The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling

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The resistance of mosquitoes to chemical insecticides is threatening vector control programmes worldwide. Cytochrome P450 monooxygenases (CYPs) are known to play a major role in insecticide resistance, allowing resistant insects to metabolize insecticides at a higher rate. Among them, members of the mosquito CYP6Z subfamily, like Aedes aegypti CYP6Z8 and its Anopheles gambiae orthologue CYP6Z2, have been frequently associated with pyrethroid resistance. However, their role in the pyrethroid degradation pathway remains unclear. In the present study, we created a genetically modified yeast strain overexpressing A. aegypti cytochrome P450 reductase and CYP6Z8, thereby producing the first mosquito P450–cytochrome P450-reductase (NADPH-cytochrome P450-reductase) complex in a yeast recombinant system. The results of the present study show that: (i) CYP6Z8 metabolizes PBAlc (3-phenoxybenzoic alcohol) and PBAld (3-phenoxybenzaldehyde), common pyrethroid metabolites produced by carboxylesterases, producing PBA (3-phenoxybenzoic acid); (ii) CYP6Z8 transcription is induced by PBAlc, PBAld and PBA; (iii) An. gambiae CYP6Z2 metabolizes PBAlc and PBAld in the same way; (iv) PBA is the major metabolite produced in vivo and is excreted without further modification; and (v) in silico modelling of substrate–enzyme interactions supports a similar role of other mosquito CYP6Zs in pyrethroid degradation. By playing a pivotal role in the degradation of pyrethroid insecticides, mosquito CYP6Zs thus represent good targets for mosquito-resistance management strategies.

Key words: cytochrome P450 monooxygenase, insecticide, metabolism, mosquito, pyrethroid, recombinant system, resistance.

INTRODUCTION

Mosquitoes transmit numerous parasites and viruses responsible for severe human diseases, such as malaria or dengue. These diseases represent an important burden in tropical and subtropical regions, predominantly affecting developing countries [1]. Indeed, half of the world’s population is at risk of malaria, whereas dengue represents a major threat in over 100 countries with more than 2.5 billion people at risk [1].

In the absence of efficient treatments or vaccines, vector control often represents the most effective means for limiting disease transmission [2]. Effective vector control largely relies on the use of insecticides targeting adults or larvae [3] and, because of their high efficiency and cheapness, chemical insecticides remain the first line of defence against mosquitoes when disease prevalence is high. Chemical insecticides used for mosquito control belong to various chemical families, from which pyrethroids are mainly used for impregnating bednets and sprayings.

However, resistance of mosquitoes to insecticides is threatening vector control programmes worldwide [4]. Resistance can be the consequence of a mutation of the protein targeted by the insecticide (target-site resistance), a lower penetration or a process of the insecticide (metabolic resistance) [5,6]. Detoxification enzymes such as cytochrome P450 monooxygenases (P450s or CYPs), GSTs and CCEs (carboxy/choline esterases) are known for their roles in insecticide metabolism in insects [7,8] and their overproduction has been frequently associated with resistance to chemical insecticides in mosquitoes [5,6].

P450s are haem-thiolate-containing enzymes present in almost all organisms and are involved in the metabolism of a wide range of molecules [9]. Most P450s involved in detoxification processes are expressed in the endoplasmic reticulum and catalyse the oxidation of xenobiotics or endogenous compounds in the presence of their obligatory electron donor CPR (NADPH-cytochrome P450-reductase) and sometimes Cyt b5 (cytochrome b5) [10]. Insect P450s are involved in metabolic resistance to various insecticides [7,9,11,12]. In mosquitoes, P450s are encoded by more than 100 CYP genes [13,14].

Following the development of transcriptomic tools in mosquitoes [14,15], several P450s overtranscribed in pyrethroid-resistant mosquitoes were identified [5,6]. Some of them have been validated as pyrethroid metabolizers such as Anopheles gambiae CYP6M2 and CYP6P3 [16,17], Anopheles funestus CYP6P9b [18] and Aedes aegypti CYP9J32 [19]. Among mosquito P450s, members of the CYP6Zs have been frequently associated with pyrethroid resistance [15,20–22]; however, this is not supported by functional studies as they do not appear to metabolize pyrethroids. Chiu et al. [23] showed that An. gambiae CYP6Z1 metabolized DDT, whereas pyrethroid metabolism was not mentioned. McLoughlin et al. [24] revealed that An. gambiae CYP6Z2 metabolized various substrates, but not pyrethroids. In Ae. aegypti, CYP6Z8 was found to be induced by insecticides and pollutants [22,25–28] and constitutively overtranscribed in...
pyrethroid-resistant populations [14,29]. This gene was also found preferentially transcribed in tissues classically involved in insecticide metabolism such as midgut and Malpighian tubules [27].

In this context, the present study aimed at characterizing Ae. aegypti CYP6Z8 substrate selectivity and its ability to metabolize insecticides. For this purpose, a yeast expression system allowing the co-expression of any mosquito microsomal P450 along with its associated CPR was developed. Using this system, we obtained a functional microsomal membrane complex of CYP6Z8 and Ae. aegypti CPR, which was used for in vitro metabolism assays. Our data indicate that although CYP6Z8 metabolizes various substrates, it is not capable of metabolizing most insecticides. However, our data reveal that CYP6Z8 and CYP6Z2, an anopheline orthologue of CYP6Z8, are likely to play a pivotal role in the clearance of pyrethroid insecticides via further catabolism of pyrethroid derivatives obtained by the action of carboxylesterases. This is significant from an operational vector control perspective as it is the first direct evidence that secondary metabolism of insecticides pyrethyroids by P450s is linked to resistance. In silico 3D-modelling of substrate–enzyme interactions supports the involvement of other mosquito CYP6Zs in this process. The findings of the present study are discussed in regard to metabolic resistance mechanisms and detoxification pathways in mosquitoes.

EXPERIMENTAL

Materials, strains and media

Enzymes were purchased from New England Biolabs, oligonucleotides were purchased from Eurogentec, chemicals were purchased from Sigma–Aldrich and culture media were purchased from Euromedex. DNA sequencing was performed by Cogenics (Genome Express). Ae. aegypti mosquitoes from the Bora-Bora strain were bred under standard insectary conditions as described in Pouparin et al. [22]. Yeast Saccharomyces cerevisiae strain W303-1B (MATα; leu2, his3, trp1, ade2-1, ura3, can6, cyr+), henceforth known as W(N), represents the wild-type strain. In silico 3D-modelling of substrate–enzyme interactions supports the involvement of other mosquito CYP6Zs in this process. The findings of the present study are discussed in regard to metabolic resistance mechanisms and detoxification pathways in mosquitoes.

Cloning of CYP6Z8 and Ae. aegypti CPR for expression in yeast

The full cDNA sequence encoding Ae. aegypti CYP6Z8 (VectorBase accession number AAEL009131-RA) was amplified by PCR using high-fidelity Taq Expand™ (Roche Applied Science) and the following primers: forward, 5′-AAGGATCC-AAAATGGCTGAAGTGAACCC-3′ (start codon underlined), and reverse, 5′-GCCGAATTCCTTACTGAGTAAAAGTGAACC-3′. The fragment was purified, cloned into the pET22b expression vector (Novagen) and resequenced. Protein expression was performed in Escherichia coli Rosetta 2 (DE3) cells as described previously [30]. Cyt b5 was then purified on a HiPrep DEAE–Sepharose FF column (GE Healthcare) and eluted by a step gradient of NaCl. The fraction containing the purified Cyt b5 was then desalted using a HiPrep Desalting column (GE Healthcare) and concentrated using an Amicon Ultra YM10 filter unit (Millipore). High-molecular-mass contaminants were eliminated by using a HiLoad Superdex 75 column. Protein was concentrated again and purity was checked by SDS/PAGE. The Cyt b5 concentration was evaluated as described previously [31].

Construction of CYP6Z8 and CPR expression plasmids

CYP6Z8 and CPR synthetic genes were subcloned into yeast replicative pyEpd60 and yeast integrative pyEpd110 plasmids (kindly provided by Dr P. Urban) as described in Pompon et al. [32]. Both vectors can be propagated in E. coli, hold an expression cassette under a glucose-repressed and galactose-inducible GAL10-CYC1 promoter and included an URA3 marker (uracil auxotrophy complementation). The plasmid pyEpd60 also contains an adenine ADE2 marker. Both plasmids were digested with BamHI and EcoRI and purified with the Gel Extraction Purification kit (Qiagen). CYP6Z8 and CPR synthetic genes were ligated to pyEpd60 and pyEpd110 respectively using T4 DNA ligase and the ligation product was used to transform DH5α chemically competent E. coli. Positive colonies were detected by PCR. Plasmids containing CYP6Z8 and CPR constructs were purified, double-digested and sequenced. Expression plasmids containing synthetic genes encoding CYP6Z8 and CPR were called pyEpd60-6Z8 and pyEpd110-CPR.

Stable integration of AeCPR into the yeast genome

The W(AeR) strain was obtained by stable integration of the AeCPR into the yeast genome. This genome integration was performed by disrupting the yeast CPR gene. Briefly, pyEpd110-CPR was first linearized by NotI and purified using a gel-extraction kit. Then, W(N) yeast were transformed as described previously using the lithium acetate/single-strand carrier DNA/PEG method [33] and spread on SGAI-agar plates. Positive colonies were streaked...
individually on SGA1-agar plates, allowed to grow, and then streaked again on N3-agar plates to ensure they can grow in the absence of glucose (rho + phenotype). Positive colonies were checked by PCR to ensure the integrity of the recombined genomic locus.

Expression of CYP6Z8 in the W(AeR) yeast strain

W(AeR) yeast were transformed as described previously [33] by pYeDP60-6Z8. Positive colonies were streaked on SGI-agar plates and N3-agar plates and then checked by PCR to ensure the presence of the CYP gene and pYeDP60-6Z8 plasmid. Afterwards, 30 ml of stationary-phase SGI culture were used to inoculate 500 ml of YPGE and the culture was allowed to grow at 30°C for 48 h. When a D600 of 4 was reached, 20 g/l galactose was added to the culture to induce CPR and CYP6Z8 expression. The culture was further allowed to grow at 30°C for 7 h with horizontal agitation at 140 rev./min before microsome extraction.

Preparation of yeast CYP6Z8 microsomes

The culture was first centrifuged at 2000 g for 18 min and the pellet washed with 1 ml of TEK [50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 100 mM KCl] per 0.5 g of cells. The pellet was resuspended in 10 ml of TES [50 mM Tris/HCl (pH 7.4), 1 mM EDTA, 0.6 M sorbitol, 1 mM PMSF and 1 mM DTT] and glass beads were added. Yeast cells were broken by vortex-mixing five times for 30 s at 4°C. TES (5 ml) was added to wash the beads and the supernatant was transferred into a clean tube. The beads were washed again twice, the supernatants pooled and centrifuged at 10000 g for 10 min. The supernatant was further ultracentrifuged at 27000 rev./min (rotor SW 28, Beckman Coulter) for 1 h. The pellet microsomes containing CYP6Z8 and CPR were resuspended in 500 μl of TEG [50 mM Tris/HCl (pH 7.4), 1 mM EDTA and 30% glycerol] per 500 ml of yeast culture, aliquoted and stored at −80°C until use [32].

Quantification of CPR and CYP6Z8 in yeast microsomes

Microsomal protein concentration was determined using the Bradford method [34]. Specific content in P450s was measured from the reduced carbon monoxide difference spectra according to Omura and Sato [35] on both W(AeR)-CYP6Z8 microsomes and W(AeR) microsomes. CPR activities were measured in W(AeR)-CYP6Z8, W(AeR) and W(N) microsomes by following the reduction of cytochrome c at 550 nm, in the presence of NADPH [36].

Activity of CYP6Z8 microsomes against standard P450 substrates

Four resorufin ethers (methoxyresorufin, ethoxyresorufin, pentoxyresorufin and benzoxylresorufin; Sigma–Aldrich) were tested as fluorogenic substrates against W(AeR)-CYP6Z8 microsomes. For each sample, 5 pmol of P450 in a total reaction volume of 200 μl was added to 0.1 M phosphate buffer (pH 7.4) containing 5 μM substrate, 0.1 mM NADPH and an electron regeneration system (3 mM glucose 6-phosphate and 0.4 unit of glucose-6-phosphate dehydrogenase), and incubated at 30°C for 60 min. The production of resorufin was monitored by measuring fluorescence at 537 nm excitation and 587 nm emission with a Varioskan Flash Multimode Reader (Thermo Fisher Scientific). A standard curve of resorufin (Sigma–Aldrich) was used to calculate product formation rate. 7-Ethoxycoumarin O-de-ethylation was monitored in a similar manner. The reaction mix was identical as above, except that 8 pmol of P450 was used in a final volume of 100 μl. After 10, 30 and 60 min, the reaction was stopped by adding 100 μl of 50:50 (v/v) glycerol/ethanol buffer and the production of 7-OH (7-hydroxycoumarin) was quantified by measuring the fluorescence at 380 nm excitation and 460 nm in comparison with an 7-OH standard (Sigma–Aldrich). The effect of the presence of Cyt b5 on CYP6Z8 activity was assessed by supplementing the incubation reaction with 2.5–50 pmol of Cyt b5. For the determination of kinetic parameters, the substrate concentration ranged from 0 to 10 μM and the incubation time was set to 15 min.

In vitro metabolism assays with non-fluorescent substrates

In vitro incubations contained 10 μM substrate, 50 pmol of Ae. aegypti CYP6Z8 in W(aeR) microsomes or AgCYP6Z2 in E. coli membranes [24] when indicated, 5 mM MgCl₂, 0.1 mM NADPH and the electron regenerative system (see above) for a total reaction volume of 100 μl. Control experiments consisted of omitting the NADPH regeneration system. After incubation at 30°C, the reaction was stopped by adding 100 μl of acetonitrile, shaking and incubation for 20 min and then centrifugation for 5 min at 16000 g to pellet microsomal proteins. The supernatant was then transferred into ultraclean glass vials and analysed by reverse-phase HPLC. The effect of the presence of Cyt b5 on CYP6Z8 activity was assessed by supplementing incubation mixtures with 80 pmol (8 equivalents) of purified Ae. aegypti Cyt b5.

Insecticide metabolism was monitored by reverse-phase HPLC on an Agilent 1260 HPLC system equipped with a Nucleodur C₁₈ Polartec 250 mm × 4.6 mm 3 μm column and a multi-wavelength photodiode array. Insecticides and potential metabolites were eluted with a gradient from 20 to 100% acetonitrile in water containing 0.1% TFA (trifluoroacetic acid) for 20 min, followed by a plateau at 100% for 4 min. For imidacloprid, a gradient from 10 to 50% acetonitrile in 20 min was used. Elution times of each compound and wavelengths used for their detection are shown in Table 1.

MS identification of PBAlc (3-phenoxybenzoic alcohol) metabolites

The LC–MS/MS analysis was performed on an Agilent 1100 HPLC coupled to a Bruker Esquire 3000 + Ion Trap mass spectrometer (Bruker Daltonics) in a positive mode (ESI +) under the following conditions: nebulizer gas 11 p.s.i. (N₂), drying gas 8 l/min, drying temperature 350°C, HV capillary 2000 V, HV End Plate Offset −500 V, capillary exit 103 V and skimmer 40 V.
Table 1 Metabolism of various xenobiotics by CYP6Z8

Turnover is measured as pmol of substrate depleted/min per pmol of P450. ND, not detected.

<table>
<thead>
<tr>
<th>Substrate Type</th>
<th>Turnover</th>
<th>Elution time (min); (wavelength, nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAlc Pyrethroid metabolite</td>
<td>1.578 ± 0.359</td>
<td>16.1 (232)</td>
</tr>
<tr>
<td>α-Naphtoflavone (αNF) Flavone</td>
<td>0.561 ± 0.022</td>
<td>19.1 (280)</td>
</tr>
<tr>
<td>Resveratrol Stilbene</td>
<td>0.463 ± 0.156</td>
<td>11.6 (300)</td>
</tr>
<tr>
<td>PBA Pyrethroid metabolite</td>
<td>0.425 ± 0.004</td>
<td>14.0 (232)</td>
</tr>
<tr>
<td>Diethylstilbestrol Stilbene</td>
<td>0.234 ± 0.072</td>
<td>18.3 (254)</td>
</tr>
<tr>
<td>Pyriproxifen Insecticide (growth regulator)</td>
<td>0.026 ± 0.001</td>
<td>18.7 (232)</td>
</tr>
<tr>
<td>Permethrin Insecticide (pyrethroid)</td>
<td>ND</td>
<td>22.0 and 22.4 (232)</td>
</tr>
<tr>
<td>Deltamethrin Insecticide (pyrethroid)</td>
<td>ND</td>
<td>21.4 (232)</td>
</tr>
<tr>
<td>PBA Pyrethroid metabolite</td>
<td>ND</td>
<td>15.9 (232)</td>
</tr>
<tr>
<td>DDT Insecticide (organochlorine)</td>
<td>ND</td>
<td>22.1 (232)</td>
</tr>
<tr>
<td>Temephos Insecticide (organophosphate)</td>
<td>ND</td>
<td>19.4 (232)</td>
</tr>
<tr>
<td>Diflubenzuron Insecticide (chitin synthesis inhibitor)</td>
<td>ND</td>
<td>16.7 (232)</td>
</tr>
<tr>
<td>Imidacloprid Insecticide (neonicotinoid)</td>
<td>ND</td>
<td>13.2 (270)</td>
</tr>
<tr>
<td>Propoxur Insecticide (carbamate)</td>
<td>ND</td>
<td>12.9 (232)</td>
</tr>
</tbody>
</table>

Induction of CYP6Z8 by hydrolysed pyrethroid metabolites

The induction of CYP6Z8 by PBAI, PBAI_d (3-phenoxybenzaldehyde) and PBA (3-phenoxybenzoic acid) was investigated by exposing third stage larvae for 24 h to 250 and 2500 nM of each compound and measuring the CYP6Z8 transcription level by reverse transcription followed by quantitative real-time PCR (RT-qPCR). Experimental procedures used for RNA extractions, reverse transcription, real-time PCR and primers used for qPCR are described in Poupardin et al. [27]. Data analysis was performed according to the ΔΔCT method taking into account PCR efficiency [37] and using the...
housekeeping gene encoding the ribosomal protein L8 (**AeRPL8**, GenBank accession no DQ440262). Three independent biological replicates using different egg batches were performed and results were expressed as the mean transcription ratio relative to controls (unexposed larvae). Transcription data were computed by using a Mann–Whitney test on transcription ratios (*P* < 0.05). Genes were considered significantly overtranscribed compared with controls when the mean transcription ratio was superior to 1.5 and the Mann–Whitney *P* value was < 0.05.

**In silico substrate docking 3D modelling**

The molecular models of CYP6Z8 and other CYP6Zs were created based on the crystal structure of CYP3A4 ([38]; PDB code 1TQN), currently the most homologous protein with a known structure, with 28% identity. Docking studies were carried out using GOLD v3.1 with the ChemScore scoring function ([39] and an active site radius of 20 Å (1 Å = 0.1 nm). Ligand structures were obtained from ChemIDPlus (http://chem.sis.nlm.nih.gov/chemidplus/). In total 50 binding modes were obtained for deltamethrin, PBAlc, PBAlD and PBA. Figures were prepared using PyMOL (http://www.pymol.org).

**RESULTS**

**CYP6Z8 sequence analysis**

*Ae. aegypti CYP6Z8* was cloned from cDNA (Bora-Bora strain) and fully sequenced (**CYP6Z8v1**, GenBank accession number JQ970488). Sequence analysis revealed 49 nucleotide variations compared with the genome sequence (gene AAEL009131, Liverpool strain, AaegL1.2 genset) (Supplementary Figure S1 at http://www.biochemj.org/bj/455/bj4550075add.htm). Of these, 13 were non-synonymous, leading to 97.14% identity with AAEL009131-PA (Supplementary Figure S2 at http://www.biochemj.org/bj/455/bj4550075add.htm). Only one non-synonymous variation was located within SRS (substrate recognition site) regions. This variation (C855A), leading to the replacement of a phenylalanine by a leucine at position 285 is not likely to affect substrate-binding properties. Indeed, structural models showed that this amino acid does not project into the active site and does not affect its dimensions. In addition, this variation was not predicted to affect the I helix position or substrate access channels (results not shown).

**Creating a yeast strain overexpressing mosquito CPR**

The W(N) yeast strain was submitted to homologous recombination with the integrative plasmid pYeDP110-CPR to obtain the W(AeR) strain which overproduces AeCPR in the presence of galactose (GAL promoter). PCR with specific primers confirmed the stable replacement of the yeast CPR gene by the insert containing the GAL promoter and AeCPR gene. Microsomes extracted from galactose-induced W(AeR) cultures showed a significant overexpression of CPR activity compared with the W(N) strain (164 ± 49 nmol of reduced cytochrome c/min per mg of protein compared with 32 ± 13 nmol/min per mg of protein).

**CYP6Z8 activity against standard P450 fluorescent substrates**

W(AeR) microsomes expressing only AeCPR did not show any significant activity against any fluorescence substrate, confirming the very low expression of endogenous yeast P450s under these conditions. W(AeR)-CYP6Z8 microsomes were able to metabolize fluorescent substrates at different rates (Table 2 and Figure 1C). Determining apparent kinetic parameters, *Km* and *Vmax*, for each fluorescent substrate showed that CYP6Z8 metabolized preferentially ethoxyresorufin and benzoyloxyresorufin (*kcat/Km* ratios of 0.49 and 0.74 respectively), whereas pentoxyresorufin and 7-ethoxycoumarin were metabolized at much lower rates (*kcat/Km* ratios of 0.0094 and 0.17 respectively). Apparent kinetic parameters for methoxyresorufin could not be determined due to a non-Michaelian behaviour of this substrate. Although Cyt b5 integration into W(AeR)-CYP6Z8 microsomes and its interaction with the P450 were observed (results not shown),
supplementing the reaction with recombinant Cyt b5 did not lead to a significant increase in activity for any of the substrates tested.

### CYP6Z8 activity against insecticides and other xenobiotics

Metabolism of insecticides from various chemical classes was assayed with W(AeR)-CYP6Z8 microsomes in the presence of absence of NADPH and the NADPH regenerating system. The degradation of the substrate and appearance of metabolites were monitored by reverse-phase HPLC (Table 1). The insecticides permethrin, deltamethrin, DDT, temephos, diflubenzuron, imidacloprid and propoxur were not significantly metabolized by CYP6Z8. However, CYP6Z8 metabolized different natural and synthetic xenobiotics such as α-naphthoflavone, resveratrol, diethylstilbestrol and to a lesser extent the insecticide pyriproxyfen. As for fluorescent substrates, the presence of Cyt b5 did not affect CYP6Z8 substrate specificity and turnover (results not shown).

As pyrethroid catabolism may involve carboxylesterase-mediated hydrolysis as a first step, the conversion of intermediate metabolites, namely PBAlc and PBAld was also examined. When PBAlc was used as a substrate (Figure 2A), no significant metabolism occurred in the absence of NADPH and the NADPH regenerating system. In the presence of NADPH, PBAlc was metabolized by CYP6Z8 at a rate of 0.42 pmol/min per pmol of P450. PBAlc turnover was not significantly affected by the presence of Cyt b5 (results not shown). Several metabolites were produced by CYP6Z8 including PBA [R, retention time] 15.8 min, PBAld (R, 16 min) and two more hydrophilic metabolites, M1 and M2 (R, 11.1 min and 12.6 min respectively). PBA accumulated gradually and was the major metabolite after 60 min incubation, followed by the M1 metabolite. PBAld never accumulated to a large extent; its proportion increased progressively over 15 min before decreasing for longer incubation times, suggesting the further conversion of this metabolite. Finally, a minor M2 metabolite gradually appeared during incubation. Kinetic parameters of PBAlc metabolism were K_m = 15.1 ± 2.9 μM, V_max = 33.4 ± 1.5 pmol/min, k_cat = 0.66 ± 0.03 min⁻¹ and k_cat/K_m = 0.046.

When PBAld was used as a substrate (Figure 2B), a significant conversion into PBA occurred in the absence of CYP6Z8, suggesting that this reduction is CYP-independent. Conversion into PBA strongly increased in the presence of CYP6Z8 and NADPH (1.98 pmol/min per pmol of P450), confirming that CYP6Z8 also metabolizes PBAld. PBAld remained a significant metabolite over time, indicating equilibrium between oxidation and reduction reactions. As for PBAlc, a minor production of M1 and M2 metabolites was noticed and PBAld turnover was not significantly affected by the presence of Cyt b5 (results not shown). Finally, no PBA metabolism occurred in the presence of NADPH, indicating that this compound is not further metabolized by CYP6Z8.

The ability of An. gambiae CYP6Z2, an anopheline orthologue of CYP6Z8, to metabolize PBAlc and PBAld was also investigated. These experiments performed with E. coli recombinant CYP6Z2 [24] under identical conditions demonstrated the ability of CYP6Z2 to metabolize both PBAlc and PBAld.

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**Table 2** CYP6Z8 specific activity against standard fluorescent P450 substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (μM)</th>
<th>V_max (nM/min)</th>
<th>k_cat (min⁻¹)</th>
<th>k_cat/K_m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoxysresorufin</td>
<td>0.13 ± 0.02</td>
<td>2.41 ± 0.05</td>
<td>0.097 ± 0.002</td>
<td>0.74</td>
</tr>
<tr>
<td>Ethoxysresorufin</td>
<td>2.39 ± 0.28</td>
<td>30 ± 1</td>
<td>1.19 ± 0.05</td>
<td>0.49</td>
</tr>
<tr>
<td>7-Ethoxycoumarin</td>
<td>5.90 ± 0.90</td>
<td>49 ± 2</td>
<td>0.98 ± 0.04</td>
<td>0.17</td>
</tr>
<tr>
<td>Pentoxysresorufin</td>
<td>13.96 ± 3.14</td>
<td>3.29 ± 0.02</td>
<td>0.13 ± 0.01</td>
<td>0.0094</td>
</tr>
<tr>
<td>Methylxysresorufin</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**ND**, not determined because of non Michaelis–Menten behaviour.
producing PBA together with minor M1 and M2 metabolites in the same way as CYP6Z8 (Supplementary Figure S3 at http://www.biochemj.org/bj/455/bj4550075add.htm).

Identification of metabolites

Positive-mode electrospray LC–MS detected PBAlc, PBAld, PBA, M1 and M2 at 183, 199, 215, 200 and 228 m/z respectively. PBAlc was detected 18 units below its theoretical mass (201 m/z), indicating its dehydration during LC–MS (Supplementary Table S1 at http://www.biochemj.org/bj/455/bj4550075add.htm). MS analyses did not allow the full identification of M1 and M2 metabolites. However, M1 and M2 metabolites did not appear to result from the direct or sequential hydroxylation of PBAlc or PBAld as no +16 m/z were observed. In addition, LC–MS m/z obtained for M1 and M2 did not match with any expected metabolite structure.

MS/MS analyses by positive-mode electrospray fragmentation detected various fragmentation ions associated with each compound (Supplementary Table S2 at http://www.biochemj.org/bj/455/bj4550075add.htm). The fragment shared by PBAld and PBA (171 m/z) is likely to correspond to diphenylether. This fragment could not be identified from M1 or M2, suggesting that these two metabolites rather originate from PBAlc. In addition, among all fragments, one was shared between PBAlc and M1 (155 m/z), suggesting a common backbone of these two compounds. This fragment was not shared with M2. Moreover, replacing H2O/0.1% TFA (pH 2.0) by 10 mM Tris buffer (pH 7.5) for HPLC analysis resulted in the shift of PBA and PBAlc peaks (results not shown), whereas PBAlc, M1 and M2 peaks were not affected (no change in signal intensity and Rf), suggesting that M1 and M2 do not possess any pH-dependent function, unlike PBAld or PBA.

In silico 3D modelling of CYP6Z–substrate interactions

In order to better understand why pyrethroids and their metabolites were metabolized or not by CYP6Zs, 3D models were built and the docking modes of PBAlc, PBAld, PBA and deltamethrin were predicted in silico. In CYP6Z8 and CYP6Z2, PBAld and PBAlc bound in two alternative modes that allow metabolism either on the phenyl or benzyl rings (Figure 3), consistent with experimental findings showing two metabolites. Given the phenyl ring structure of PBAld and PBAlc, interactions with aromatic side-chain amino acids are of potential interest in substrate binding and metabolism. A cluster of aromatic side chains are predicted in the active site of CYP6Z8, Phe102, Phe110, Phe115, Tyr208 and Phe211, which project into the active site (Figure 3). Residues that project into the active site are labelled. Superscript text refers to amino acids belonging to SRS1 (1), FG loop (FG) and SRS5 (5) regions.

In vivo assays and regulation of CYP6Z8 expression by pyrethroid metabolites

Incubating alive Ae. aegypti third instar larvae with PBAlc for 24 h revealed a concomitant decrease in PBAlc in the growing medium and an increase in PBA together with other minor metabolites, suggesting that the PBA produced is directly excreted by larvae without further modification (Figure 5).

In order to assess whether CYP6Z8 transcription is regulated by its substrates and/or products, larvae were exposed for 24 h to various concentrations of PBAlc, PBAld and PBA. The transcription levels of CYP6Z8 compared with unexposed larvae were then compared using qPCR (Supplementary Figure S4 at http://www.biochemj.org/bj/455/bj4550075add.htm). CYP6Z8 transcription was significantly induced by all compounds (2.0–3.4-fold) with a moderate dose-dependent effect.

DISCUSSION

The present study aimed at co-expressing Ae. aegypti CYP6Z8 and AeCPR in an heterologous system, characterizing its activity towards known P450 substrates and investigating its role in the metabolism of insecticides.

CYP6Z8 sequence analysis revealed several variations in the Bora-Bora strain compared with the genome sequence (Liverpool strain, VectorBase AeagL1.2 genset). In total 49 nucleotide variations, including 13 variations leading to amino acid changes, were found, confirming the high polymorphism of mosquito CYPs. These non-synonymous variations did not occur in the conserved P450 signature motifs [12]. Only one amino acid change occurred in an SRS at a position not strictly conserved among CYP6Zs. As confirmed by 3D modelling, this replacement (F285L) occurring in the oxygen-binding motif (SRS4) is likely to affect CYP6Z8 substrate specificity, as physicochemical properties of these two amino acids are similar (Figure 4).

The yeast P450 expression system developed by Pompon et al. [32] was chosen to create a novel genetically modified yeast strain allowing the co-expression of any mosquito P450 together with
An. gambiae P450. This was recently confirmed by the successful expression of any microsomal mosquito CPR by mosquito CPR under a galactose-inducible promoter. The results of the present study show that replacing yeast CPR by mosquito CPR under a galactose-inducible promoter enables the W(AeR) strain to overexpress mosquito CPR at a high level. Transforming the W(AeR) strain with an expression plasmid carrying the CYP6Z8 gene under the same promoter allowed us to produce a functional mosquito CPR–P450 membrane system. As CPR is highly conserved in mosquitoes, this new tool should produce a functional mosquito CPR–P450 membrane system. As CPR is highly conserved in mosquitoes, this new tool should thereby provide a useful alternative method with the advantage of having euakaryotic cellular and redox machinery plus organelle structure appropriate for the translation of euakaryotic P450s.

The results of the present study clearly indicate that our recombinant CYP6Z8 is functional and able to metabolize various standard P450 substrates. Kinetic data suggest that benzylxoyresorufin is preferred, followed by ethoxyresorufin. Supplementing reactions with purified Ae. aegypti Cyt b5 did not affect CYP6Z8 substrate specificity and activity. Whether CYP6Z8 does not strongly interact with Cyt b5 or the co-expression of AeCPR and CYP6Z8 produces such a high P450/CPR ratio making the presence of an extra electron donor, such as Cyt b5, not necessary requires further investigation [43].

Then, the ability of CYP6Z8 to metabolize various xenobiotics, including insecticides, was investigated. As for An. gambiae CYP6Z2, CYP6Z8 did not metabolize permethrin and deltamethrin [23,24]. In contrast with CYP6Z1, CYP6Z8 was not capable of metabolizing DDT either. However, CYP6Z8 metabolized α-naphthoflavone and the stilbene resveratrol, as did CYP6Z2 [24].

In mammals it has been demonstrated that pyrethroids can be hydrolysed by carboxylesterases leading to the production of PBAle and PBAld and that these metabolites can be further processed by P450s into PBA [44–46]. Recently, in vitro metabolism assays with microsomes extracted from Ae. aegypti larvae suggested that this detoxification pathway occurs in mosquitoes [47]. Because CYP6Zs are frequently overtranscribed in mosquito populations resistant and/or exposed to pyrethroids, the ability of CYP6Z8 to metabolize common pyrethroid metabolites was investigated further. Our results clearly demonstrate that CYP6Z8 is capable of metabolizing PBAle into PBA, with PBAld being a transitory metabolite (Figure 6). Similarly, we showed that its An. gambiae orthologue CYP6Z2 can also metabolize PBAle in the same manner, supporting the functional orthology of these two mosquito P450s. To our knowledge, CYP6Z8 and CYP6Z2 are the first insect P450s shown to metabolize pyrethroid metabolites generated by carboxylesterase hydrolysis.

Two minor more hydrophilic metabolites (M1 and M2) were also detected. Examination of M1 and M2 metabolites by LC–MS and MS/MS did not allow the absolute identification of their chemical structure. The behaviour of PBAle in positive-mode electrospray was not trivial as the parental ions (183 m/z) did not correspond to the theoretical mass (201 m/z), but suggested its dehydration. Despite this, no direct additions of alcohol function were detected. Examination of M1 and M2 metabolites by LC–MS and MS/MS did not allow the absolute identification of their chemical structure. The behaviour of PBAle in positive-mode electrospray was not trivial as the parental ions (183 m/z) did not correspond to the theoretical mass (201 m/z), but suggested its dehydration. Despite this, no direct additions of alcohol function was detected. The results of the

![Figure 4 Molecular models of CYP6Zs](image)

Top panel: comparison of deltamethrin, PBAle and PBAld docking in AeCYP6Z8 and AgCYP6Z2 (highest-ranked poses). Bottom panel: alignment of AeCYP6Z8 (green), AeCYP6Z2 (blue), AeCYP6Z4 (yellow), AeCYP6Z3 (pink), AeCYP6Z1 (dark blue) and AgCYP6Z2 (orange). The table shows a comparison of residues projecting into the active site. Residues are labelled according to the position on CYP6Z8. Ae, Ae. aegypti; Ag, An. gambiae.

![Figure 5 In vivo metabolism of PBAle](image)

HPLC chromatograms showing excreted metabolites after exposing Ae. aegypti larvae alive or dead for 24 h to 2 mg/l PBAle. PBAle and main metabolites are indicated by arrows.
present study suggest that the attack of pyrethroid metabolites generated by esterase hydrolysis by CYP6Zs is different.

Because PBA is not metabolized by CYP6Z8, M1 and M2 can only represent metabolites of PBAlc or PBAld. Common fragmentation ions were found between M1 and PBAlc, suggesting a similar backbone. In addition, M1 and M2 HPLC $R_t$ were not affected by pH variations as opposed to PBAld and PBA, confirming that they do not carry acid or aldehyde functions. Overall, these results support the hypothesis of M1 originating from PBAlc and M2 being a secondary metabolite of M1 (Figure 6). Finally, in vivo experiments revealed that PBA is the major metabolite produced and is excreted from mosquitoes without further modification. In contrast, M1 and M2 metabolites were not excreted from mosquitoes. Instead, two more polar metabolites (M1* and M2* in Figure 5) separated by a comparable Δ$R_t$ were observed, suggesting that M1 and M2 are further processed before being excreted.

In silico 3D modelling of CYP6Z–substrate interactions confirmed our experimental findings on CYP6Z8 and CYP6Z2 with good binding scores and limited clashes of pyrethroid metabolites in the active site. Comparing 3D models between various Aedes and Anopheles CYP6Zs supports the capacity of other mosquito CYP6Zs to metabolize these and produce similar metabolites (Figure 4). The inability of CYP6Z8 and CYP6Z2 to metabolize deltamethrin was supported by 3D models showing good binding scores but high clashes between active-site residues and substrate or within substrate itself. Bulky residues in the FG loop and SR51 and SR55 of mosquito CYP6Zs are probably preventing the metabolism of large pyrethroids. Indeed, while known pyrethroid metabolizers such as CYP6M2, CYP6B8 and CYP3A1 may require aromatic residues in the active site at position Phe115 to π-stack with the phenyl ring, and/or Phe102 to bind with the benzyl ring [17,49,50], the extensive network of aromatic residues in the CYP6Z subfamily may restrict the binding of the large pyrethroids, but stabilize the binding of smaller compounds such as PBAlc or PBAld.

Overall, the results of the present study lift the veil on the pivotal role of CYP6Z8 and CYP6Z2 in pyrethroid biodegradation in mosquitoes and clarify why they have frequently been associated with resistance [15,21,22,29,51], whereas their capacity to metabolize pyrethroid insecticides could not be validated [23,24]. This is the first direct evidence that secondary metabolism of insecticide pyrethroids by P450s is linked to resistance. Our results strongly support the role of these P450s following the action of carboxylesterases in order to clear mosquito body from these metabolites. Although hydrolysed pyrethroid metabolites such as PBAlc and PBAld are far less toxic than intact pyrethroids, their accumulation is likely to be detrimental to mosquitoes. Therefore the role of CYP6Zs in the mosquito response to pyrethroids is not negligible and the over-transcription of these genes in natural populations should be considered as supporting evidence of metabolic resistance.

Genes encoding carboxylesterases have been frequently found to be overtranscribed in pyrethroid-resistant populations and their role in pyrethroid biodegradation has been established in vitro [47]. However, to our knowledge, no particular mosquito carboxylesterase has yet been validated as a pyrethroid metabolizer. A better understanding of insecticide degradation pathways in mosquitoes will allow for the pinpointing of action points to develop new strategies to overcome resistance mechanisms. In this frame, estimating the relative importance of mosquito detoxification enzymes in resistance is also important, but not trivial. Any enzyme involved in the insecticide degradation pathway may have a different importance in the resistant phenotype depending on its expression profile, the step catalysed, its substrate specificity and turnover rate, and the toxicity and lipophilicity of the metabolites produced. This certainly represents the next research challenge for understanding how mosquitoes adjust their metabolism to resist insecticides and better manage these resistance mechanisms.

AUTHOR CONTRIBUTION
Alexia Chandor-Proust contributed to conceiving the study, performed experiments, analysed results and wrote the paper. Jaclyn Bibby and Mark Paine performed the structural modelling work and helped draft the paper. Myriam Régent-Kloeckner performed qPCR experiments and contributed to in vivo experiments. Jessica Roux contributed to protein expression work. Emilie Guittard-Grilat performed CYP6Z cloning, Rodolphe Poupardin and Muhammad Asam Riaz helped conceive the study. Chantal Dauphin-Villemant contributed to cloning and in vitro experiments and helped draft the paper. Stéphane Reynaud contributed to the study design, data analysis and helped write the paper. Jean-Philippe David conceived and co-ordinated the study, analysed results and wrote the paper. All authors read and approved the final paper.

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Role of mosquito CYP6Zs in pyrethroid detoxification


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SUPPLEMENTARY ONLINE DATA

The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling

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Figure S1 Alignment of cloned CYP6Z8 cDNA compared with the genome sequence

Coding sequence cloned from Bora-Bora strain cDNA was compared with the cDNA sequence extracted from VectorBase (AaegL1.2 geneset, Liverpool strain). Differences are shaded.

Figure S2 Alignment of Ae. aegypti CYP6Z8 protein sequences

Differences between CYP6Z8 obtained from the genome sequence (AaegL1.2 geneset, Liverpool strain) and CYP6Z8 cloned from the Bora-Bora strain are shaded. The degree of sequence conservation is indicated by standard motifs. Substrate-binding site regions are indicated by boxes.

Figure S3 Analysis of PBAlc metabolism by An. gambiae CYP6Z2

HPLC chromatograms (off-set) showing the time course of PBAlc metabolism by AgCPR-CYP6Z2 membranes. The lowest off-set corresponds to the negative control (− NADPH) followed by reactions in the presence of NADPH stopped after 10 and 60 min. PBAlc, PBA and metabolite peaks are indicated.
Third-stage larvae were exposed for 24 h to three increasing doses of each compound. Transcription levels were measured by qPCR on pools of larvae from three independent replicates and expressed as means ± S.E.M. relative to controls (unexposed larvae). The significance of transcription ratios relative to controls were assessed by a Mann–Whitney test (n = 3). *P < 0.05.

Table S1 LC–MS analysis of PBAlc and PBAld metabolites
Masses of [M + H]+ ions were measured by HPLC coupled with MS (electrospray positive mode). Theoretical expected mass in this mode, dehydrated mass and observed mass are indicated. -, no dehydration is observed; ?, whether dehydration occurs or not is unknown.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Theoretical mass</th>
<th>Dehydration (−18)</th>
<th>Observed mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAlc</td>
<td>201</td>
<td>183</td>
<td>183</td>
</tr>
<tr>
<td>PBAld</td>
<td>199</td>
<td>−</td>
<td>199</td>
</tr>
<tr>
<td>PBA</td>
<td>215</td>
<td>−</td>
<td>215</td>
</tr>
<tr>
<td>M1</td>
<td>Unknown</td>
<td>?</td>
<td>200</td>
</tr>
<tr>
<td>M2</td>
<td>Unknown</td>
<td>?</td>
<td>228</td>
</tr>
</tbody>
</table>

Table S2 MS/MS analysis of PBAlc and PBAld metabolites
Fragmentation ions observed in MS/MS (electrospray positive mode) are shown for each compound. Parental ions ([M + H]+) are shown in bold. Common fragments are underlined.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Observed mass of fragmentation ions</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBAlc</td>
<td>183 165 155</td>
</tr>
<tr>
<td>PBAld</td>
<td>199 171 171</td>
</tr>
<tr>
<td>PBA</td>
<td>215 171 171</td>
</tr>
<tr>
<td>M1</td>
<td>228 211 193</td>
</tr>
<tr>
<td>M2</td>
<td>200 193 175</td>
</tr>
</tbody>
</table>

Table S3 Docking binding scores
Binding scores of the best ranked poses (kJ/mol) of PBAlc, PBAld, PBA and deltamethrin in members of the CYP6Z family. Ae, Ae. aegypti; Ag, An. gambiae.

<table>
<thead>
<tr>
<th>Family</th>
<th>PBAlc</th>
<th>PBAld</th>
<th>PBA</th>
<th>Deltamethrin</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgCYP6Z1</td>
<td>34.49</td>
<td>34.64</td>
<td>31.99</td>
<td>37.24</td>
</tr>
<tr>
<td>AgCYP6Z2</td>
<td>37.09</td>
<td>36.46</td>
<td>33.43</td>
<td>42.69</td>
</tr>
<tr>
<td>AgCYP6Z3</td>
<td>36.73</td>
<td>36.18</td>
<td>33.69</td>
<td>43.4</td>
</tr>
<tr>
<td>AgCYP6Z4</td>
<td>34.22</td>
<td>32.17</td>
<td>31.55</td>
<td>42.76</td>
</tr>
<tr>
<td>AeCYP6Z6</td>
<td>34.25</td>
<td>34.43</td>
<td>30.59</td>
<td>45.24</td>
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<tr>
<td>AeCYP6Z8_bora</td>
<td>37.63</td>
<td>35.71</td>
<td>32.28</td>
<td>42.93</td>
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<tr>
<td>AeCYP6Z8_liv</td>
<td>36.34</td>
<td>36.42</td>
<td>31.97</td>
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<tr>
<td>AeCYP6Z9</td>
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<td>30.6</td>
<td>28.68</td>
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