# Cloning and characterization of two glutathione S-transferases from a DDT-resistant strain of *Anopheles gambiae*

Hilary RANSON*, La-aied PRAPANTHADARA† and Janet HEMINGWAY*‡

*Department of Pure and Applied Biology, University of Wales College of Cardiff, PO Box 915, Cardiff, CF1 3TL, Wales, U.K., and †Research Institute for Health Sciences, Chiangmai University, PO Box 80CMU, Chiangmai, 50200, Thailand

Two cDNA species, *aggst1-5* and *aggst1-6*, comprising the entire coding region of two distinct glutathione *S*-transferases (GSTs) have been isolated from a 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDT) resistant strain (ZANDS) of *Anopheles gambiae*. The nucleotide sequences of these cDNA species share 80.2% identity and their derived amino acid sequences are 82.3% similar. They have been classified as insect class I GSTs on the basis of their high sequence similarity to class I GSTs from *Drosophila melanogaster* and *Musca domestica* and they are localized to a region of an *An. gambiae* chromosome known to contain further class I GSTs. The genes *aggst*1-5 and *aggst*1-6 were expressed at high levels in *Escherichia coli* and the recombinant GSTs were purified by affinity chromatography and characterized. Both agGST1-5 and agGST1-6 showed high activity with the substrates 1-chloro-2,4-dinitrobenzene and 1,2-dichloro-4-nitrobenzene but negligible activity with the mammalian theta class substrates, 1,2-epoxy-3-(4-nitrophenoxy)-propane and *p*-nitrophenyl bromide. Despite their high level of sequence identity, agGST1-5 and agGST1-6 displayed different kinetic properties. Both enzymes were able to metabolize DDT and were localized to a subset of GSTs that, from earlier biochemical studies, are known to be involved in insecticide resistance in *An. gambiae*. This subset of enzymes is one of three in which the DDT metabolism levels are elevated in resistant insects.

## INTRODUCTION

The glutathione S-transferase (GST) enzymes are a major family of detoxification enzymes, which possess a wide range of substrate specificities (reviewed in [1]). Most organisms possess multiple GSTs belonging to two or more classes. Interest in GSTs in insects is focused on their role in insecticide resistance; many resistant insects have been shown to contain elevated levels of GST activity. For example, the overexpression of a GST is thought to be responsible for resistance to organophosphates in a strain of *Musca domestica* [2], and elevated levels of GST activity have also been found in 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)-ethane (DDT)-resistant strains of *Aedes aegypti* [3]. However, although several GSTs from resistant strains of insects have been cloned, a direct link between a cloned insect GST and insecticide metabolism has only been demonstrated for one enzyme from *Drosophila* [4].

The GSTs of *Anopheles gambiae* are of interest because of their involvement in DDT resistance in this important malaria vector. A comparison of the partly purified GSTs from a susceptible (G3) and a DDT resistant (ZANDS) strain of *An. gambiae* demonstrated that DDT resistance is associated with quantitative increases in multiple enzymes. These elevated GST levels metabolized DDT at different rates; the enzymes showing the highest DDTase activity were predicted to be members of the insect class I family on the basis of their physical properties and substrate specificities [5]. The presence of multiple GSTs with overlapping substrate specificities in all fractions after several column chromatography steps meant that it was difficult to obtain pure preparations of enzyme. Therefore a molecular cloning approach was applied to the study of the class I GSTs of *An. gambiae*.

We initially isolated genomic clones containing *An. gambiae* class I GSTs from an insecticide-susceptibile strain by exploiting similarities to other insect class I GSTs [6]. We now report the cloning and heterologous expression of two novel class I GSTs from an insecticide-resistant *An. gambiae* strain. The relationship of these enzymes to other insect and mammalian GSTs and their involvement in insecticide resistance is discussed.

## MATERIALS AND METHODS

### Mosquito strains

A susceptible strain of *An. gambiae*, G3, was colonized from the Gambia in 1975 and maintained without selection pressure. A DDT-resistant strain of *An. gambiae* (ZANDS) from Zanzibar, Tanzania, was colonized in 1982. This strain was maintained under larval DDT selection pressure resulting in a 20-fold resistance to DDT.

### Isolation of *aggst1-5* and *aggst1-6* cDNA species

We have previously reported the isolation of a partial *An. gambiae* cDNA, *aggst1-1*, from the susceptible G3 strain, which encodes the 142 N-terminal amino acid residues of a class I GST [6]. An oligonucleotide (5′-ATGGATTTTTATTACCTACCCGG-3′) encompassing the initiation codon of *aggst1-1* was designed and used to isolate a cDNA containing the full GST coding region from ZANDS fourth instar larvae by 3′ rapid amplification of cDNA ends (RACE) techniques [7]. The resultant PCR products were subcloned into pBluescript (Stratagene) and four individual PCR products were sequenced. An alignment of the sequences of these four PCR products revealed the presence of two distinct cDNA

Abbreviations used: CDNB, 1-chloro-2,4-dinitrobenzene; DCNB, 1,2-dichloro-4-nitrobenzene; DDE, 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane; DDT, 1,1,1-trichloro-2,2-bis-(*p*-chlorophenyl)-ethane; GST, glutathione S-transferase; RACE, rapid amplification of cDNA ends.

‡ To whom correspondence should be addressed.

The nucleotide sequence results reported here will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers Z81291 and Z81292.

species, which we have named *aggst1-5* and *aggst1-6* in line with the nomenclature adopted earlier [6].

### Preparation of expression constructs

Pairs of PCR primers spanning the coding region of *aggst1-5* and *aggst1-6* were designed that would permit the insertion of these cDNA species into an expression vector. Because both cDNA species had identical N-termini, a single forward primer (5′-CGGAATTC*GGATCC*ATGGATTTTTATTACCTACCCG-3′) was designed for the production of both constructs. The positioning of the *Bam*HI site (in italics) directly upstream from the translation initiation ATG (underlined) ensured that the constructs were translated in the correct frame. The 8 bp linker sequence preceding the *Bam*HI site was included so that the PCR fragments could be efficiently digested with *Bam*HI. The last 22 bp of the forward primer are identical with the first 22 base pairs of *aggst1-5* and *aggst1-6*. The reverse primer for *aggst1-5* had the sequence 5′-CGGAATTC*GGATCC*TTACTTCTCA-AAGTACTTCTTG-3′ and the reverse primer for *aggst1-6* had the sequence 5′-CGGAATTC*GGATCC*TTAGGACAGGAA-TTTTGCCTTG-3′. The *Bam*HI site of each reverse primer is shown in italics and the stop codon of each cDNA is underlined.

These primer sets were utilized in a PCR reaction to re-amplify the cDNA species from the pBluescript plasmids described above. The 50 $\mu$l PCR reaction contained 1 ng of target plasmid, 100 ng of each primer, 0.5 mM each dNTP, 2 mM $MgCl_2$, 1.5 units of *Taq* DNA polymerase and was buffered in *Taq* DNA polymerase buffer [50 mM KCl/10 mM Tris/HCl (pH 9.0 at 25 °C)/0.1 % Triton X-100]. Amplification (35 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min) were carried out in a Hybaid OmnE thermal cycler. A single product of approx. 650 bp was obtained from each PCR reaction. These PCR products were subcloned into pBluescript and sequenced in both directions to ensure that no errors had been introduced during PCR amplification. The inserts were then isolated from the plasmids by digestion with *Bam*HI in preparation for ligation into the expression vector.

The plasmid vector, pET3a (Novagen), was used for the expression of the recombinant proteins in *Escherichia coli*. Induction of the bacteriophage T7 promoter in this vector results in the production of high levels of recombinant proteins preceded by a 13-residue tag from the N-terminus of the T7 gene 10 protein. The *Bam*HI-digested PCR products described above were ligated into the *Bam*HI site of pET3a and the ligation products were used to transform *E. coli* XL-1 Blue. Colonies containing the appropriate insert in the correct orientation were identified by restriction digestion. The two expression constructs produced in this way were named pX*aggst1-5* and pX*aggst1-6*.

### Induction of expression

*E. coli* BL21(DE3)pLysS transformed with pX*aggst1-5*, pX*aggst1-6* or a control plasmid that consisted of a non-recombinant pET3a were grown to an attenuance of 0.6 and then induced by the addition of 0.4 mM isopropyl $\beta$-D-thiogalactoside. Incubation was continued for a further 3 h at 37 °C, after which the cells were harvested by centrifugation (5000 ***g*** for 5 min) and washed in buffer A [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/150 mM NaCl]. After a single round of freeze–thawing, the cells were resuspended in buffer B [50 mM Tris/HCl (pH 7.4)/1 mM EDTA/200 mM NaCl/1 mg/ml lysosyme/10 mM 2-mercaptoethanol] and sonicated 15 times for 15 s in a Soniprep 150 at setting 18. Dithiothreitol (10 mM) was added and the cell debris was removed by centrifugation (30000 ***g*** for 20 min).

### Purification of expressed proteins

The enzymes were purified from the cell supernatant by affinity chromatography on an *S*-hexylglutathione column as described previously [8]. The bound proteins were eluted with 5 mM *S*-hexylglutathione. The fractions containing active enzymes were passed through two NAP-5 columns and a PD-10 column (Pharmacia) to remove the *S*-hexylglutathione, and the enzymes were stored in 50 mM sodium phosphate (pH 7.4)/10 mM dithiothreitol/40 % (v/v) glycerol.

### Determination of enzyme activity

Specific activities were determined spectrophotometrically as described [9]. All measurements were performed at 22 °C in 0.1 M phosphate buffer. The activity with 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and 10 mM glutathione was measured by $A_{340}$ at pH 6.5. The activity with 1 mM 1,2-dichloro-4-nitrobenzene (DCNB) and 5 mM glutathione was measured by $A_{345}$ at pH 7.5. *p*-Nitrophenethyl bromide was used at 0.1 mM in the presence of 5 mM glutathione and the reaction was monitored at 310 nm; 1,2-epoxy-3-(*p*-nitrophenoxy)propane (1 mM) was used with 5 mM glutathione and monitored at 360 nm. Glutathione peroxidase activity with cumene hydroperoxide was determined by the method of Wendel [10].

DDT metabolism assays were performed as previously described [8]. Controls for non-enzymic dehydrochlorination were performed in all experiments.

### Kinetic studies

The initial rate of the enzymic reaction was measured at 0.5–10 mM glutathione as the CDNB concentration was varied from 0.05 to 2 mM. $K_m$ and $V_{max}$ were determined by non-linear regression analysis [11].

### Determination of protein concentration and SDS/PAGE

Protein was assayed by using the Bio-Rad protein reagent, with BSA as the standard protein [12]. The purity and subunit size of the enzyme preparations were determined by SDS/PAGE with Bio-Rad low-range standards as molecular mass markers.

### Partial purification of *An. gambiae* G3 GSTs

*An. gambiae* GSTs were partly purified from crude homogenates of fourth instar larvae from the DDT susceptible strain, G3, by Q-Sepharose, affinity and hydroxyapatite chromatography as described previously [8].

### Raising of polyclonal antibodies and immunoblotting

Polyclonal antibodies were raised against the recombinant GSTs agGST1-5 and agGST1-6 in New Zealand White rabbits by standard procedures. The recombinant proteins and the partly purified GSTs from G3 larvae were resolved by SDS/PAGE and blotted on a nitrocellulose membrane (Amersham) by using a HYBRI.DOT® manifold (Gibco BRL). The filter was probed with the GST polyclonal antibodies and detection was performed with the enhanced chemiluminescence detection system (Amersham).

## RESULTS

### Cloning of two class I GSTs from *An. gambiae*

We have previously described the isolation of a partial cDNA, *aggst1-1*, from the G3 strain of *An. gambiae*, which encodes the

**Table 1 Comparison of the amino acid sequences of AGGST1-5 and AGGST1-6 with other insect and mammalian GSTs**

Percentage similarity was calculated with the LASERGENE package (DNASTAR Ltd.). The amino acid sequences are as follows: agGST1-5 and agGST1-6 *Anopheles gambiae* (this study); GSTD1 *Drosophila melanogaster* [13]; mdGST-1 *Musca domestica* [14]; agGST1-2 *An. gambiae* [6]; dmGST-2 *D. melanogaster* [25]; agGST2-1 *An. gambiae* [26]; human alpha GSTA2-2 [27]; human pi [28]; human mu GSTM5-5 [29]; human theta GSTT2 [30].

| | Amino acid sequence similarity (%) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | GSTD1 | mdGST-1 | agGST1-2 | dmGST-2 | agGST2-1 | Human alpha | Human pi | Human mu | Human theta |
| agGST1-5 | 68.9 | 69.7 | 49.3 | 13.9 | 14.8 | 14.4 | 14.0 | 12.4 | 24.4 |
| agGST1-6 | 67.5 | 68.8 | 49.8 | 14.4 | 16.7 | 13.0 | 13.0 | 12.9 | 27.3 |

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agGST1-5 M D F Y Y L P G S A P C R A V Q M T A A A V G V E L N L K L  30
agGST1-6 M D F Y Y L P G S A P C R A V Q M T A A A V G V E L N L K L  30

agGST1-5 T D L M K G E H M K P E F L K I N P Q H C I P T L V D N G F  60
agGST1-6 T D L M K G E H M K P E F L K L N P Q H C I P T L V D N G F  60

agGST1-5 A L W E S R A I C T Y L A E K Y G K D D K L Y P K D P Q K R  90
agGST1-6 A L W E S R A I Q I Y L A E K Y G K D D K L Y P K D P Q K R  90

agGST1-5 A V V N Q R M Y F D M G T L Y Q R F A D Y Y Y P Q I F A K Q 120
agGST1-6 A V V N Q R L Y F D M G T L Y Q R F A D Y H Y P Q I F A K Q 120

agGST1-5 P A N P E N E Q K M K D A V D F L N T F L D G H K Y V A G D 150
agGST1-6 P A N P E N E K K M K D A V G F L N T F L E G Q E Y A A G N 150

agGST1-5 S L T I A D L S I L A T I S T Y D V A G F D L A K Y Q H V A 180
agGST1-6 D L T I A D L S L A A T I A T Y E V A G F D F A P Y P N V A 180

agGST1-5 V W Y E N I R K E A P G A A I N Q A G I E E F K K Y F E K   209
agGST1-6 A W F A R C K A N A P G Y A L N Q A G A D E F K A K F L S   209
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**Figure 1 Alignment of derived amino acid sequences of agGST1-5 and agGST1-6**

Identical residues are boxed.

N-terminus of a class I GST [6]. To obtain a cDNA comprising the full coding region of this GST from an insecticide-resistant strain, we designed a primer complementary to the first 22 base pairs of *aggst1-1* and used this primer in a 3′ RACE PCR with cDNA prepared from *An. gambiae* ZANDS. Sequencing of the products of this PCR reaction revealed the presence of two distinct cDNA species, *aggst1-5* and *aggst1-6*. The nucleotide sequences of these two cDNA species were 80.2 % identical and they presumably represent the transcription products of closely related genes within a gene family. The genes *aggst1-5* and *aggst1-6* were predicted to encode class I GSTs on the basis of their sequence similarity to other class I insect GSTs (Table 1). The derived amino acid sequences of these two genes show the greatest similarity to the class I GSTs, GSTD1 and mdGST-1 isolated from *Drosophila melanogaster* and *M. domestica* respectively [13,14] and lower levels of similarity to a previously identified class I GST from *An. gambiae*, agGST1-2 [6]. There are very low levels of sequence identity between the two classes of insect GST such that the *An. gambiae* class I GSTs identified in this study had higher levels of similarity to the mammalian class theta GSTs than to the insect class II GSTs (Table 1).

An alignment of the derived amino acid sequences of *aggst1-5* and *aggst1-6* is shown in Figure 1. The two proteins, agGST1-5 and agGST1-6, are 82.3 % similar overall but they show a much higher degree of variation at the C-terminus than at the N-terminus (96.1 % similarity between residues 1 and 78 compared with 73 % similarity between residues 85 and 209). Studies on the crystal structures of mammalian and insect GSTs have shown

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agGST1-1 ATGGATTTTTATTACCTACCCGGATCTGCCCCGTGCCGTGCCGTC 45
agGST1-5 ---------------------------------------------
agGST1-6 ---------------------------------------------

agGST1-1 CAGATGACGGCGGCCGCCGTTGGCGCTGAGCTGAACCTGAAGCTC 90
agGST1-5 --------------G----------T-------------------
agGST1-6 -------------------------T-------------------

agGST1-1 ACCGACCTGATGAAGGGCGAGCACATGAAGCCGGAGTTCCTGAAG 135
agGST1-5 ---------------------------------------------
agGST1-6 ---------------------------------------------

agGST1-1 CTCAATCCTCAACACTGCATTCCAACGCTGGTCGACAATGGATTC 180
agGST1-5 A-T--C--A-----------------------------------
agGST1-6 ---------------------------------------------

agGST1-1 GCCCTGTGGGAGTCGCGTGCCATTCAGATCTATCTGGCGGAGAAG 225
agGST1-5 --------------C--C--T--CTGT-CT---------------
agGST1-6 ---------------------------------------------

agGST1-1 TACGGCAAGGACGACAAGCTGTACCCGAAGGACCCGCAGAAGCGT 270
agGST1-5 ---------------------------C-----------------
agGST1-6 ---------------------------------------------

agGST1-1 GCCGTCGTAAACCAGCGACTGTACTTCGACATGGGCACTCTATAC 315
agGST1-5 -----A------------A--------------------------
agGST1-6 ---------------------------------------------

agGST1-1 CAGCGGTTCGCTGACTACCACTATCCTCAAATCTTTGCCAAGCAG 360
agGST1-5 -----C-----A-----TT----------------C---------
agGST1-6 -----C-----------------------------------------

agGST1-1 CCGGCCAACCCGGAAAATGAGAAGAAGATGAAGGATGCGGTCGGT 405
agGST1-5 --------T--A--------AC---------------A--T-AC
agGST1-6 ---------------------------------------------

agGST1-1 TTCCTGAACACCTTCCTGGAC 426
agGST1-5 --T-----------------
agGST1-6 --------------------G
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**Figure 2 Comparison of the N-terminal nucleotide sequences of *aggst1-5* and *aggst1-6* with the sequence of the partial cDNA *aggst1-1***

A dash represents identity.

that most of the active-site residues are contained within the N-terminal domain and hence this region of the protein is more strictly conserved between GSTs [15–17]. The C-terminal domain is under less tight constraint and the high degree of sequence divergence within this region leads to the variation in substrate specificities exhibited by individual enzymes.

The high degree of conservation of GSTs at the N-terminus makes it difficult to predict which, if either, ZANDS cDNA represents the full-length version of *aggst1-1* from G3. An alignment of the nucleotide sequences of the three cDNA species is shown in Figure 2. The nucleotide sequence of the first 426 bp of coding sequence of *aggst1-6* is 99.3 % identical with the corresponding region of *aggst1-1*, whereas the equivalent region of *aggst1-5* shows a lower level of identity (91.8 %). Hence on the basis of this alignment it seems that *aggst1-6* and *aggst1-1* might be allelic variants. However, as the 3′ ends of GST genes show a higher degree of divergence only an examination of the complete

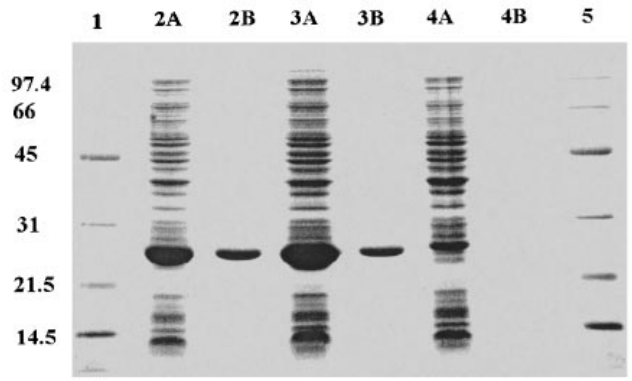


**Figure 3 SDS/PAGE gel of cell lysates (lanes A) and purified GSTs (lanes B) eluted from an *S*-hexylglutathione affinity column**

Lanes 1 and 5 contain molecular mass markers, as indicated by the scale (in kDa) at the left. The cell lysate shown in lane 2 contains recombinant agGST1-5, lane 3 contains recombinant agGST1-6 and lane 4 is a control lane that does not contain recombinant proteins. A 7.5 $\mu$l sample of an induced culture was loaded on each lane A. Approximately 1 $\mu$g of purified protein was loaded onto lanes 2B and 3B. Only trace amounts of protein were recovered after affinity chromatography of the control sample. This sample was concentrated to the same degree as for the recombinant proteins and 10 $\mu$l of the concentrated sample was applied to lane 4B.

gene sequences from which these cDNA species are derived would clarify the relationship between the three cDNA species.

### Expression of agGST1-5 and agGST1-6 in *E. coli*

Induction of the expression constructs pX*aggst1-5* and pX*aggst1-6* resulted in the production of high yields of recombinant protein. Approx. 5 mg of agGST1-5 was routinely obtained from 50 ml of culture, whereas the yield from agGST1-6 was almost twice as high. The purified enzymes were shown to be homogeneous preparations by Coomassie staining of overloaded SDS/PAGE gels (results not shown). Because *E. coli* possesses native GSTs that bind to glutathione affinity columns [18] these bacterial GSTs could conceivably be co-purified with the recombinant GSTs described in this study. However, when the supernatant from a control culture was applied to the *S*-hexylglutathione under the conditions described above, only trace amounts of protein and no detectable GST activity was eluted with 5 mM glutathione (Figure 3).

The subunit sizes of agGST1-5 and agGST1-6 are predicted to be 23.885 and 23.460 kDa respectively on the basis of their amino acid compositions. The recombinant proteins had subunit sizes of 26 kDa as determined by SDS/PAGE. The increase in subunit size is due to a 1.4 kDa vector-derived tag that is present on the N-terminus of the expressed proteins (see the Materials and methods section).

### Characterization of the recombinant GSTs

The substrate specificities of the recombinant GSTs are shown in Table 2: agGST1-5 and agGST1-6 have high activities with CDNB and detectable activity with DCNB and cumene hydroperoxide. However, both enzymes have very low levels of activity with the mammalian theta class GST substrates, *p*-nitrophenyl bromide and 1,2-epoxy-3-(*p*-nitrophenoxy)propane. DDTase activity of the enzymes was measured as nmol of 1,1-dichloro-2,2-bis-(*p*-chlorophenyl)ethane (DDE) formed/mg of protein under assay conditions identical to those used previously for studies on anopheline mosquitoes [5,19]. The activities of agGST1-5 and agGST1-6 towards DDT were comparable with that of purified *An. dirus* GST 4a [19] but lower than the activity reported for the partly purified GSTs from the DDT-resistant ZANDS strain of *An. gambiae* [5].

**Table 2 Substrate specificities of the recombinant *Anopheles gambiae* GSTs agGST1-5 and agGST1-6**

The activities are the means $\pm$ S.E.M. for at least three separate assays. The concentrations of substrates used are described in the text.

| | Activity ($\mu$mol/min per mg of protein) | |
|---|---|---|
| Substrate | agGST1-5 | agGST1-6 |
| CDNB | 56.44 $\pm$ 8.71 | 195.14 $\pm$ 11.95 |
| DCNB | 0.326 $\pm$ 0.035 | 0.636 $\pm$ 0.026 |
| *p*-Nitrophenethyl bromide | $<$ 0.15 | $<$ 0.15 |
| 1,2-Epoxy-3-(*p*-nitrophenoxy)propane | $<$ 0.007 | 0.102 $\pm$ 0.016 |
| Cumene hydroperoxide | $<$ 0.13 $\pm$ 0.012 | 0.977 $\pm$ 0.062 |
| | Activity (nmol of DDE formed/mg of protein) | |
| DDT | 4.8 $\pm$ 0.09 | 7.71 $\pm$ 0.72 |

Despite their high level of sequence identity, agGST1-5 and agGST1-6 show distinct substrate specificities. The different properties of these two enzymes are further exemplified by a comparison of the kinetic properties (Table 3). Comparison of the $K_m$ of both enzymes for glutathione and CDNB indicates that the two enzymes have similar affinities for these substrates. However, the value for $K_{cat}$ is 3.3-fold higher for agGST1-6 compared with agGST1-5 and hence agGST1-6 is a more efficient enzyme at turning over CDNB.

### Immunological cross-reactivity between recombinant GSTs and GSTs partly purified from *An. gambiae*

In a previous study, multiple peaks of GST activity have been partly purified from *An. gambiae*. Three peaks of GST activity (4, 5 and 6) were shown to have a role in resistance by comparison of the enzymes isolated from the susceptible G3 and from a DDT-resistant ZANDS strain of *An. gambiae* [5]. The ability of the recombinant proteins agGST1-5 and agGST1-6 to bind to *S*-hexylglutathione affinity columns and the high levels of activity of these enzymes with CDNB suggested that they belonged to the peak 4 cluster of *An. gambiae* enzymes. To verify this, the GSTs from *An. gambiae* were separated biochemically (Figure 4). The three broad peaks of activity (4, 5 and 6) were resolved by SDS/PAGE and blotted on nitrocellulose membranes, which were probed with antisera against the expressed proteins. The

**Table 3 Kinetic parameters for the recombinant *Anopheles gambiae* GSTs agGST1-5 and agGST1-6**

Values are the mean for two separate assays.

| | | | $K_m$ (mM) | | $K_{cat}/K_m$ ($mM^{-1} \cdot s^{-1}$) | |
|---|---|---|---|---|---|---|
| Enzyme | $V_{max}$ ($\mu$mol/min per mg) | $K_{cat}$ ($s^{-1}$) | Glutathione | CDNB | Glutathione | CDNB |
| agGST1-5 | 83.51 | 40.47 | 0.822 | 0.099 | 49 | 410 |
| agGST1-6 | 348.35 | 136.2 | 0.807 | 0.123 | 120 | 792 |

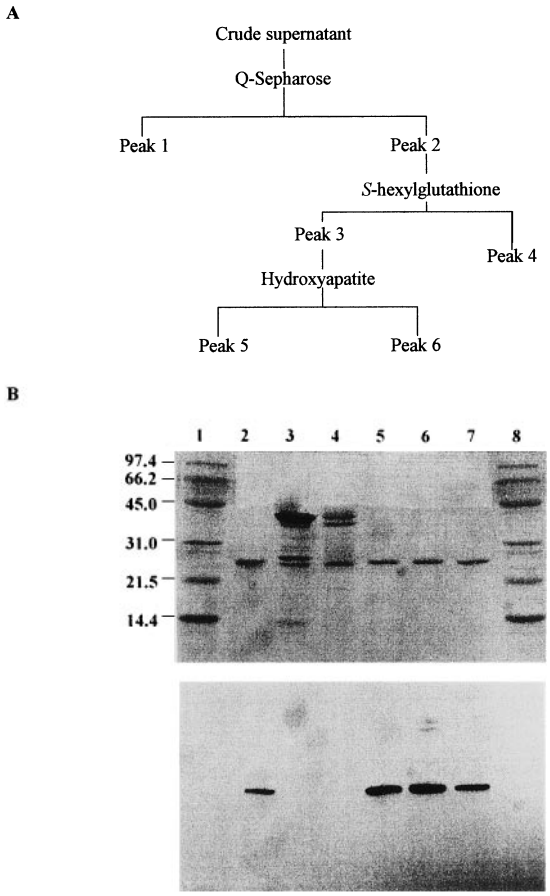


**Figure 4 Immunocrossreactivity of recombinant GSTs**

(**A**) GSTs from crude homogenates of *Anopheles gambiae* G3 larvae were separated into three peaks of activity (peaks 4, 5 and 6) as described previously [8]. In each case, bound enzymes are shown on the right-hand branch. (**B**) The partly purified and the recombinant *Anopheles gambiae* GSTs were resolved by SDS/PAGE. The top gel was stained with Coomassie Blue R-250, whereas the bottom gel was transferred to a nitrocellulose membrane and probed with antisera raised against agGST1-6. Lanes 1 and 6 in the top panel of (**B**) contain molecular mass markers, as indicated by the scale (in kDa) at the left. Lane 2 contains 200 ng of peak 4 fraction of GSTs from *An. gambiae* G3, lane 3 contains 400 ng of the peak 5 fraction, and lane 4 contains 400 ng of the peak 6 enzyme fraction. Lanes 5, 6 and 7 contain 100 ng of the recombinant GSTs, agGST1-5 (lane 7) and agGST1-6 (lanes 5 and 6).

results from probing with anti-agGST1-6 are shown in Figure 4. Similar results were obtained with antisera against agGST1-5. The results in Figure 4 confirm that agGST1-5 and agGST1-6 are members of the peak 4 subset of *An. gambiae* enzymes.

## DISCUSSION

Two novel cDNA species, *aggst1-5* and *aggst1-6*, encoding class I GSTs have been isolated from a DDT resistant strain of *An. gambiae*. These GSTs were isolated with a primer designed to mimic the partial cDNA *aggst1-1* from the G3 susceptible strain [6]. The two GST cDNA species from the resistant strain have a high level of sequence identity with *aggst1-1* and indeed it is possible that one of these cDNA species (*aggst1-6*) represents a full-length transcript from the same locus as *aggst1-1*.

*In situ* hybridization of *aggst1-1* with *An. gambiae* polytene chromosomes identified a single region of complementarity on chromosome 2R division 18A [6]. Because an *aggst1-1* probe would also detect *aggst1-5* and *aggst1-6* the genes from which these two cDNA species are derived must be situated within this region. A further class I GST gene, *aggst1-2*, and multiple GST pseudogenes have so far been identified within this section of the chromosome and we have evidence to suggest the existence of additional genes within this region ([6], and unpublished work). The family of class I GSTs from *An. gambiae* encodes a diverse group of enzymes because the GST encoded by *aggst1-2* is less than 50% similar to *aggst1-5* and *aggst1-6*. Hence the arrangement of *An. gambiae* class I GST genes seems to be similar to that of the GST D genes of *D. melanogaster* [20], in which eight divergent intronless genes are found within a 60 kb DNA segment. Other insect species have been shown to contain multiple class I GSTs and in the housefly there is also evidence that these genes may be tightly linked [21].

To determine the role of these GSTs in insecticide resistance, the enzymes were expressed in *E. coli* and the recombinant proteins were characterized. The GSTs were expressed with a 13-residue leader tag attached to the N-termini. The presence of this tag did not prevent the folding of the recombinant proteins and it enabled very high levels of protein expression to be achieved (100–200 mg/litre of culture). The specific activities of agGST1-5 and agGST1-6 with the general GST substrate, CDNB, were high (56.4 and 195.1 $\mu$mol/min per mg respectively) and comparable to the activities described for an *M. domestica* class I GST, mdGST-1 (130 $\mu$mol/min per mg) and *D. melanogaster* GST D1 (58.1 $\mu$mol/min per mg) [2,4]. However, the activities of the GSTs from different insect species with DCNB showed considerable variation with the activity being highest in the *An. gambiae* GSTs, agGST1-5 and agGST1-6, and undetectable in mdGST-1. Several studies have suggested that GST activity as measured with the substrate DCNB is correlated positively with resistance (see, for example, [2,8,22]). Therefore the very high levels of activity with DCNB exhibited by agGST1-5 and agGST1-6 indicate that these enzymes might be involved in insecticide resistance.

Comparison of the amino acid sequences of insect and mammalian GSTs shows that the insect class I GSTs are most closely related to the mammalian theta class ([23], and present study). We therefore examined the activity of the *An. gambiae* GSTs against *p*-nitrophenethyl bromide and 1,2-epoxy-3-(*p*-nitrophenoxy)propane, both of which are known substrates of mammalian theta GSTs [24]. Neither agGST1-5 nor agGST1-6 had significant activity with either of these substrates. Furthermore theta GSTs are characterized by low CDNB activities and an inability to bind to glutathione or *S*-hexylglutathione affinity columns, and neither of these properties is exhibited by the *An. gambiae* GSTs described here. Hence, although insect class I GSTs are more closely related to the theta class than to any other class of mammalian or insect GSTs, the two classes do not share similar physical or catalytic properties.

DDT dehydrochlorinase activity was detected for both agGST1-5 and agGST1-6 and this is the first demonstration that pure homodimeric GST preparations from *An. gambiae* can metabolize DDT. Recombinant GSTD1 from *D. melanogaster* is also able to metabolize DDT, and the presence of increased levels of GSTD1 in a DDT-resistant strain of *D. melanogaster* suggests that this enzyme is involved in resistance [4]. The closely related *D. melanogaster* GST, GSTD21 was unable to metabolize DDT, indicating that DDT dehydrochlorinase activity is not an inherent property of all insect class I GSTs. Comparison of the relative efficiencies of the DDT dehydrochlorination reactions of GSTs from *D. melanogaster* and *An. gambiae* is difficult to make because of the different nature of the detection assays. The method we used to measure rates of DDT dehydrochlorination was identical with that used earlier for other *Anopheline* mos-

quitoes. This was done specifically to allow us to link the studies on recombinant GSTs to the earlier biochemical studies on the same insect strains.

The GSTs from *An. gambiae* have been separated by sequential column chromatography into peaks of activity, each containing multiple enzymes. Three of these peaks (peaks 4, 5 and 6) have been shown to be involved in DDT resistance by comparing the enzymes isolated from a susceptible and a DDT-resistant strain [5]. To ascertain whether the recombinant GSTs described in this study were likely to be involved in resistance we probed the partly purified enzymes with antibodies against the recombinant agGST1-5 and agGST1-6. The results from this experiment identified these recombinant GSTs as belonging to the peak 4 group of *An. gambiae* enzymes.

The DDT dehydrochlorinase activity of partly purified peak 4 GSTs has been determined for enzymes from both the susceptible (G3) and resistant (ZANDS) strains. The peak 4 enzymes from G3 showed no detectable DDT dehydrochlorinase activity [5,8], whereas activities in the ZANDS strain ranged from 22 to 942 (when converted to nmol of DDE/mg of protein) [5]. The peak 4 recombinant enzymes, agGST1-5 and agGST1-6, from ZANDS had DDT dehydrochlorinase activities of 4.8 and 7.7 nmol of DDE/mg of protein respectively, which is lower than the specific activities of the partly purified resistant enzymes although higher than the values for partly purified G3 enzymes. This variation is probably due to the presence of multiple GSTs within each partly purified ZANDS fraction, some of which must presumably have higher specific activities with DDT. These ZANDS enzymes may be homo- or hetero-dimers of different GST subunits or be heterodimers containing agGST1-5 and/or agGST1-6. Alternatively it is possible that cofactors or post-translational modifications of the GSTs might enhance the native enzyme's activity towards DDT. However, this seems unlikely as the recombinant enzyme activities towards other GST substrates are comparable to those of the native enzymes, indicating that the recombinant proteins are correctly folded and fully active.

Although the peak 4 enzymes are elevated in resistant insects they account for only 6% of the total DDT metabolism in resistant individuals [5]. However, although agGST1-5 and agGST1-6 do not represent the major DDT-metabolizing enzymes in the ZANDS strain, the demonstration that they are able to metabolize DDT and their localization to a subset of enzymes that are elevated in resistant insects indicate that we have identified a GST class involved in resistance. Further characterization and expression work of the GSTs in this family will determine whether this is the only GST family conferring resistance in *An. gambiae*.

In summary, the current study has for the first time demonstrated that pure homodimeric preparations of *An. gambiae* GSTs are able to metabolize DDT. The GSTs described in this study are involved in insecticide resistance but they have lower specific activities towards DDT than some of the isoenzymes reported in earlier biochemical studies. AgGST1-5 and agGST1-6 are members of a large *An. gambiae* class I GST family [5]. We have shown earlier that the genes encoding these class I GSTs are arranged sequentially along the chromosome [6]. Further sequencing of this region will reveal the precise number of class I GST genes present in *An. gambiae*. The expression of newly identified GST genes can be rapidly and efficiently achieved with the procedures described here. In this way all the enzymes involved in DDT resistance can be identified.

We thank Dr. J. Hill for his helpful advice on the expression of GSTs *in vitro*, and Dr. E. Liddel for raising antibodies. This work was supported by The Wellcome Trust.

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Received 28 August 1996/5 December 1996; accepted 8 January 1997