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Cytochrome P450 6M2 from the malaria vector *Anopheles gambiae* metabolizes pyrethroids: Sequential metabolism of deltamethrin revealed

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ABSTRACT

Resistance to pyrethroid insecticides in the malaria vector *Anopheles gambiae* is a major threat to malaria control programmes. Cytochome P450-mediated detoxification is an important resistance mechanism. CYP6M2 is over-expressed in wild populations of permethrin resistant *A. gambiae* but its role in detoxification is not clear. CYP6M2 was expressed in *Escherichia coli* and a structural model was produced to examine its role in pyrethroid metabolism. Both permethrin and deltamethrin were metabolized. Rates were enhanced by *A. gambiae* cytochrome b_5 with kinetic parameters of $K_M = 11 \pm 1$ μM and $k_{cat} = 6.1 \pm 0.4$ per min for permethrin (1:1 *cis–trans*) and $K_M = 2.0 \pm 0.3$ μM and $k_{cat} = 1.2 \pm 0.1$ per min for deltamethrin. Mass spectrometry and NMR analysis identified 4′-hydroxy deltamethrin and hydroxymethyl deltamethrin as major and minor deltamethrin metabolites respectively. Secondary breakdown products included cyano(3-hydroxyphenyl)methyl deltamethrate and deltamethric acid. CYP6M2 was most highly transcribed in the midgut and Malpighian tubules of adult *A. gambiae*, consistent with a role in detoxification. Our data indicates that CYP6M2 plays an important role in metabolic resistance to pyrethroids and thus an important target for the design of new tools to combat malaria.

1. Introduction

Malaria is a devastating global health problem that affects over 500 million people and causes over 1 million deaths each year. In sub-Saharan Africa the mosquito *Anopheles gambiae* is the principal vector of the human malaria parasite *Plasmodium falciparum*. Vector control, through insecticide treated bed nets (ITNs) or indoor residual spraying (IRS), currently offers the most effective means of preventing malaria transmission. However, these methods rely on the use of pyrethroid insecticides, such as permethrin or deltamethrin, which are the only class of insecticide approved for use on ITNs. Pyrethroid resistance in *A. gambiae* is spreading rapidly across Africa (Roll-Back-Malaria et al., 2005), thus threatening the efficiency of malaria control by insecticide-treated materials (Hargreaves et al., 2000; N'Guessan et al., 2007).

Resistance to pyrethroids in *A. gambiae* is generally attributed to either target site insensitivity, metabolic resistance by detoxification enzymes, or both (Hemingway and Ranson, 2000). The former is associated with single point mutations of the voltage-gated sodium channel and well characterised (Martinez-Torres et al., 1998; Ranson et al., 2000). In contrast, relatively little is known about the mechanisms of metabolic resistance, in particular those associated with cytochromes P450 (Li et al., 2007; Ranson et al., 2002): a diverse family of heme-containing enzymes present in most organisms that catalyze the monooxygenation of a broad range of xenobiotic and endogenous compounds. *A. gambiae* contains 111 P450 genes (Ranson et al., 2002; Ranson et al., 2004), thus pin-pointing the individual enzymes involved in pyrethroid

Abbreviations: b5, histidine-tagged *A. gambiae* cytochrome b_5; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; CYP, cytochrome P450; EDTA, ethylenediaminetetraacetic acid; G6PDH, glucose-6-phosphate dehydrogenase; IPTG, isopropyl β-D-1-thio-galactopyranoside; MES, 2-(N-morpholino)ethanesulfonic acid.

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metabolism is a challenging but crucial step in understanding the molecular mechanisms of insecticide resistance (Hemingway et al., 2006).

Numerous P450 genes have recently been shown to be transcriptionally upregulated in permethrin resistant populations of *A. gambiae*, potentially causing enhanced metabolic detoxification. These include; CYP6Z1 (Nikou et al., 2003), CYP6Z2 (McLaughlin et al, 2008), CYP325A3 (Awolola et al., 2008; David et al., 2005), CYP6M2 (Djouaka et al., 2008; Müller et al., 2007), and CYP6P3 (Djouaka et al., 2008; Müller et al., 2008). All except CYPM2 and CYP325A3 have been functionally expressed *in vitro*, but as yet only CYP6P3 is proven to metabolize pyrethroids (Müller et al., 2008).

Here we have focussed on CYP6M2 [VectorBase ID: AGAP008212] since it is consistently over-expressed in permethrin resistant populations of *A. gambiae* including: Odumassy and Dodowa in southern Ghana (Müller et al., 2007, 2008), Akron and Gdedjromede in south-eastern Benin (Djouaka et al., 2008), and Ojoo in south west Nigeria (Djouaka et al., 2008). Such a strong association with permethrin resistance suggests a key functional role in detoxification.

In this study, our aim was to determine whether CYP6M2 is capable of metabolizing pyrethroid insecticides and thereby involved in the resistance phenotype. Aided by structural modeling and transcriptional mapping studies we have investigated the mechanism of deltamethrin [(*S*)-α-cyano-3-phenoxybenzyl (1*R*,3*R*)-*cis*-2,2-dimethyl-3-(2,2-dibromovinyl)-cyclopropanecarboxylate] breakdown by CYP6M2, a P450 strongly associated with metabolic resistance in the major malaria vector *A. gambiae*.

2. Materials and methods

2.1. Reagents

Oligonucleotides and chemicals used in this research were obtained from Sigma–Aldrich unless otherwise indicated. Enzymes for DNA manipulation were supplied by New England Biolabs, insecticides 1:1 *cis–trans* permethrin and deltamethrin from ChemService, and HPLC solvents from Fisher Scientific.

2.2. Cloning CYP6M2 for expression in Escherichia coli

The cDNA sequence encoding CYP6M2 (GenBank ID: AgaP_AGAP008212) was isolated by RT-PCR using RNA purified from *A. gambiae* RSP (MRA-334) as described (Nikou et al., 2003). For P450 expression, CYP6M2 was fused to a bacterial ompA leader sequence and expressed using pCW-ori+ as previously described (McLaughlin et al., 2008; Pritchard et al., 1997). First, *CYP6M2* was amplified with an alanine-proline linker at the 5′ end and an *Eco*RI restriction site downstream of the stop codon using forward primer ECG18 (5′-GCACCAATGTTTAGCTTGTTGGATTTCAC-3′, codons for alanine–proline are underlined) and GAATTCTAAATCTTATCCACCTTCAACCAC-3′, *Eco*RI site underlined). Second, a gene fragment containing the coding sequence for the ompA signal peptide with downstream alanine-proline linker and the first 16 nucleotides of *CYP6M2* were amplified using forward primer ECG76 (5′-GGAGGTCATATGAAAAAGACAG-3′, *Nde*I site underlined), reverse primer ECG77 (5′-CCAACAAGCTAAACATTGGTGCGGCCTGCGCTACGGTAGCGAAAC-3′, the region corresponding to the reverse complement of alanine–proline codons and the start of *CYP6M2* is underlined) and pB13::ompA-CYP6Z2 (McLaughlin et al., 2008) as the PCR template. Third, the PCR products from the first and second steps were used as the templates in a fusion PCR reaction with primers ECG76 and ECG19 to generate the full-length *ompA*-AP-*CYP6M2* coding sequence. All three PCR steps used the hi-fidelity KOD DNA polymerase (Novagen) according to manufacturer's instructions. The final *ompA*-AP-*CYP6M2* product was ligated into a modified pCW-ori+ vector plasmid, pB13 (Pritchard et al., 1998), via *Nde*I and *Eco*RI sites to produce pB13::CYP6M2. DNA sequencing was performed to check for PCR errors. The predicted CYP6M2 peptide sequence was identical to that of the VectorBase sequence (VectorBase ID: AGAP008212-PA), except that an aspartate residue at position 286 is a glutamate in this study. This is a bona fide natural variant as the residue was present in two independent clones and observed in the genomic DNA sequence of field caught mosquitoes. It is unlikely to affect P450 activity as this is conservative change (glutamate and aspartate residues contain negatively charged side-chains).

2.3. Preparation of membranes with CYP6M2 and CPR

Competent *E. coli* DH5α cells were co-transformed with pB13::cyp6m2 and pACYC-AgCPR (McLaughlin et al, 2008) for co-expression of CYP6M2 and *A. gambiae* CPR. Expression, membrane isolation and determination of P450 content was carried out as previously described (McLaughlin et al., 2008; Müller et al., 2008). Cultures, generally 0.2 l, were incubated at 25 °C for P450 and CPR expression with cells harvested 22 h after induction with 1 mM IPTG. Membrane preparations were stored in aliquots at −70 °C (P450 was prone to degradation if freeze-thawed or stored at higher temperatures) and assayed for: total protein concentration (Bradford assay with BSA standards), P450 concentration (Omura and Sato, 1964), and CPR activity by monitoring cytochrome *c* reduction (Strobel and Dignam, 1978).

2.4. Preparation of His-tagged A. gambiae cytochrome b_5

The preparation of a histidine-tagged *A. gambiae* cytochrome b_5 (b5) followed the approach used by Holmans et al. (1994). The b5 gene was amplified from a previously cloned full-length template (Nikou et al., 2003, GenBank ID: AY183376) using KOD DNA polymerase and the following primers: 5′-TTTATTA**ATGCACCATCACCAT**ATGTCGGAAGTGAAAACGTACTC-3′ (*Ase*I site underlined and followed by the start codon and four histidine codons in bold), and 5′-TTTGAAT**TCA**CTGGGTGAAGTAGAACCGATAG-3′ (*Eco*RI site underlined, reverse complement of stop codon in bold). The PCR product was digested with *Ase*I and *Eco*RI, before ligation with pB13 that had been digested with *Nde*I (complementary cohesive end for *Ase*I) and *Eco*RI to create construct, pB13::4H-cyt-b5. The integrity of the construct was verified by DNA sequencing.

2.4.1. Purification of A. gambiae cytochrome b_5

E. coli DH5α transformed with pB13::4H-cyt-b5 was grown in 1 l of Terrific Broth with 50 μg/l ampicillin, and membranes expressing b5 prepared as described above. The resulting red-brown cell pellet was then processed according to the method outlined by Holmans et al. (1994) apart from a 50 °C incubation step following the cell lysis, as this degraded most of the functional *A. gambiae* b5. Solubilized b5 was stored in b5 buffer (20% (v/v) glycerol, 10 mM Tris–HCl at pH 8, 0.05% (w/v) CHAPS, 0.5 mM EDTA and 0.1 mM DL-dithiothreitol). The preparation was analysed for b5 concentration by reduced vs. oxidised spectroscopy (A424-A409, Ex. Coef. = 185 cm^{-1} mM^{-1}, Omura and Sato, 1964), total protein concentration (Bradford assay with BSA standards), and purity by polyacrylamide gel electrophoresis (Bis–Tris 4–12% gradient gel with MES running buffer; NuPAGE, Invitrogen).

2.5. Pyrethroid metabolism

The pyrethroids were dissolved in ethanol immediately before use and diluted to a working concentration in 20% ethanol such that