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Characterization of *Anopheles dirus* Glutathione Transferase Epsilon 4

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ABSTRACT: The coding sequences of a wild type glutathione transferase (GST) Epsilon 4 and three isoenzymes were obtained by RT-PCR from a Thai malaria mosquito, *Anopheles dirus*. After confirmation by sequencing, the RT-PCR products were subcloned into an expression vector and proteins were expressed, purified, and biochemically characterized to study the function of these enzymes and for comparison with two orthologs from *An. gambiae* (agGSTE4-4) and *Aedes aegypti* (aaGSTE4-4). The results showed that *An. dirus* GST Epsilon 4 (adGSTE4-4) shares more than 85% amino acid sequence similarity with agGSTE4-4 and aaGSTE4-4. However, adGSTE4-4 possesses a greater catalytic efficiency (k_{cat}/K_m) for 1-chloro-2,4-dinitrobenzene as well as greater activities for several other substrates compared with agGSTE4-4 and aaGSTE4-4. Moreover, adGSTE4-4 enzyme possesses peroxidase and DDT dehydrochlorinase activities while these activities were not observed for agGSTE4-4. In addition, adGSTE4-4 binds two pyrethroid insecticides (permethrin and l–cyhalothrin) with a relatively high affinity. We conclude that adGSTE4-4, unlike agGSTE4-4, can contribute to DDT resistance by DDT dehydrochlorinase activity as well as to pyrethroid resistance by sequestration and protection against oxidation from secondary pyrethroid metabolites via its peroxidase activity.

KEYWORDS: Glutathione transferase; Epsilon class; *Anopheles dirus*; DDT dehydrochlorinase; pyrethroid insecticide.

INTRODUCTION

Glutathione transferases (GSTs: E.C. 2.5.1.18) are a diverse family of multifunctional enzymes that play roles in metabolism, transportation, xenobiotic compound detoxification, and cell mediation against oxidative stress[1]. They are found in almost all living organisms and catalyze a conjugation reaction by transferring the thiol group of reduced glutathione (GSH) to an electrophilic substrate, making the resultant products more water soluble and excretable. GSTs are composed of two subunits and each subunit consists of two domains containing a highly conserved glutathione binding site (or G site) and a hydrophobic substrate binding site (or H site). Currently, more than 40 GST genes have been detected and grouped into at least 13 different classes[1,2]. Generally, the intra-class GSTs have greater than 40% amino acid sequence identity whereas the inter-class GSTs have amino acid sequence identity less than 30%[3]. The insect GSTs are of particular interest because they have the potential to confer resistance to all major groups of insecticides. GST-based resistance has been detected by elevated levels of GST activity in strains of insects resistant to organophosphates[4], organochlorines[5], and pyrethroids[6]. The mechanisms that GSTs use to detoxify insecticides are direct metabolism of insecticides or the secondary products, prevention of the oxidative damage induced by insecticide and sequestration[7]. Recently, the complete genome of *Anopheles gambiae* (an African malaria mosquito) was obtained and GST genes in this species have been annotated into at least six different classes (namely Theta, Sigma, Zeta, Omega, Delta, and Epsilon). Two insect specific classes, Delta and Epsilon, encode two-thirds of the *An. gambiae* cytosolic GSTs[2]. Moreover, a cluster of eight insect-specific epsilon class GSTs (*gste1 - gste8*) have been identified and co-localized with a DDT resistance locus, *rtd1*, on polytene chromosome arm 3R division 33B[8]. In addition, a quantitative PCR assay showed five of the eight epsilon GSTs (namely *gste1, gste2, gste3, gste4*, and *gste7*) are expressed at significantly greater levels in the DDT resistant strain[2]. Earlier studies had shown that GSTE2-2 was very efficient at metabolizing DDT, GSTE1-1 possessed peroxidase activity whereas GSTE4-4 and GSTE8-8 had no detectable DDT dehydrochlorinase or peroxidase activities[9]. In our laboratory, we are interested in Epsilon class GSTs in

the malaria mosquito, *Anopheles dirus*. Malaria is a major public health problem in Thailand, resulting in 325 reported deaths and 37,335 cases in 2003[10]. Furthermore, a comparison between the genomes of *An. dirus* and *An. gambiae* showed that the two Anopheline species possess a similar pattern of GST isoenzymes, although the individual enzymes differ significantly at the functional level[11]. For example, when comparing the equivalent alternatively spliced GSTs from *An. dirus GST1AS1* and *An. gambiae GST1a*, the amino acid sequence identities for adGSTD1-1 versus agGSTD1-6, and adGSTD3-3 versus agGSTD1-5 are 93% and 92%, respectively[12], but the catalytic efficiencies toward 1-chloro-2,4-dinitrobenzene (CDNB) are 16.5-fold and 1.5-fold different, respectively[13,14]. Despite the high level of sequence identity between GST enzymes from *An. dirus* and *An. gambiae*, the enzymes display different kinetic properties and substrate specificities that may lead to differences at the functional level. The aim of this study was to obtain and characterize Epsilon GSTs from the Thai malaria vector, *An. dirus*. To be in alignment with a proposed universal GST nomenclature the enzyme reported here was named adGSTE4-4 (in insect GST nomenclature, "E" refers to the Epsilon class and "4-4" refers to the homodimeric isoenzyme[15,3]). Here we report the amino acid sequences of GST Epsilon 4 (adGSTE4-4) enzyme and three variant isoenzymes from *An. dirus,* and compare with the orthologous enzymes from *An. gambiae* and *Aedes aegypti*.

Materials and Methods

Mosquito Strain

An *Anopheles dirus* B colony established at the Department of Parasitology, Faculty of Medicine, Chiang Mai University, was used in this study. The colony was identified on the basis of its morphological and chromosomal characteristics[16].

RT-PCR and cDNA Sequencing of *An. dirus gste4*

Total RNA was extracted from 0.1 g of fourth instar larvae of *An. dirus* using TRI reagent (SIGMA, USA), according to the manufacturer's instructions. Then mRNA was reverse transcribed into cDNA using ImProm-II™ Reverse Transcriptase (Promega, USA) and oligo(dT) primer (5'-CGGTCGACATATG$(dT)_{18}$-3') following the instructions from Promega. PCR used cDNA as a template, a 5' primer started at ATG codon based on *An. gambiae gste4* sequence (5'-ATGCCGA ACATCAAGCTGTAC-3'), a 3' oligo(dT) primer, and *Taq* DNA Polymerase (Promega, USA). PCR (consisted of 35 cycles of 94°C for 10 s, 55°C for 30 s, 72°C for 1 min) was performed in a Perkin–Elmer thermocycler (USA). Then RT-PCR product was directly sequenced in both directions using a Bigdye™ terminator cycle sequencing kit (Perkin Elmer, USA). Multiple alignments of nucleotide sequences and translated amino acid sequences were analyzed using the ClustalX program.

5'Rapid Amplification of cDNA Ends (RACE) of *An. dirus gste4*

To determine 5' amino acid sequence identity of *An. dirus gste*, 5' RACE was performed according to the 5' RACE System for Rapid Amplification of cDNA Ends, Version 2.0 instruction manual (GIBCO BRL, USA). Total RNA was extracted and reverse transcribed into cDNA as previously described using *adgste4* gene specific primer 1 (5'-ATTCGT CGCCGCATAGTAGGG-3') to obtain full-length *An. dirus gste4* coding sequence.

Cloning, Expression and Purification of *An. dirus gste4*

Full-length coding region of *An. dirus gste4* was amplified by RT-PCR as described above using *Taq* DNA Polymerase, 3' primer containing *Bam*HI site (5'-GC**GGATCC**TCACTTTGCTTTAGCACGGTTC-3'), and two different 5' primers containing *Bam*HI site (5'-AAAC**CCATGG**ATCCATGCCGAA CATCAAGCTG-3') or *Nde*I site (5'-CG**CATATG**CCAAACATTAAGCTGTA CACGG-3'). These two RT-PCR products were subcloned into two different sites (*Bam*HI site or *Nde*I–*Bam*HI site) of the pET3a expression vector (Novagen, USA) to generate adGSTE4-4 enzymes with or without a T7tag on the N-terminus, respectively. Then recombinant plasmids were analyzed by restriction digestion, sequenced and transformed into *E. coli* BL21(DE3)pLysS. Proteins were expressed as previously described[17] and soluble target proteins were purified using cation exchange chromatography using a SP-XL column, followed by hydrophobic interaction chromatography using a HiTrap Phenyl Sepharose column (Amersham Biosciences, USA). The SP-XL column was equilibrated with 50 mM phosphate buffer pH 7 and lysate was applied to the column. The adGSTE4-4 enzymes did not bind to the SP-XL column, so flow-through was collected and NaCl added to a final concentration of 3 M before loading to a HiTrap Phenyl Sepharose column pre-equilibrated with 50 mM phosphate buffer, pH 7, containing 3 M NaCl. Protein was eluted with a linear gradient from 2 - 0.75 M NaCl in 50 mM phosphate buffer, pH 7, containing 10 mM dithiothreitol (DTT). The major activity of adGSTE4-4 enzymes were eluted in 50 mM phosphate buffer, pH 7, containing 1 or 0.75 M NaCl. Purified enzymes were concentrated and desalted by Hitrap desalting columns (Amersham Biosciences, USA) as previously described[18]. GSTs were observed to be homogenous by SDS-PAGE. The enzymes were stored in 50 mM phosphate buffer, pH 6.5, 10 mM DTT, 50% (v/v)

glycerol at -20°C. Protein concentrations were determined by Bio-Rad protein reagent (Bio-Rad) with bovine serum albumin as standard protein[19].

Enzyme Characterization

Steady-state kinetics were performed by varying concentrations of 1-chloro-2,4-dinitrobenzene (CDNB) and glutathione (GSH) in 0.1 M phosphate buffer, pH 6.5, as previously described to obtain kinetic parameters, k_{cat}, K_m and k_{cat}/K_m, of wild type adGSTE4-4 and variant enzymes [17]. Substrate specificity of the enzymes were determined as previously described with five different substrates; CDNB, 1,2-dichloro-4-nitrobenzene (DCNB), ethacrynic acid (EA), *p*-nitrophenethyl bromide (PNPB) and *p*-nitrobenzyl chloride (PNBC)[17]. A thermal stability assay was performed to determine half life of wild type and variant enzymes by incubating enzymes (1 mg/ml in 0.1 M potassium phosphate, pH 6.5, containing 5 mM DTT and 1 mM EDTA) at 45°C and enzyme aliquots were assayed for activity at different time-points as previously described[17]. Insecticide K_i experiments for permethrin and λ-cyhalothrin (SUPELCO, USA) were performed by varying CDNB concentrations with 30 mM GSH in the presence and absence of the pyrethroid insecticides. The initial rate of reaction was used to construct a double reciprocal plot, 1/*V* versus 1/*S*, and the inhibition constant (K_i) was determined as previously described[20,13]. Peroxidase activities of wild type and variant enzymes were determined at 340 nm by coupling the reduction of cumene hydroperoxide (CHP) to the oxidation of NADPH by oxidized GSH with glutathione reductase as previously described[21]. DDT dehydrochlorinase activity was determined by conversion of DDT to 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDE) detected by high-

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                        *        20         *        40         *        60         *        80
adGSTE4-4 WT       : MPNIKLYTAKLSPPGRAVELTGKALGLEFDISPINLIAGDHLREEFRKLNPQHTIPLIDD-AGTIVYESHAIIVYLVTKY
:  79
adGSTE4-4 Pro14Leu : .............L.....................................................-...................
:  79
adGSTE4-4 Glu44Lys : ..........................................K.................-...................
:  79
adGSTE4-4 Ile131Thr: ..........................................................-...................
:  79
agGSTE4-4          : ................S....A......L..V......QE..T.A...............-N.....D....N.......
:  79
aaGSTE4-4          : .GK.Q................A.....DL..H...........P..V...........V.ED.....D............
:  80
adGSTD3-3          : ---.DF.YLPG.A.C......AA.....L..KLT.....E...P..L......C..T.V.-N.FA....R..CT..AE..
:  76


                              *       100         *       120         *       140         *       160
adGSTE4-4 WT       : G--ADDSLYPSDAVTRSKVNAALHFDSGVLFARLRFYLEPILYYGSTETPQEKIDNLYRAYELLNATLVD--DYIVGSRL
: 155
adGSTE4-4 Pro14Leu : .--.....................................................................--........
: 155
adGSTE4-4 Glu44Lys : .--.....................................................................--........
: 155
adGSTE4-4 Ile131Thr: .--.....................................................T...............--........
: 155
agGSTE4-4          : .KPEG.......V.Q.A................F...........A...................D....--E....NE.
: 157
aaGSTE4-4          : A--K......K.IA..A.............................PD...D...YAC.......D....--E....N..
: 156
adGSTD3-3          : .--K..K...K.PQK.AV..QR.Y..M.T..Q.FAD.YY.Q..AKQPAN-A.NEKK.KD.VDF..-.F..GHK..A.DS.
: 152


                              *       180         *       200         *       220
adGSTE4-4 WT       : TLADLSCVASIASMHAIFPIDAGKYPKLLAWVERIAKLPYYAATNQAGAEELAQLYHAKLAENRAKAK : 223
adGSTE4-4 Pro14Leu : ..................................................................... : 223
adGSTE4-4 Glu44Lys : ..................................................................... : 223
adGSTE4-4 Ile131Thr: ..................................................................... : 223
agGSTE4-4          : ............................AG..K........E...R..........R...E...TN.. : 225
aaGSTE4-4          : ..............Y........A.....A......E.....KG...E......A..RD.......GK. : 224
adGSTD3-3          : ......V....S.YDVAG-FELA...H.A..Y..TR.EAPG..I....I..FRKY.EK---------- : 209
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Fig 1. Amino acid sequence alignment of *An. dirus* GSTE4-4 wild type (adGSTE4-4 WT) and variants (Pro14Leu, Glu44Lys, and Ile131Thr), *An. gambiae* GSTE4-4 (agGSTE4-4), *Aedes aegypti* GSTE4-4 (aaGSTE4-4), and *An. dirus* GSTD3-3 (adGSTD3-3). Gaps introduced to maximize sequence similarity are shown by a horizontal dash while a dot represents the same amino acid. GenBank accession numbers for the sequences are: adGSTE4-4, **DQ168030**; agGSTE4-4, **AY070254**; aaGSTE4-4, **AY819709**; adGSTD3-3, **AF273039**.

Table 1. Substrate specificities, kinetic parameters, and half life of *Anopheles dirus* and *Anopheles gambiae* GSTE4-4 enzymes.

	adGSTE4-4 WT	adGSTE4-4 T7WT	adGSTE4-4 Pro14Leu	adGSTE4-4 Glu44Lys	adGSTE4-4 Ile131Thr	agGSTE4-4
Substrate specificity						
CDNB (μmol/min/mg)	79.95 ± 0.81	77.03 ± 1.11	1.43 ± 0.08	71.21 ± 3.72	53.56 ± 2.71	16.50 ± 1.02
DCNB (μmol/min/mg)	0.324 ± 0.011	0.462 ± 0.011	0.002 ± 0.000	0.381 ± 0.016	0.276 ± 0.021	0.070 ± 0.010
PNPB (μmol/min/mg)	0.113 ± 0.009	0.123 ± 0.011	not detected	0.099 ± 0.005	0.128 ± 0.007	-
PNBC (μmol/min/mg)	0.140 ± 0.006	0.133 ± 0.008	not detected	0.124 ± 0.004	0.134 ± 0.001	-
EA (μmol/min/mg)	0.034 ± 0.006	0.095 ± 0.007	0.007 ± 0.002	0.029 ± 0.005	0.007 ± 0.004	-
CHP (nmol/min/mg)	118.02 ± 6.19	-	not detected	74.90 ± 6.46	not detected	not detected
DDTase activity (nmol of DDE/mg)	2.56 ± 0.32	-	-	-	-	not detected
Kinetic parameters						
V_{max} (μmol/min/mg)	94.83 ± 5.06	87.55 ± 1.73	1.65 ± 0.04	83.17 ± 0.83	69.20 ± 1.05	16.30 ± 1.59
k_{cat} (S^{-1})	39.28	36.26	0.69	34.45	28.66	13.74
K_m for GSH (mM)	4.53 ± 1.04	7.15 ± 0.18	11.26 ± 1.30	3.81 ± 0.15	5.12 ± 0.21	5.38 ± 0.79
k_{cat}/K_m for GSH ($S^{-1}.mM^{-1}$)	8.7	5.1	0.06	9	5.6	2.5
K_m for CDNB (mM)	0.076 ± 0.010	0.060 ± 0.006	0.144 ± 0.017	0.070 ± 0.010	0.040 ± 0.004	0.131 ± 0.026
k_{cat}/K_m for CDNB ($S^{-1}.mM^{-1}$)	517	604	4.8	492	717	105
K_i for permethrin (μM)	66.27 ± 2.75	-	not inhibited	53.28 ± 3.42	669.28 ± 27.42	-
K_i for λ-cyhalothrin (μM)	115.43 ± 15.16	-	not inhibited	72.20 ± 1.66	433.79 ± 11.63	-
Half life at 45°C (min)	21.48 ± 0.40	18.93 ± 1.78	32.16 ± 2.53	13.93 ± 0.61	6.97 ± 0.85	-

The data are means ± standard deviation for at least three separate experiments. adGSTE4-4 T7WT indicates wild type GSTE4 containing the T7 tag on the N-terminus.
A dash indicates data not determined.
For substrate specificity, the concentrations used were: 1 mM CDNB (1-chloro-2,4-dinitrobenzene); 1 mM DCNB (1,2-dichloro-4-nitrobenzene); 0.1 mM PNPB (*p*-nitrophenethyl bromide); 0.1 mM PNBC (*p*-nitrobenzyl chloride); 0.2 mM EA (ethacrynic acid); and 1 mM CHP (cumene hydroperoxide). *An. gambiae* data are given for comparison[9].

performance liquid chromatography as described previously[13].

Results

Cloning, Expression and Purification of adGSTE4-4 Enzymes

The full-length coding sequence of *An. dirus gste4* contains 672 bp translated to give a polypeptide of 223 amino acids which is the same length as *Aedes aegypti gste4*[22] but two amino acids less than *An. gambiae gste4*[2]. A comparison of *An. dirus gste4* with *An. gambiae* and *Ae. aegypti* enzymes shows amino acid sequence identity/similarity for the enzymes of 81%/90% and 74%/85%, respectively (Fig. 1). In the 52 region *An. dirus gste4* had only two nucleotides differ from *An. gambiae gste4* although the same amino acids were still encoded. However, from 12 full-length sequenced clones of *An. dirus gste4*, three clones showed variations in amino acid sequences; the first (adGSTE4-4 Pro14Leu) had Pro14 changed to Leu, the second (adGSTE4-4 Glu44Lys) had Glu44 changed to Lys, and the third (adGSTE4-4 Ile131Thr) had Ile131 changed to Thr (Fig. 1). These variants and wild type *adgste4* were subcloned into the *Nde*I-*Bam*HI site of pET3a expression vector to generate enzymes without a fusion tag. Additionally a wild type *adgste4* sequence was subcloned into the *Bam*HI site of pET3a to generate adGSTE4-4 enzyme with a T7 tag on the N-terminus. This was to observe the effect of the T7 tag on enzyme properties and for comparison with *An. gambiae* GSTE4-4 enzyme which possessed a T7 tag[9]. Five adGSTE4-4 enzymes, wild type with or without T7 tag and three variants without T7 tag, were expressed as soluble proteins at high levels. These enzymes were purified by using cation exchange chromatography followed by hydrophobic interaction chromatography, which gave a 3-fold greater yield for wild type adGSTE4-4 enzyme without T7 tag when compared with purification by S-hexylglutathione affinity chromatography (data not shown).

Substrate Specificity, Kinetic Properties, and Half Life Stability of adGSTE4-4 Enzymes

Six GST substrates were used to determine substrate specificity (Table 1). *An. dirus* adGSTE4-4 had 5-fold greater activities for CDNB and DCNB than the *An. gambiae* enzyme. Moreover, wild type adGSTE4-4 exhibited peroxidase and DDT dehydrochlorinase activities whereas these activities for *An. gambiae* agGSTE4-4 were undetectable. These results showed variation in substrate specificities among wild type adGSTE4-4 with or without T7tag, adGSTE4-4 variants, and the orthologous enzyme from *An. gambiae*. The kinetic property differences between these two enzymes may originate from either the 14 N-terminus amino acids (T7tag) of agGSTE4-4 or the 41 amino acids that are different between agGSTE4-4 and wild

type adGSTE4-4. However, the kinetic properties between wild type adGSTE4-4 with and without T7 tag were similar, which suggests the T7 tag had little effect on adGSTE4-4 kinetic properties, supporting the idea that the enzyme differences originate with the sequence differences.

When comparing wild type adGSTE4-4 with the variant enzymes, we observed that adGSTE4-4 Pro14Leu exhibited the lowest enzyme activities for all substrates tested including a dramatically decreased catalytic efficiency toward CDNB (107-fold). Thus Pro14 appeared to have a major impact on enzyme catalysis. In contrast, adGSTE4-4 Glu44Lys had similar kinetic properties to the wild type enzyme. Whereas adGSTE4-4 Ile131Thr had no detectable peroxidase activity and showed 5-fold lower activity for EA than the wild type.

The insecticide inhibition kinetics of permethrin and λ-cyhalothrin were shown to be a non-competitive inhibitor for two variants (adGSTE4-4 Glu44Lys, and adGSTE4-4 Ile131Thr). However, for wild type adGSTE4-4 permethrin and λ-cyhalothrin were found to be a non-competitive inhibitor and mixed inhibitor, respectively. A comparison of K_i values for permethrin and λ-cyhalothrin illustrates differences between various *An. dirus* enzymes (Table 1), the most notable one being adGSTE4-4 Pro14Leu which showed no inhibition with either permethrin or λ-cyhalothrin.

The half lives of wild type adGSTE4-4 with and without T7 tag at 45°C were similar (Table 1). The half-life of adGSTE4-4 Pro14Leu increased 1.5-fold whereas those of adGSTE4-4 Glu44Lys and adGSTE4-4 Ile131Thr decreased 1.5- and 3-fold, respectively, when compared with the wild type enzyme. These residue changes therefore showed only small affects on the structural stability of the enzyme.

Discussion

Despite the high degree of sequence identity between the two orthologous GSTE4-4 enzymes from *An. dirus* and *An. gambiae*, they displayed differences in kinetic properties and substrate specificity, especially for peroxidase and DDT dehydrochlorinase activities which were only observed for the *An. dirus* enzyme. In contrast to *An. gambiae* agGSTE2-2, agGSTE4-4 and agGSTE8-8 enzymes, which have undetectable peroxidase activities, *An. dirus* adGSTE4-4 possesses peroxidase activity comparable to the *Ae. aegypti* aaGSTE2-2 (0.11 mmol/min/mg), but 88.7-fold greater activity than agGSTE1-1 from the DDT resistant strain of *An. gambiae* (1.33 nmol/min/mg)[9,22]. This peroxidase activity has been shown to be protective against the damage caused by lipid peroxidation products induced by exposure to pyrethroid insecticides[23]. In contrast, several GSTs from the DDT resistant strain of *An. gambiae*, agGSTE1-1, agGSTE4-4, and agGSTE8-8, showed no detectable DDT dehydrochlorinase activity whereas, *An. dirus* adGSTE4-4 possessed DDT dehydrochlorinase activity (2.56 nmole of DDE/mg). However, this enzyme has 1000-fold lower DDT dehydrochlorinase activity than those of agGSTE2-2 and aaGSTE2-2 from the DDT resistant strains of *An. gambiae* (2.77 nmole of DDE/μg) and *Ae. aegypti* (4.16 nmole DDE/μg), respectively[9,22]. Previously it has been reported that GST enzymes protect against pyrethroid toxicity in insects by sequestering the insecticide[6]. In the pyrethroid inhibition study, *An. dirus* GSTE4-4 enzyme was affected by a non-competitive and mixed type inhibition by permethrin and λ-cyhalothrin, respectively, which demonstrates that both pyrethroids interacted with the enzyme although not in the active site. The K_i value of adGSTE4-4 for permethrin indicates that it has similar permethrin binding affinity to *An. dirus* Delta class GSTs for which the K_i values range from 9 – 53 mM[13].

Three variants of adGSTE4-4 enzymes (Pro14Leu, Glu44Lys, and Ile131Thr) were also obtained by RT-PCR. As no Epsilon GST structure is currently available we used the structure of adGSTD3-3[24] to model the variant residue positions (Fig. 2). Pro14Leu is near the critical active site serine residue involved in thiol deprotonation of GSH. The Pro14 residue is a highly conserved active-site residue in Delta, Epsilon, Theta, and several unclassified GSTs in insects[2,22,25], suggesting its importance for backbone orientation. As proline causes a kink in the peptide backbone, when changed to leucine it would therefore change the conformation of the active site pocket, impacting on substrate specificity as well as catalysis. In addition, the Pro14Leu variant enzyme was not inhibited by permethrin or λ-cyhalothrin. It appears that the Pro14Leu variant enzyme lost the pyrethroid binding site due to a change in the enzyme topology. The second variant enzyme, containing a Glu44Lys change had the same kinetic properties and permethrin binding affinity as wild type, but surprisingly had peroxidase activity slightly lower than the *An. dirus* wild type enzyme, which was similar to *Ae. aegypti* GSTE2-2. Glu44Lys had a 56.3-fold greater peroxidase activity than agGSTE1-1 from a resistant strain of *An. gambiae*[9,22]. Moreover, Glu44Lys variant enzyme had a 1.6-fold greater λ-cyhalothrin binding affinity when compared to the wild type adGSTE4-4. The Glu44 residue is a conserved negative charge residue in the Epsilon class GSTs[2]. It is difficult to interpret the contribution of the Glu44 residue to peroxidase activity and specificity as no Epsilon structure is currently available. However the model suggests Glu44 is in α helix 2 which is known to be involved in the active site through GSH binding but also influencing specificity

of the hydrophobic substrate as well[26]. In contrast, the third variant enzyme, Ile131Thr in α helix five, showed no peroxidase activity and had 10- and 3.7-fold lower binding affinities to permethrin and λ-cyhalothrin, respectively. The above data suggests one putative surface site of interaction for the pyrethroids is a hydrophobic groove consisting of four motifs from both domains 1 and 2. These four motifs include 1) residues 32-41 in the loop between β2 sheet and α helix, 2) the C-terminus of α4 and loop residues between and including the N-terminus of α5, 3) the C-terminus of α6 and 4) the C-terminus of α8. This area would be affected through structural adjustments brought about by the residue changes observed in this study.

In conclusion, the Epsilon 4 GST of *An. dirus* shares more than 85% amino acid sequence similarity with the two orthologs from *An. gambiae* and *Ae. aegypti*. However, adGSTE4-4 possesses greater catalytic efficiency (k_{cat}/K_m) for CDNB as well as greater activity for several other substrates. In contrast, the *An. dirus* adGSTE4-4 enzyme possesses peroxidase and DDT dehydrochlorinase activities while these activities were not observed for the *An. gambiae* enzyme. In addition, we have shown the *An. dirus* enzyme binds two pyrethroid insecticides (permethrin and λ–cyhalothrin) with relatively high affinity. From these results, we conclude that *An. dirus* GST Epsilon 4 enzyme can contribute to DDT resistance by DDT dehydrochlorinase activity and to pyrethroid insecticide resistance by sequestration as well as protect against oxidation from the secondary pyrethroid metabolites via its peroxidase activity.

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References

1. Hayes JD, Flanagan JU and Jowsey IR (2005) Glutathione transferases. *Annu Rev Pharmacol Toxicol* **45**, 51-88.
2. Ding Y, Ortelli F, Rossiter LC, Hemingway J and Ranson H (2003) The *Anopheles gambiae* glutathione transferase supergene family: annotation, phylogeny and expression profiles. *BMC Genomics* **4**, 35-50.
3. Chelvanayagam G, Parker MW and Board PG (2001) Fly fishing for GSTs: a unified nomenclature for mammalian and insect glutathione transferases. *Chem Biol Interact* **133**, 256-60.

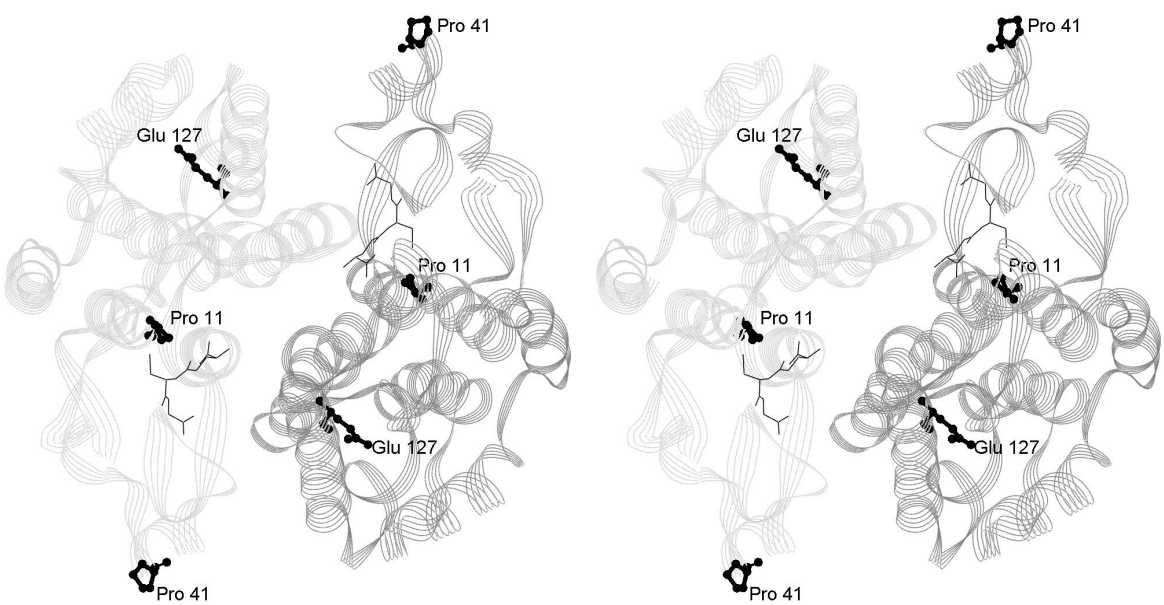

Fig 2. Stereo view of three amino acid residues in adGSTD3-3[24]. These three amino acids (Pro11, Pro41, and Glu127) are equivalent, as shown by amino acid alignment (Fig. 1), to the variant amino acid residues in adGSTE4-4: Pro14Leu, Glu44Lys, and Ile131Thr, respectively. This stereo view is looking at the two-fold axis on to the active sites. The three residues are shown as black ball-and-stick. GSH is shown in black stick in the active sites. The two subunits are shown in light gray and dark gray. The figure was generated using the program DS Viewer Pro.

4. Fournier D, Bride J-M, Poirié M, Bergé J-B and Plapp FW (1992) Insect glutathione S-transferases. Biochemical characteristics of the major forms from houseflies susceptible and resistant to insecticides. *J Biol Chem* **267**, 1840-5.
5. Grant DF and Hammock BD (1992) Genetic and molecular evidence for a *trans*-acting regulatory locus controlling glutathione S-transferase-2 expression in *Aedes aegypti*. *Mol Gen Genet* **234**, 169-76.
6. Kostaropoulos I, Papadopoulos AI, Metaxakis A, Boukouvala E and Papadopoulou-Mourkidou E (2001) Glutathione S-transferase in the defence against pyrethroids in insects. *Insect Biochem Molec Biol* **31**, 313-9.
7. Hemingway J, Hawkes NJ, McCarroll L and Ranson H (2004) The molecular basis of insecticide resistance in mosquitoes. *Insect Biochem Molec Biol* **34**, 653-65.
8. Ranson H, Jensen B, Wang X, Prapanthadara L, Hemingway J and Collins FH (2000) Genetic mapping of two loci affecting DDT resistance in the malaria vector *Anopheles gambiae*. *Insect Molecular Biology* **9**, 499-507.
9. Ortelli F, Rossiter LC, Vontas J, Ranson H and Hemingway J (2003) Heterologous expression of four glutathione transferase genes genetically linked to a major insecticide-resistance locus from the malaria vector *Anopheles gambiae*. *Biochem J* **373**, 957-63.
10. (2005) *http://w3.whosea.org/EN/Section10/Section21/Section340_4027 htm*
11. Prapanthadara L, Promtet N, Koottathep S, Somboon P and Ketterman AJ (2000) Isoenzymes of glutathione S-transferase from the mosquito Anopheles dirus species B: the purification, partial characterization and interaction with various insecticides. *Insect Biochem Molec Biol* **30**, 395-403.
12. Pongjaroenkit S, Jirajaroenrat K, Boonchauy C, Chanama U, Leetachewa S, Prapanthadara L and Ketterman AJ (2001) Genomic organization and putative promotors of highly conserved glutathione S-transferases originating by alternative splicing in *Anopheles dirus*. *Insect Biochem Molec Biol* **31**, 75-85.
13. Jirajaroenrat K, Pongjaroenkit S, Krittanai C, Prapanthadara L and Ketterman AJ (2001) Heterologous expression and characterization of alternatively spliced glutathione *S*-transferases from a single *Anopheles* gene. *Insect Biochem Molec Biol* **31**, 867-75.
14. Ranson H, Prapanthadara L and Hemingway J (1997) Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of *Anopheles gambiae*. *Biochem J* **324**, 97-102.
15. Mannervik B, Awasthi YC, Board PG, Hayes JD, Di Ilio C, Ketterer B, Listowsky I et al. (1992) Nomenclature for human glutathione transferases. *Biochem J* **282**, 305-6.
16. Baimai V (1989) Speciation and species complexes of the anopheles malaria vectors in Thailand.*The third conference on malaria research, Thailand*, 146-52.
17. Vararattanavech A and Ketterman A (2003) Multiple roles of glutathione binding-site residues of glutathione S-transferase. *Protein and Peptide Letters* **10**, 441-8.
18. Udomsinprasert R and Ketterman AJ (2002) Expression and characterization of a novel class of glutathione S-transferase from *Anopheles dirus*. *Insect Biochem Molec Biol* **32**, 425-433.
19. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**, 248-54.
20. Dixon M and Webb EC (1979) Enzyme inhibition and activation.**3**, 332-467.
21. Wendel A (1981) Glutathione peroxidase. *Methods Enzymol* **77**, 325-33.
22. Lumjuan N, McCarroll L, Prapanthadara L, Hemingway J and Ranson H (2005) Elevated activity of an epsilon class glutathione transferase confers DDT resistance in the dengue vector, *Aedes aegypti*. *Insect Biochem Molec Biol* **35**, 861-71.
23. Singh SP, Coronella JA, Beneš H, Cochrane BJ and Zimniak P (2001) Catalytic function of *Drosophila melanogaster* glutathione S-transferase DmGSTS1-1 (GST-2) in conjugation of lipid peroxidation end products. *Eur J Biochem* **268**, 2912-23.
24. Oakley AJ, Harnnoi T, Udomsinprasert R, Jirajaroenrat K, Ketterman AJ and Wilce MCJ (2001) The crystal structures of glutathione S-transferases isozymes 1–3 and 1–4 from *Anopheles dirus* species B. *Protein Science* **10**, 2176-85.
25. Sawicki R, Singh SP, Mondal AK, Beneš H and Zimniak P (2003) Cloning, expression and biochemical characterization of one Epsilon-class (GST-3) and ten Delta-class (GST-1) glutathione S-transferases from *Drosophila melanogaster*, and identification of additional nine members of the Epsilon class. *Biochem J* **370**, 661-9.
26. Ricci G, Caccuri AM, Lo Bello M, Rosato N, Mei G, Nicotra M, Chiessi E et al. (1996) Structural flexibility modulates the activity of human glutathione transferase P1-1. Role of helix 2 flexibility in the catalytic mechanism. *J Biol Chem* **271**, 16187-92.