 mM DTT, 40% (v/v) glycerol at −20°C. Protein was assayed by the method of Bradford using the Bio-Rad protein reagent with BSA as the standard protein (Bradford, 1976). The purity and subunit size of the enzyme preparations were confirmed by SDS-PAGE with Bio-Rad broad-range standards as molecular mass markers.

2.4. Characterization of expressed enzyme

The method for determination of GST activity with 1-chloro-2, 4-dinitrobenzene (CDNB) was described previously (Habig et al., 1974). The activity with 3 mM CDNB and 16 mM glutathione was monitored at 340 nm using a SpectraMax 250 (Molecular Devices) in 0.1 M phosphate buffer pH 6.5. This is the standard assay used for the adGST4-1 enzyme.

The kinetic studies were performed by varying the concentration of CDNB from 0.1 to 3.2 mM and glutathione from 0.25 to 20 mM. The V_{max} and K_m were determined by non-linear regression analysis using GraphPad Prism 2.01 software.

The inhibition studies were performed with the standard assay in the presence of various compounds as inhibitors. Determinations of IC_{50} were performed with ethacrynic acid and S-hexylglutathione by varying the inhibitor concentrations as previously described (Prapanthadara et al., 1996).

3. Results

3.1. Isolation and cloning of adgst4-1

Several positive signal fragments were detected after the recombinant bacteriophage was double digested with restriction enzymes, *Sal*I and *Xho*I. These fragments were subcloned into pBluescriptII SK$^+$ and sequenced. The program BLAST was used to analyze the 3188 bp contiguous sequence. The full-length gene contained two coding exons and one 59 bp intron (Fig. 1). The 794 bp down stream sequence was also analyzed for other GST coding sequences. The BLAST program was used to determine whether there were more coding exons that might yield multiple alternatively spliced products as previously observed (Pongjaroenkit et al., 2001). To determine the GST classification, the translated amino acid sequence was compared to other insect GSTs in the Genbank database by using BLAST search programs (Fig. 2). The amino acid sequence alignment was also performed to generate percent identities and similarities (Table 1). The percent identities are 33–43% and 11–12% for adGST4-1 and insect GSTs Delta class (Class I) and Sigma class (Class II), respectively. However, the percent similarities with GST Delta class were about 50–60%. Sequence alignment with other *An. dirus*, adGST1-1, 1-2, 1-3, and 1-4, showed adGST4-1 to have sequence

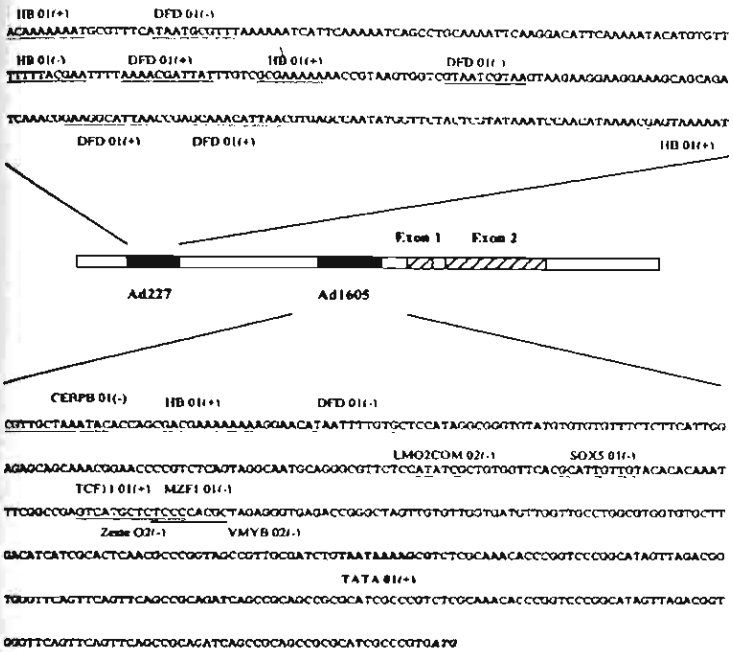

Fig. 1. A schematic of the *adgst4-1* gene isolated from an *An. dirus* genomic library. The genomic sequence for *adgst4-1* of 3188 bp is available in Genbank accession number AY014405. The two putative promoters, distal and proximal, are shown as black boxes with the protein coding exons shown as hatched boxes. Identified regulatory elements are underlined and labeled. The TATA box is shown in bold. The start site for translation is shown in bold italics.

variation even in the N-terminus which is highly conserved within a class (Fig. 2).

3.2. Promoter prediction

The 1702 bp upstream sequence of *adgst4-1* was analyzed and characterized by using a combination of two programs, TSSW (human RNA polymerase II recognition using the TRANSFAC database; http://dot.imgen.bmc.tmc.edu:9331/gene-finder/gf.html) and the MatInspector program (www.gsf.de2cgi-bin/matsearch.pl). The promoter regions were predicted by the TSSW program and the putative elements were predicted by the MatInspector program. Two putative promoters located at positions 227 and 1605 were analyzed and named Ad227 and Ad1605, respectively (Fig. 1). A TATA binding site was determined at position 1605 upstream of the coding gene. The proximal promoter Ad1605 was identified as the *adgst4-1* promoter. This putative promoter contained nine different regulatory protein binding sites (Fig. 1). These binding sites or regulatory elements may control expression of the GST gene in a tissue or stage specific manner. Additionally, the distal Ad227 promoter contained multiple Hunchback and *Dfd* recognition sites.

3.3. Expression and purification of adGST4-1

A 630 bp product from mRNA was obtained by PCR using two primers specific to the 5′ and 3′ end of the genomic sequence. The PCR product contained only coding sequence. This cDNA was subcloned into pET3a expression vector to produce a GST with a subunit size of 24,237 Da. The yield obtained after purification by glutathione ligand affinity chromatography was about 35% of the total *E. coli* lysate activity or approximately 20 mg l^{-1} of culture. During the purification of the recombinant enzyme it was observed that this GST possessed a lower affinity of interaction with the affinity column compared to other *An. dirus* GSTs in our laboratory. However, a high degree of purification, >99%, could be obtained as shown by SDS-PAGE (Fig. 3).

3.4. Characterization of adGST4-1 recombinant protein

The kinetic parameters of adGST4-1 for GSH and CDNB were determined (Table 2). Comparison of adGST4-1 with other *An. dirus* GSTs (adGST1-1, 1-2, 1-3, and 1-4) showed the adGST4-1 K_m for glutathione and CDNB was relatively high indicating low binding affinities for these substrates (Table 2). The V_m and K_m data for adGST4-1 could only be estimated because of the limitation of CDNB solubility. This also makes the k_{cat} an estimate and it is shown only for purposes of comparison. Although these kinetic parameters are estimates due to physical limitations, the values obtained were reproducible as shown in Table 2. The plots of V versus S also show the data obtained were approaching saturation thereby contributing to the reproducibility (Fig. 4). The parameters obtained, k_{cat}, k_{cat}/K_{mGSH} and k_{cat}/K_{mCDNB}, are relatively low compared to the other *An. dirus* GSTs indicating a very slow rate of turnover for CDNB and GSH. Several other substrates were used to determine activity including 1,2-dichloro-4-nitrobenzene, *p*-nitrophenethyl bromide and ethacrynic acid. However, adGST4-1 had no detectable activity for these substrates.

3.5. Inhibition study of adGST4-1

The inhibition of several compounds on CDNB conjugating activity of adGST4-1 is shown in Table 3. All compounds inhibited CDNB conjugating activity of the different *An. dirus* GSTs although to different extents. The extent of each insecticide inhibition appears to be very similar for each enzyme except the inhibition by permethrin and λ-cyhalothrin of adGST4-1 shows a significant difference from the other three adGSTs enzymes. For adGST4-1, ethacrynic acid was a good inhibitor of CDNB conjugating activity.

The IC_{50} for ethacrynic acid and S-hexylglutathione,

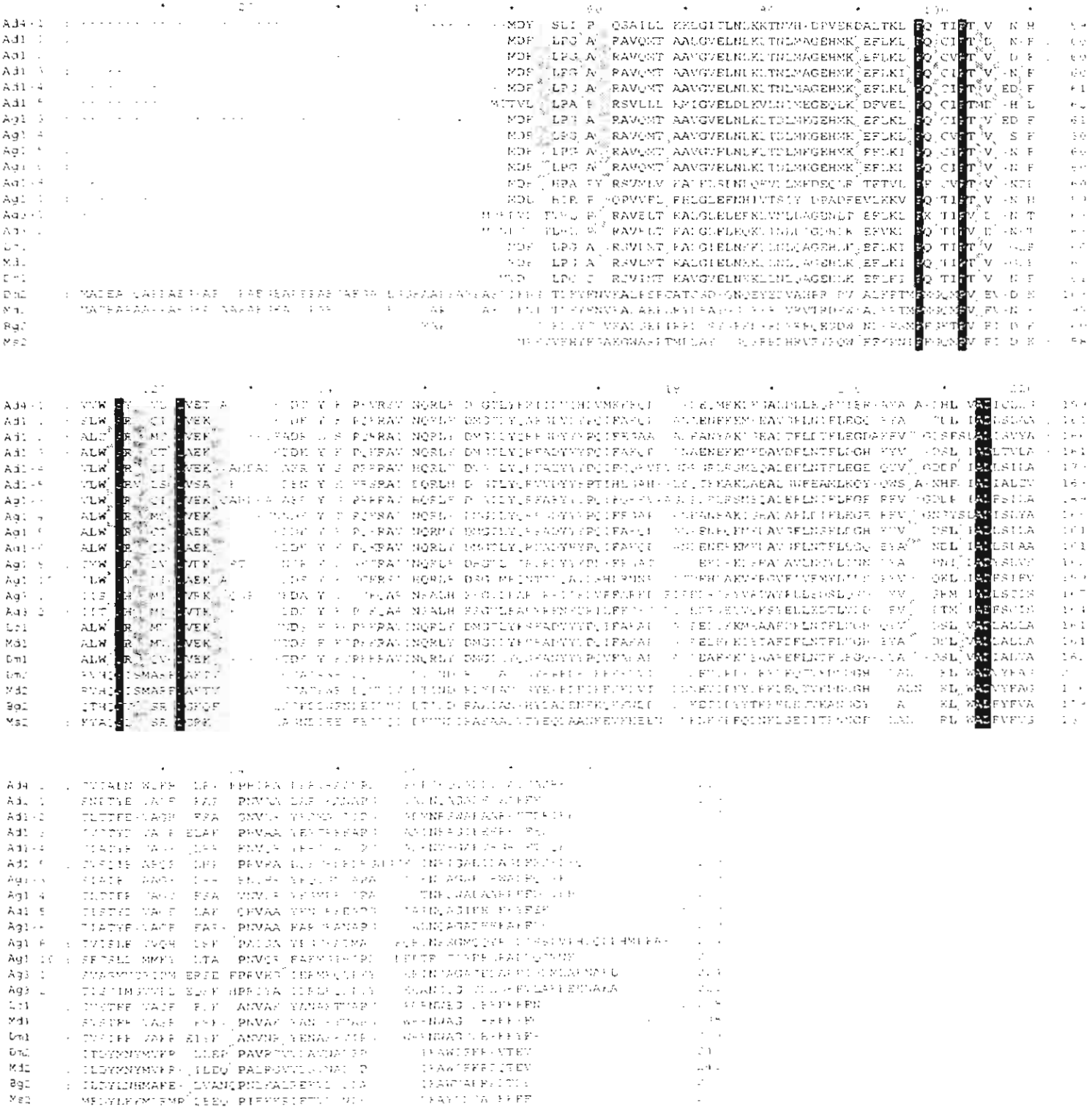

Fig. 2. The deduced amino acid sequence alignment of multiple adGSTs gene. Shading indicates degree of conservation of residue where black is 100% conserved and dark grey is >80% conserved and light grey is 60–80% conserved. The figure was produced by GeneDoc version 2.5.

23.2±3.9 μM and 126±30 μM, respectively, were determined by interpolation from the plots of fractional activity (y) versus log inhibition concentration (log [I]). This value is 100- and 13-fold greater than what was observed for adGST1-1 (Prapanthadara et al., 1996).

4. Discussion

4.1. Promotor prediction

Previously we have reported an *An. dirus* GST gene that was alternatively spliced to generate four different

Table 1
Comparison of the amino acid sequence of adGST4-1 with other insect GSTs

	Ad1-1	Ad1-2	Ad1-3	Ad1-4	Ad1-5	Ag1-3	Ag1-4	Ag1-5	Ag1-6	Ag1-8	Ag1-10	Ag3-1	Ag3-2	Lc1	Md1	Dm1	Dm2	Md2	Bg2	Ms2
	43%	33%	42%	35%	37%	33%	34%	40%	42%	37%	41%	29%	30%	38%	40%	39%	14%	15%	17%	13%
Ad4-1	60%	55%	60%	55%	58%	53%	57%	59%	61%	56%	66%	49%	52%	58%	58%	59%	27%	29%	30%	26%
		61%	72%	63%	44%	60%	62%	76%	91%	41%	38%	38%	36%	67%	68%	65%	13%	12%	16%	15%
Ad1-1		74%	87%	73%	63%	72%	74%	86%	95%	59%	54%	53%	54%	78%	78%	77%	26%	26%	29%	27%
			63%	62%	40%	60%	85%	61%	61%	40%	31%	32%	33%	58%	56%	56%	10%	9%	14%	14%
Ad1-2			78%	74%	59%	72%	91%	79%	75%	57%	53%	50%	50%	73%	72%	72%	22%	23%	29%	28%
				64%	42%	60%	62%	92%	80%	40%	37%	36%	36%	70%	70%	70%	13%	12%	16%	14%
Ad1-3				77%	62%	76%	78%	97%	90%	62%	55%	55%	56%	82%	81%	81%	25%	26%	30%	26%
					39%	84%	68%	63%	65%	37%	33%	34%	34%	57%	56%	57%	12%	10%	15%	14%
Ad1-4					56%	90%	74%	76%	75%	55%	50%	52%	54%	73%	73%	74%	22%	23%	29%	25%
						38%	40%	41%	43%	41%	32%	32%	34%	40%	41%	41%	9%	9%	12%	12%
Ad1-5						57%	62%	63%	65%	58%	50%	52%	56%	59%	59%	59%	20%	23%	25%	24%
							61%	62%	62%	36%	35%	33%	33%	54%	52%	54%	11%	10%	16%	14%
Ag1-3							74%	76%	78%	55%	51%	51%	53%	72%	71%	72%	21%	22%	29%	24%
								63%	65%	42%	34%	33%	34%	59%	57%	58%	11%	10%	14%	13%
Ag1-4								80%	76%	58%	56%	52%	51%	74%	73%	74%	22%	23%	28%	26%
									82%	40%	37%	34%	35%	68%	68%	68%	13%	12%	16%	14%
Ag1-5									91%	62%	53%	54%	56%	82%	81%	82%	24%	26%	29%	26%
										41%	38%	36%	36%	67%	68%	67%	13%	12%	16%	15%
Ag1-6										61%	55%	53%	53%	80%	81%	80%	25%	25%	29%	27%
											30%	31%	32%	41%	42%	41%	10%	10%	14%	14%
Ag1-8											52%	48%	52%	60%	60%	60%	23%	24%	28%	25%
												30%	30%	32%	32%	32%	12%	12%	18%	14%
Ag1-10												49%	50%	55%	55%	54%	25%	28%	32%	27%
													65%	35%	36%	35%	10%	10%	14%	14%
Ag3-1													83%	54%	55%	56%	22%	24%	30%	27%
														34%	35%	36%	9%	9%	13%	12%
Ag3-2														56%	56%	56%	23%	24%	30%	27%
															92%	82%	9%	8%	14%	13%
Lc1															96%	92%	23%	24%	29%	26%
																84%	10%	9%	15%	14%
Md1																92%	23%	25%	30%	28%
																	10%	10%	15%	13%
Dm1																	24%	26%	30%	28%
																		81%	38%	30%
Dm2																		84%	53%	44%
																			40%	32%
Md2																			58%	40%
																				60%
Bg2																				59%

The top number in each cell represents the percent amino acid sequence identities and the bottom number represents the percent similarity. The amino acid sequences were obtained from the GenBank database. The sequences are Ad4-1 (AY014406), Ad1-1 (AF273041), Ad1-2 (AF273038), Ad1-3 (AF273039), Ad1-4 (AF273040), Ad1-5 (AF251478), Ag1-3 (AAC79999.1), Ag1-4 (AAC79998), Ag1-5 (Q93112), Ag1-6 (Q93113), Ag1-8 (AF316637), Ag1-10 (AF316638), Ag3-1 (AF316635), Ag3-2 (AF316636), Lc1 (P42860), Md1 (P28338), Dm1 (P20432), Md2 (P46437), Dm2 (P41043), Bg2 (O18598), Ms2 (P46429). The abbreviations are Ad for *Anopheles dirus*, Ag for *Anopheles gambiae*, Lc for *Lucilia cuprina*, Md for *Musca domestica*, Dm for *Drosophila melanogaster*, Bg for *Blattella germanica*, Ms for *Manduca sexta*.

protein products (Pongjaroenkit et al., 2001). We have now obtained a novel GST gene that codes for a single expressed protein product which we name adGST4-1. The 3188 bp genomic sequence contained two coding exons and 1702 bp 5′ flanking sequence that was analyzed for promoters and regulatory elements which may control expression of this gene. Response elements are short conserved sequences that regulate expression of a gene. Several of these elements were identified in two possible promoters (Fig. 1). Most putative elements contained in the Ad227 and Ad1605 promoters have been shown in other species to be functionally involved in developmental stage regulation as well as responding to xenobiotic modulation.

Although, GSTs are involved in protecting an organism from toxic and mutagenic xenobiotics, it has been reported that the over-expression of the Pi class GST has been associated with tumor development and carcinogenesis (Batist et al., 1986). Therefore, understanding the transcriptional regulatory mechanism of these genes is of interest. Several regulating elements of GSTs have been identified. In mammals, the GSTP1 promoter included a putative AP-1 response element as well as a negative regulatory element in a multidrug resistant derivative of a human mammary carcinoma cell line (Moffat et al., 1994). Sp1 binding sites, the GC box motif, have also been shown to play a role in regulating basal levels of GSTP1 transcription (Moffat et al., 1996).

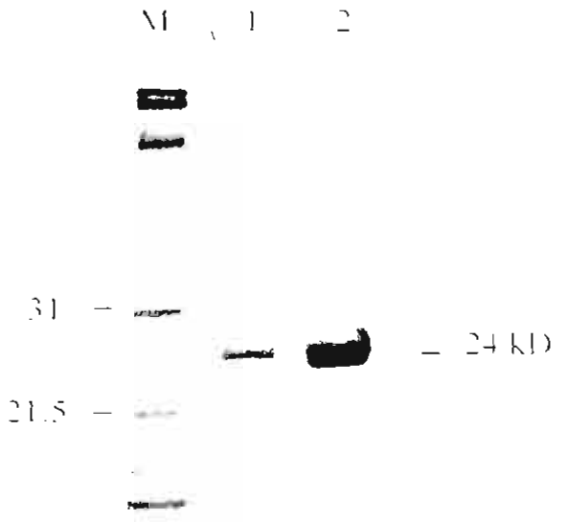

Fig. 3. Purification of recombinant protein adGST4-1. Lane M is molecular weight markers as shown in kD. Lane 1 is 3 μg and Lane 2 is 6 μg of the purified recombinant adGST4-1.

The rat GST-P gene is regulated by two enhancers and a silencer (Sakai et al., 1988). The protein bound to the silencer sequence belongs to the CCAATT/enhancer-binding protein (C/EBP) family (Osada et al., 1995). A rat Alpha class GST has been shown to be negatively regulated by C/EBP protein interaction with an antioxidant/electrophile response element (ARE/EpRE) in vascular smooth muscle cells to function as oxidative stress protection for blood vessels (Chen and Ramos, 2000).

TCF11 is a widely expressed transcription factor that binds to a subclass of AP-1 sites. The complexes of TCF11/LCR-F1/Nrf1 form heterodimers with a small Maf protein to increase stringency of specific binding to the NF-E2 site, the antioxidant response element and the heme-responsive element, and contribute to negative regulation of this specific target site (Johnsen et al., 1998). In human Pi-class GST, disruption of a putative AP-1 response element (Xia et al., 1991) within the *GSTP1* promoter abrogated *GSTP1* transcription while increased levels of GSTP1 transcription can play a major role in regulating overexpression of GSTP1-1 in multidrug-resistant cell line (Moffat et al., 1994).

Many of the putative elements contained in both *adgst4-1* promoters are DNA-binding regions for transcription factors expressed during a developmental or cell differentiation stage (Martinez-Arias and Lawrence, 1985; Siegfried and Perkins, 1990; Blair, 1994; Stauber et al., 2000). The data suggest that adGST4-1, a phase II detoxication enzyme, is expressed and regulated by

Table 2
Kinetic parameters of *Anopheles dirus* GSTs (the data are the mean±standard error of at least three separate experiments)

Kinetic parameters	**Ad4-1**	Ad1-1	Ad1-2	Ad1-3	Ad1-4
V_m (μmol $^{-1}$ min $^{-1}$ mg)	**2.2±0.3**	12.9±0.6	63.9±3.50	67.5±1.97	40.3±1.89
K_{mGSH} (mM)	**1.8±0.4**	0.86±0.2	1.30±0.15	0.40±0.05	0.83±0.08
K_{mCDNB} (mM)	**5.3±0.8**	0.10±0.03	0.21±0.03	0.10±0.01	0.52±0.67
k_{cat} (s $^{-1}$)	**0.9**	5.03	25.9	26.9	16.9
k_{cat}/K_{mGSH} (mM $^{-1}$ s $^{-1}$)	**0.5**	5.86	20	67	20
k_{cat}/K_{mCDNB} (mM $^{-1}$ s $^{-1}$)	**0.2**	48.4	121	269	32

Table 3
Inhibition of *Anopheles dirus* recombinant GSTs CDNB activity by various compounds (the GSH and CDNB concentrations were 16 and 3 mM, respectively. The data are the mean±standard deviation of at least three separate experiments, each of which was performed in duplicate)

Compounds	Concentration	% Inhibition			
	(mM)	**Ad4-1**	Ad1-2	Ad1-3	Ad1-4
DCNB	0.1	**29.8±0.2**	21.1±12.0	9.3±2.5	28.3±6.6
p-Nitrophenyl bromide	0.1	**22.5±2.2**	7.1±12.3	25.5±4.2	31.0±6.4
Cumene hydroperoxide	2.5	**43.6±5.7**	51.9±9.1	17.8±7.3	30.1±5.1
p-Nitrobenzyl chloride	1.0	**16.5±3.7**	29.1±10.5	46.1±2.5	49.8±6.7
Ethacrynic acid	0.001	**8.6±2.1**	74.7±3.0	30.9±4.5	34.5±5.3
	0.01	**39.5±5.0**	97.9±0.4	79.2±2.3	77.6±3.0
	0.1	**88.5±1.5**	100±0.0	100±0.0	100±0.0
Permethrin	0.01	**11.8±3.4**	47.4±8.5	17.4±4.7	66.3±14.4
	0.1	**28.0±3.6**	89.0±0.8	100±0.0	100±0.0
λ-cyhalothrin	0.01	**15.2±5.1**	40.1±11.3	25.0±11.8	91.5±7.6
	0.1	**25.6±1.0**	86.3±3.9	100±0.0	100±0.0

these putative elements. The distal Ad227 promoter located upstream of the Ad1605 promoter may act as an enhancer/repressor to regulate the expression of adGST4-1 as has been reported for a Pi class rat GST (Sakai et al., 1988).

4.2. Protein characterization

As suggested by amino acid sequence identities and similarities compared with other insect GSTs Class I and Class II (Table 1), adGST4-1 is very distinctive. The percent similarity of 50–60% shows that adGST4-1 is more similar to insect GST Class I or Delta class. Comparison of amino acid sequence with other *An. dirus* GSTs, 1-1 to 1-4 (Fig. 2), shows adGST4-1 has a high variation in the N-terminal, contributing to the low affinity binding with the GSH ligand during purification.

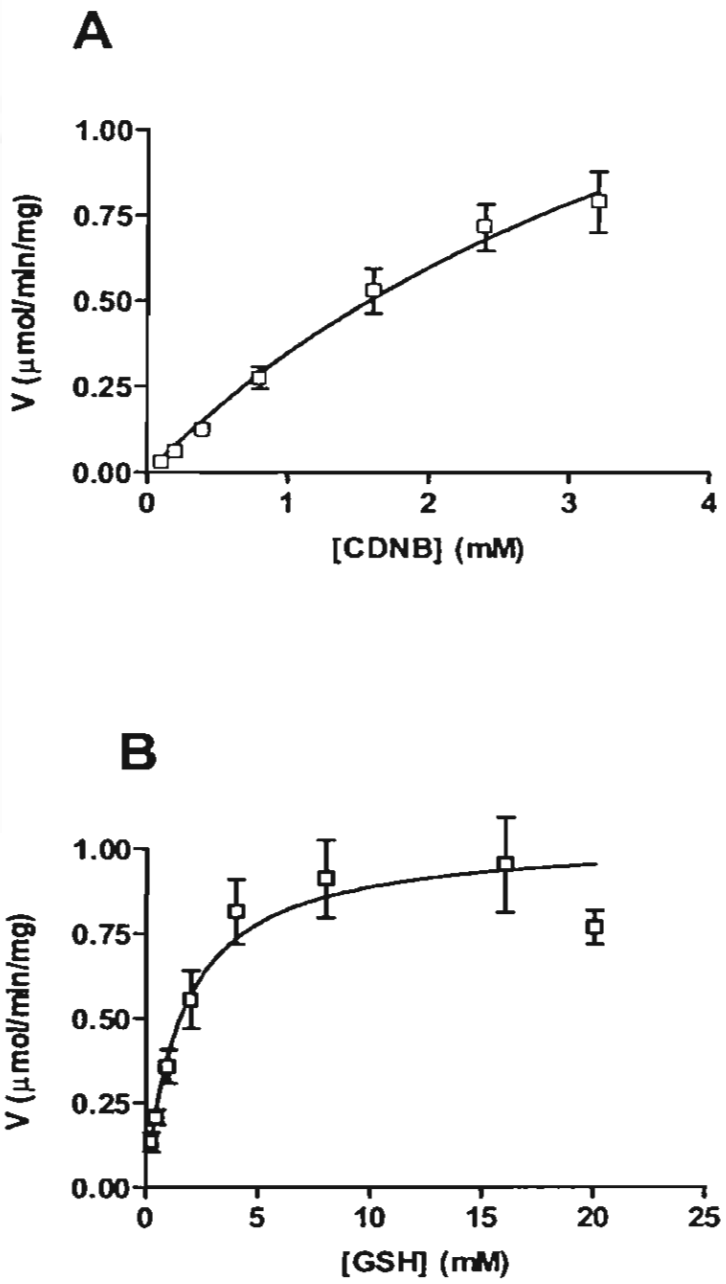

Fig. 4. Plot of velocity versus substrate concentration for kinetic constant determination of adGST4-1. (A) The glutathione concentration was fixed at 16 mM and the CDNB concentration was varied from 0.1 to 3.2 mM. (B) The CDNB concentration was fixed at 3 mM and the glutathione concentration was varied from 0.25 to 20 mM. The kinetic constants were calculated by non-linear regression. The data shown are means±SEM error bars from four independent experiments.

Studies of the enzymatic properties indicate that adGST4-1 is very different from the other known adGSTs (Table 2). The model substrate, CDNB, could be turned over only at a slow rate as described by the very low k_{cat} and k_{cat}/K_{mCDNB}. This enzyme has behavior similar to several mammalian GSTs which lack activity toward CDNB such as GST Theta class (Meyer et al., 1991), GST Zeta class (Board et al., 1997) and the new GST Omega class (Board et al., 2000). While the other *An. dirus* GSTs are more similar to mammalian GSTs in class Alpha (Schramm et al., 1984; Stenberg et al., 1991), Mu (Schramm et al., 1984; Vorachek et al., 1991) and Pi (Widersten et al., 1992). The inhibition studies (Table 3) shows that the CDNB activity of adGST4-1 can be inhibited by several compounds. Although there was no detectable enzyme activity the inhibition indicates that there is some interaction between adGST4-1 and these compounds especially for ethacrynic acid which has been shown to bind to an effector site in GST Pi class as well (Phillips and Mantle, 1993). The inhibition of CDNB conjugation by pyrethroid compounds, permethrin and λ-cyhalothrin, was also obviously different for adGST4-1 compared to other adGSTs indicating a greater affinity of interaction. The greater IC_{50} values for ethacrynic acid and S-hexylglutathione for adGST4-1 compared to the previous data for adGST1-1 (Prapanthadara et al., 1995) indicate a lower interaction with these compounds and show the inhibition characteristics of a homodimer or single affinity site for interaction (Tahir and Mannervik, 1986). Despite low CDNB conjugating activity, adGST4-1 may possess some activity for a compound that is significant in a metabolism pathway as shown by other GST classes. For example, recombinant human omega class GST (GSTO1-1) exhibited a glutathione-dependent thiol transferase activity and catalyzed glutathione-dependent reduction of dehydroascorbate (Board et al., 2000) or the novel function of human Theta GSTT2-2 with 1-menaphthylsulphate demonstrating it to be a glutathione-dependent sulphatase (Tan et al., 1996). In conclusion, the recent reports on diverse roles of GSTs in regulation of Jun N-terminal kinase (Adler et al., 1999) or in tyrosine catabolism (Dixon et al., 2000) indicate that there may be GST proteins with little traditional GST activity but having other physiological functions still to be elucidated.

Acknowledgements

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Future Plans

We will continue to perform structure function studies to increase our understanding of which amino acid residues affect enzyme specificity. We are also interested in determining the mechanism of how the elucidated amino acids contribute to the observed specificity. In addition we are also studying non-enzymatic roles of the GSTs in their interaction and regulation of the Jun N-terminal Kinase (JNK) pathway.

Publications

We have published the seven manuscripts presented in this Final Annual Report.

1. Udomsinprasert, R., Bogoyevitch, M.A. and Ketterman, A.J. (2004) Reciprocal Regulation of Glutathione *S*-Transferase Spliceforms and the *Drosophila* c-Jun N-terminal Kinase Pathway Components. *In press. Biochem. J. Immediate Publication*, DOI:10.1042/BJ20040519 *Impact Factor: 4.589*

2. Winayanuwattikun, P. and Ketterman, A.J. (2004) Catalytic and structural contributions for glutathione binding residues in a delta class glutathione S-transferase. *In press. Biochem. J. Immediate Publication*, DOI:10.1042/BJ20040697 *Impact Factor: 4.589*

3. Wongtrakul, J., Udomsinprasert, R. and Ketterman, A.J. (2003) Non-active site residues Cys69 and Asp150 affected the enzymatic properties of glutathione *S*-transferase AdGSTD3-3. *Insect Biochem. Molec. Biol.* **33**, 971-979. *Impact Factor: 2.350*

4. Vararattanavech, A. and Ketterman, A.J. (2003) Multiple roles of glutathione binding-site residues of glutathione S-transferase. *Protein and Peptide Lett.*, **10**, 441-448. *Impact Factor: 0.622*

5. Wongsantichon, J., Harnnoi, T. and Ketterman, A.J. (2003) A sensitive core region in the structure of glutathione S-transferases. *Biochem. J.* **373**, 759–765. *Impact Factor: 4.589*

6. Wongtrakul, J., Sramala, I. and Ketterman, A.J. (2003) A non-active site residue cysteine 69, of glutathione S-transferase AdGSTD3-3 has a role in stability and catalytic function. *Protein and Peptide Lett.*, **10**, 375-385. *Impact Factor: 0.622*

7. Udomsinprasert, R., and Ketterman, A.J. (2002) Expression and Characterization of a Novel Class of Glutathione S-Transferase from *Anopheles dirus*. *Insect Biochem. Molec. Biol.* **32**: 425-433. *Impact Factor: 2.350*

In the last two years we have presented ten posters of the work described in this report.

1. Piromjitpong, J., Wongsantichon, J., Winayanuwattikun, P. and Ketterman, A.J. (2003) Characterization of the contributions of the milieu of amino acids in effecting enzyme properties of glutathione S-transferases. 29th Congress on Science and Technology of Thailand, 20-22 October 2003 Khon Kaen University. SB-97P.

2. Ketterman, A. J., Harnnoi, T., Wongsantichon, J. (2003) A sensitive residue position in an inner core region in domain 2 of glutathione S-transferases. ComBio 2003 Melbourne Australia 28 Sept – 2 Oct 2003. POS-TUE-007

3. Winayanuwattikun, P. and Ketterman, A.J. (2002) Characterization of amino acid residues in the active site of *Anopheles dirus* species b glutathione S-transferase isoform 1-3. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 12

4. Vararattanavech, A., Wongsantichon, J. and Ketterman, A.J. (2002) Characterization of residues involved in the active site of glutathione S-transferase. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 13

5. Wongtrakul, J. and Ketterman, A.J. (2002) Aspartate 150 outside the active site has effect on the specificity of glutathione S-transferase adGST1-3. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 14

6. Wongsantichon, J. and Ketterman, A.J. (2002) A sensitive region in the structure of glutathione S-transferases. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 15

7. Udomsinprasert, R. and Ketterman, A.J. (2002) Studies of diptera glutathione S-transferase interaction with the stress response Jun-N-terminal kinase. 4th HUGO Pacific Meeting and 5th Asia-Pacific Conference on Human Genetics, 27-30 October, 2002. Pattaya, Thailand. IF - 16

8. Ketterman A.J., Wongsantichon J., Vararattanavech A. and Winayanuwattikun P. (2002) Site-directed mutagenesis of active site residues of glutathione S-transferase. ComBio 2002 Sydney Australia 29 Sept – 3 Oct 2002. POS-WED-012

9. Wongtrakul, J. and Ketterman, A.J. (2002) Cysteine 69 outside the active site has effect on the catalytic activity of glutathione S-transferase adgst1-3. At the Protein Science Network Symposium 2002, Protein Structure and Molecular Enzymology. held 29-30 August 2002 at the Center for Protein Structure and Function, Faculty of Science, Mahidol University.

10. Vararattanavech, A., Wongsantichon, J., Winayanuwattikun, P. and Ketterman, A.J. (2002) Characterization of residues involved in the active site of glutathione S-transferase. At the Protein Science Network Symposium 2002, Protein Structure and Molecular Enzymology, held 29-30 August 2002 at the Center for Protein Structure and Function, Faculty of Science, Mahidol University.

Invited Speaker

I have been an invited speaker to speak at the Department of Biochemistry, University of Western Australia.

Collaborations Domestic and International

I have several working collaborations. One collaboration is with Dr. L. Prapanthadara and Dr. Jeerang Wongtrakul at the Research Institute for Health Sciences, Chiangmai University, Chiangmai, Thailand. Drs. Prapanthadara and Wongtrakul are involved with the enzyme characterization studies and also in supplying my laboratory with *An. dirus* mosquitoes. A second collaboration is with Dr. Saengtong Pongjareankit at Maejo University. A third collaboration is with Dr. Matthew C.J. Wilce in the Structural Biology Unit, Department of Pharmacology/Crystallography Centre, University of Western Australia. A fourth collaboration is with Dr. Aaron J. Oakley at Australian National University, Canberra Australia. Drs. Wilce and Oakley are crystallographers and are currently crystallizing and elucidating the tertiary structure of the *An. dirus* recombinant GSTs that we are studying.

Collaborations within Institute

We have formed a scientific support network consisting of the following six ajarns and their respective groups (at this time this includes 30 people): Drs. C. Angsuthanasombat, G. Katzenmeier, A. Ketterman, C. Krittanai, C. Ongvarrasopone and D. Smith.

Problems

There are no unusual problems.

Comments and Suggestions

It would be helpful to receive the next funding budget quickly.