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Strategies for the construction of insect P450 fusion enzymes

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Abstract: Cytochrome P450 monooxygenases (P450s) are ubiquitous enzymes with a broad substrate spectrum. Insect P450s are known to catalyze reactions such as the detoxification of insecticides and the synthesis of hydrocarbons, which makes them useful for many industrial processes. Unfortunately, it is difficult to utilize P450s effectively because they must be paired with cytochrome P450 reductases (CPRs) to facilitate electron transfer from reduced nicotinamide adenine dinucleotide phosphate (NADPH). Furthermore, eukaryotic P450s and CPRs are membrane-anchored proteins, which means they are insoluble and therefore difficult to purify when expressed in their native state. Both challenges can be addressed by creating fusion proteins that combine the P450 and CPR functions while eliminating membrane anchors, allowing the production and purification of soluble multifunctional polypeptides suitable for industrial applications. Here we discuss several strategies for the construction of fusion enzymes combining insect P450 with CPRs.

Keywords: biotechnology; cytochrome P450; fusion proteins; insects; protein expression.

1 Introduction

The cytochrome P450 monooxygenases (P450s) are a ubiquitous superfamily of heme-thiolated monooxygenases that have been strongly conserved during evolution [1]. P450s fulfil diverse functions, including the metabolism of xenobiotics and drugs [2], steroid biosynthesis [3] and the assimilation of carbon sources for growth [4]. They were first discovered in 1958 and were isolated from mammalian liver microsomes in 1964 [5–7]. More than 1000 P450 families have been described and ~21,000 different P450s are known, with the number still growing [8]. A P450 nomenclature was introduced in 1987 comprising the prefix CYP followed by a family number, a subfamily letter and finally a number representing the specific enzyme, e.g. CYP6A1 [9]. All P450s form similar secondary and tertiary structures [10–12] which combine with a heme cofactor that forms the catalytic reaction center. Heme comprises a central iron atom bound to a protoporphyrin ring by four nitrogen ligands forming a planar structure. The iron atom is additionally coordinated by an evolutionarily conserved axial cysteine ligand (FXXGXXXCXG) located near the C-terminus of the P450 [1]. The iron atom is often associated with water as a sixth ligand. The heme cofactor is responsible for Soret band absorbance at 420 nm when it is coordinated with water. The absorbance signal shows a typical shift to 450 nm when carbon monoxide is bound at the opposite axial position, which is the basis of the superfamily name: pigment 450 [6].

1.1 Reactions catalyzed by cytochrome P450 monooxygenases

P450s cannot catalyze reactions on their own because they need to accept electrons from a cytochrome P450 reductase (CPR) redox partner, as shown in Figure 1. The reaction requires two electrons, derived from a nicotinamide cofactor [13]. Prosthetic groups, such as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) derived from riboflavin, serve as electron transfer centers. P450s activate molecular oxygen and catalyze unique one-step C–H bond oxidations by the insertion of one oxygen atom into the substrate while the other forms water [14, 15]. They can catalyze diverse reactions (Figure 2) and the nature of these reactions and the corresponding substrates does not appear to depend on the sequence of the P450 or its evolutionary proximity to other P450s [1]. Insects and mammals produce approximately 80 different P450s but in plants there is much greater diversity, e.g. there are 286 P450s in *Arabidopsis thaliana*. Plant P450s are designated as A-type or non-A-type. The former are specific to plants and fulfil functions such as herbicide metabolism and the synthesis of volatiles involved in plant–insect interactions, whereas the latter are more like the P450s in other

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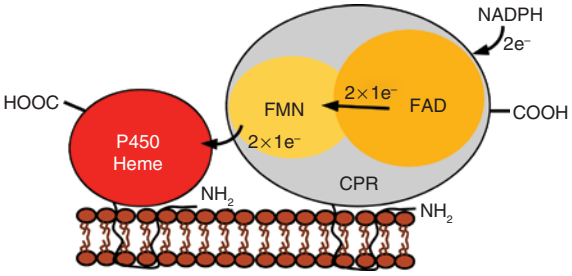

Figure 1: Schematic representation of the P450-redox partner interaction. P450, cytochrome P450 monooxygenase; CPR, cytochrome P450 reductase; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; NADPH, reduced nicotinamide adenine dinucleotide phosphate.

phyla and fulfil similar roles, e.g. sterol and fatty acid metabolism [17]. The most common reaction is monooxygenation, resulting in the hydroxylation or epoxidation of a carbon center (Figure 2A). Others include oxidation and dealkylation at a heteroatom, and complex reactions such as single electron reductions, desaturations and ring modifications alongside typical oxidations [18] (Figure 2B). These reactions are often regiospecific and stereospecific, and thus difficult to replicate by total chemical synthesis. The selectivity of P450s is one reason they are valued in the context of industrial processes.

1.2 Insect P450s and their potential applications

Insect P450s are structurally and functionally similar to their better-characterized mammalian counterparts. More than 100 enzymes have been identified from diverse insect species, and many are components of important metabolic pathways [19]. They facilitate growth, development, feeding [1] and the degradation of endogenous compounds and xenobiotics [20]. An important example of the role of insect P450s in development is provided by the so-called Halloween genes: *phantom* (*CYP306A1*), *disembodied* (*CYP302A1*), *shadow* (*CYP315A1*), and *shade* (*CYP314A1*). These are responsible for the four last hydroxylation steps that convert steroid precursors into ecdysteroids, with *CYP314A1* catalyzing the final conversion of ecdysterone to its more active derivative 20-hydroxyecdystone [21]. The importance of insect P450s in xenobiotic degradation is exemplified by their role in resistance to plant toxins and pesticides, which is often achieved by the overexpression of P450 genes targeting these compounds [1, 22]. Insect P450s can be microsomal or mitochondrial. Microsomal P450s depend on a CPR or

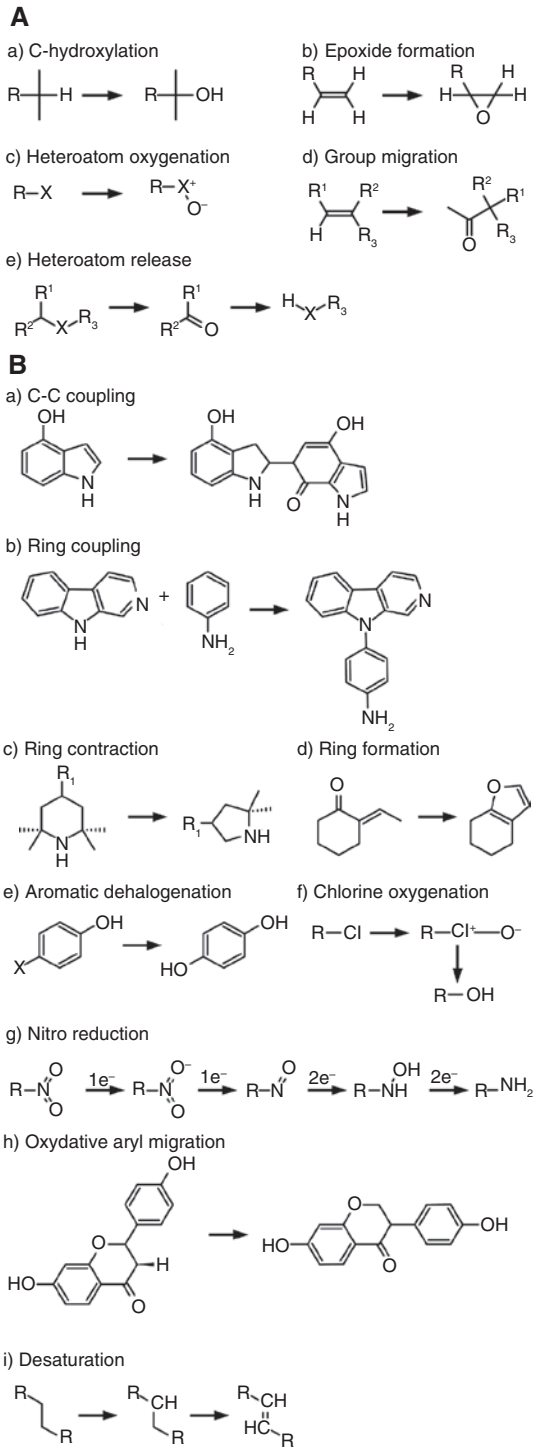

Figure 2: (A) Common and (B) uncommon reactions catalyzed by cytochrome P450 monooxygenases [16].

cytochrome b_5 (or both) as an electron donor, whereas mitochondrial P450s depend on an adrenodoxin-like ferredoxin coupled to adrenodoxin reductase. Six P450 families are known in insects, five of which are not found in other organisms (*CYP6*, *CYP9*, *CYP12*, *CYP18* and *CYP28*). The expression levels of P450s in insects can vary

across different life stages [23] and they are expressed in several tissues, such as the Malpighian tubules, fat body and midgut [24]. There is no clear phylogenetic distinction between the P450s involved in physiological homeostasis and those required for detoxification, suggesting that the function of P450s can change over evolutionary timescales [24].

The first purified insect CPR was prepared from the common housefly (*Musca domestica*) and was shown to be similar to mammalian CPRs [25]. The first purified insect P450 was the xenobiotic-degrading enzyme CYP6A1 also from the housefly, which was reconstituted in an *Escherichia coli* system allowing the identification of aldrin and heptachlor as substrates [26]. CYP6A1 has a spacious heme active site which is compatible with several substrate geometries and orientations [27]. Similarly, CYP6G1 from the fruit fly (*Drosophila melanogaster*) can be classed as a multi-pesticide degrading enzyme because it confers resistance towards several structurally unrelated compounds [28]. Insects are known to produce hydrocarbons that prevent desiccation and that also function as contact pheromones [29]. The underlying biosynthesis pathway involves the decarbonylation of aldehydes to form alkanes by CO_2 cleavage. The first P450 shown to catalyze this unusual reaction was *D. melanogaster* CYP4G1 [30].

The large number of different P450s produced by insect species combined with the diversity of insects offers a vast library of enzymes with a broad range of catalytic activities. These could be used to investigate the origin of pesticide resistance and to facilitate the development of new insecticides that inhibit P450s [24]. Furthermore, insect P450s could also be deployed as industrial enzymes for the production of fine chemicals and pharmaceuticals that cannot be produced economically by total chemical synthesis [31] or for the development of new processes based on bioelectrocatalysis [32]. Finally, they could be used for remediation purposes, such as the removal of pesticide residues and other xenobiotics from wastewater and the environment [33].

1.3 Challenges hindering the production of recombinant P450s

The functional analysis and exploitation of insect P450s is challenging because they are membrane-bound enzymes that require various electron donors [1]. The standard approach to determine the catalytic activity of P450s is heterologous expression and reconstitution [22], but the enzymes must be reconstituted in a system containing detergents and phospholipids for solubilization, and the correct CPR components and cofactors must be supplied [34, 35]. P450s do not tolerate solvents well, so many efforts to solubilize the enzymes render them inactive [31]. Because P450s have diverse substrates, the activity of a recombinant enzyme can only be established by screening a library of substrates, some of which are also only sparing soluble [31]. Finally, the industrial application of P450s is also hampered by the need for the expensive electron donor nicotinamide adenine dinucleotide phosphate (NADPH) [31, 36, 37]. Catalytically active insect P450s have been expressed in *E. coli* [38] and insect cell lines [30] but in all cases the yields were insufficient for industrial application.

1.4 Addressing the challenges by constructing P450 fusion enzymes

The challenges described above can potentially be overcome by constructing P450 fusion enzymes that incorporate the necessary CPR components and eliminate sections of the polypeptide that hinder expression and reconstitution, such as the membrane anchor. Nature has provided promising evidence to support this approach because several natural P450 fusion enzymes have already been discovered [39] as listed in Table 1. CYP102A1 and CYP11B2 have been studied in detail to develop strategies for the design of artificial fusion proteins and protein evolution methods, and these are discussed below.

1.4.1 CYP102A1 (BM3)

Bacillus megaterium CYP102A1 (also known as BM3) is a fatty acid hydroxylase which catalyzes reactions without the assistance of additional proteins. It is a natural fusion protein, in which the N-terminal catalytic P450 domain is covalently attached via a linker to the C-terminal redox domain, constituting an entire class II P450 system [46] typical of microsomal eukaryotic P450s [47]. As shown in Figure 3, the 66 kDa redox domain (*B. megaterium* reductase, BMR) binds FAD and FMN as prosthetic groups, making it functionally similar to eukaryotic CPRs [46]. Indeed, BMR shares ~33% sequence identity with mammalian hepatic CPR. The 55 kDa P450 domain shares ~25% sequence identity with fatty acid ω-hydroxylases of the CYP4 family [48].

Electron transfer from the BMR domain to the heme group influences the enzyme activity. The linker length is more important for enzyme activity than the amino acid composition because it determines the correct

Table 1: Examples of natural P450 fusion enzymes.

Name	Origin	Domains (N→C)	Reference
CYP102A1/BM3	*Bacillus megaterium*	P450-FMN-FAD	[40]
CYP11B2/RhF	*Rhodococcus* sp. strain NCIMB 9784	P450-FMN-Fe/S Red	[41]
CYP51fx	*Methylococcus capsulatus*	P450-Fdx	[42]
Xp1A	*Rhodococcus rhodochrous*	FMN-P450	[43]
CYP221A1	*Pseudomonas fluorescens*	Acyl CoS-DeH-P450	[44]
CYP5253A1	*Mimivirus*	P450-?	[45]
CYP55A1	*Fusarium oxysporum*	P450	[39]

The domain structure is shown from the N-terminus. P450, cytochrome P450 monooxygenase; FMN, flavin mononucleotide; FAD, flavin adenine dinucleotide; FMN-Fe/S Red, FMN-containing reductase with a [2Fe2S] ferredoxin-like center; Fdx, ferredoxin domain; Acyl CoS-DeH, P450-acyl-CoA dehydrogenase; ?, protein of unknown function containing several putative post-translational modification sites.

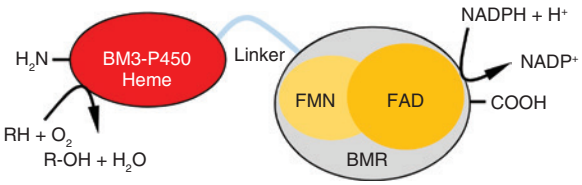

Figure 3: Schematic representation of the natural P450-redox partner fusion enzyme BM3. BMR, *Bacillus megaterium* reductase domain; P450, cytochrome P450 monooxygenase domain; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; $NADP^+$ and NADPH, oxidized and reduced nicotinamide adenine dinucleotide phosphate, respectively.

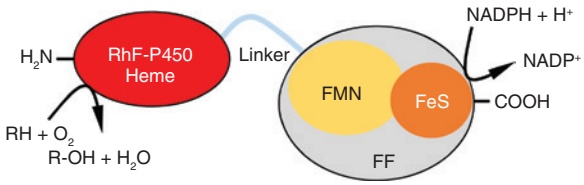

Figure 4: Schematic representation of the natural P450-redox partner fusion enzyme RhF. FF, FMN-FeS didomain.

relative positions of each domain [49]. BM3 has the highest known substrate turnover rate of any P450, oxidizing arachidonic acid at a rate of 5000 min^{-1} [50]. The P450 and BMR domains retain their activities when they are separated and presented as two proteins and form regiospecific products as similar to those synthesized by the full-length enzyme, but the rate of product formation is lower [51]. BM3 is an important model for P450 research due to its self-sufficiency, high substrate turnover and solubility [46].

1.4.2 CYP116B2 (RhF)

Rhodococcus CYP116B2 (RhF) is a natural fusion enzyme comprising an N-terminal P450 domain fused to a C-terminal FMN-FeS didomain (FF) by a 22-amino-acid linker, as shown in Figure 4 [52]. Its physiological role is currently unclear because no natural substrate is known. It offers an ideal candidate P450 for direct protein evolution because it shows high substrate promiscuity, which is unusual for P450s. The enzyme catalyzes *O*-dealkylation, aromatic hydroxylation, olefin epoxidation and the asymmetric sulfoxidation of low-molecular-weight substituted aromatics [53].

1.4.3 Artificial P450 fusion proteins

The engineering of P450s has focused on the optimization of the heme domain to improve or expand its catalytic performance, to overcome the need for a separate electron donor, to remove the need for NADPH, and to remove the membrane anchor [32]. Early strategies included enzymatic [54] and photochemical [55] cofactor regeneration, or chemical [56] and electrochemical [57] cofactor substitution. However, the construction of artificial fusion proteins to create self-sufficient P450s with integral reductases has received attention more recently [58]. Three major strategies have been developed: LICRED, PUPPET and "Molecular Lego".

LICRED is a high-throughput method for the development of P450 fusion proteins in which a vector containing a ligation-independent cloning (LIC) site adjacent to the CYP116B2 reductase domain (RED) allows the rapid insertion of P450 genes [59, 60]. PUPPET is a platform in which three proliferating cell nuclear antigen (PCNA) fusion proteins are used to form a heterotrimer that recruits P450 into a complex with its electron donors, hence PUPPET refers to "proliferating cell nuclear antigen-utilized protein complex of P450 and its two electron transfer-related proteins" [61, 62]. Finally, the "Molecular Lego" method involves the combination of P450 enzymes with the BMR domain from the natural protein BM3 described above, to make recombinant enzymes

with novel catalytic activities [63]. Soluble, self-sufficient human P450-BMR fusion enzymes have been created by removing the hydrophobic N-terminal membrane anchor domain from the insoluble human enzyme before fusion to the BMR sequence (Figure 5). This made it possible to purify the enzyme without detergents while retaining catalytic activities similar to wild-type P450s [64]. In addition to these three strategies, another method involves the exchange of shorter amino acid sequences between BM3 and novel insect P450s, an approach known as scanning chimeragenesis [65].

2 Results

2.1 Construction of insect P450 fusion enzymes using the "Molecular Lego" approach

2.1.1 Cloning strategy

The "Molecular Lego" approach described above for human P450s is also ideal for the preparation of soluble and self-sufficient insect P450 fusion proteins because the BMR domain has a similar catalytic mechanism to the housefly CPR [1]. Two candidate insect P450s were therefore selected as shown in Table 2. In each case, codon

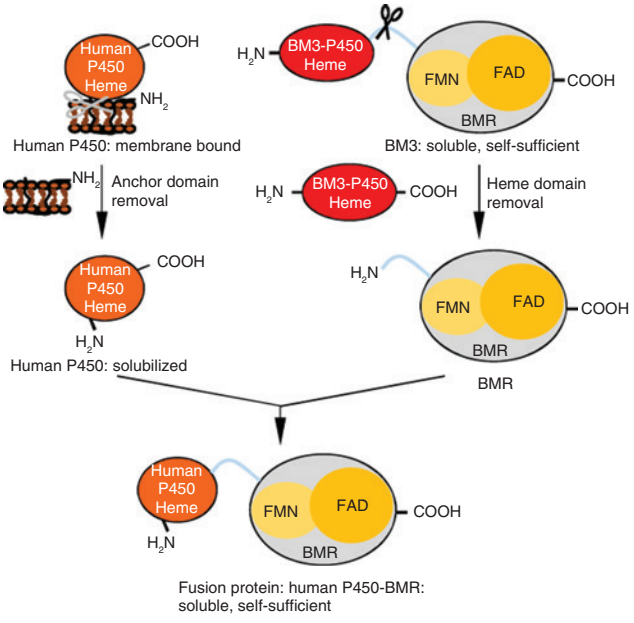

Figure 5: Schematic representation of the "Molecular Lego" principle for the construction of soluble, self-sufficient human P450-BMR fusion enzymes [32].

Table 2: Characteristics of the insect P450s used for the preparation of fusion proteins.

P450	Origin	Substrates	GenBank
CYP6A1	*Musca domestica*	Heptachlor, aldrin [66]	M25367.1
CYP6G1	*Drosophila melanogaster*	Imidacloprid, dichloro-diphenyltrichloroethane, methoxychlor [67, 68]	AAF58557.1

Figure 6: N-terminal sequence alignment of the CYP6A1 and CYP6G1 proteins. The slashes highlighted in green indicate the start of the truncated sequences after removal of the membrane anchor domain. Black outline represents identity between residues. Gray outline represents similarity between residues.

usage was optimized for *E. coli* and the N-terminus was truncated by approximately 20 amino acids to remove the membrane anchor domain. It is important not to remove too many residues because this can compromise P450 activity, e.g. residues 21–82 of human CYP2E1 are necessary for heme incorporation and correct heme pocket folding [64]. Furthermore, the N-terminus should not include any hydrophobic amino acids, so the precise number of residues for removal was determined based on studies of human P450 enzymes used for the construction of fusion proteins [35]. This comparison suggested that residues 1–23 probably functioned as the membrane anchor and should be dispensable in terms of catalytic activity, so N-terminal trimming was carried out as shown in Figure 6. The linker determines the interaction between the P450 and BMR domains thus facilitating electron flow [69–71] and reducing its length by just six residues can abolish the activity of the fusion enzyme [49]. We therefore chose a linker containing 29 amino acids. The two P450-BMR fusion proteins were then prepared using the cloning strategy explained in Figure 7.

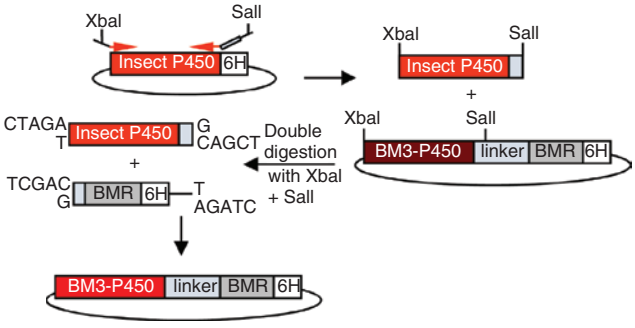

Figure 7: Cloning strategy used to construct the insect P450-BMR fusion enzymes. 6H, polyhistidine tag.

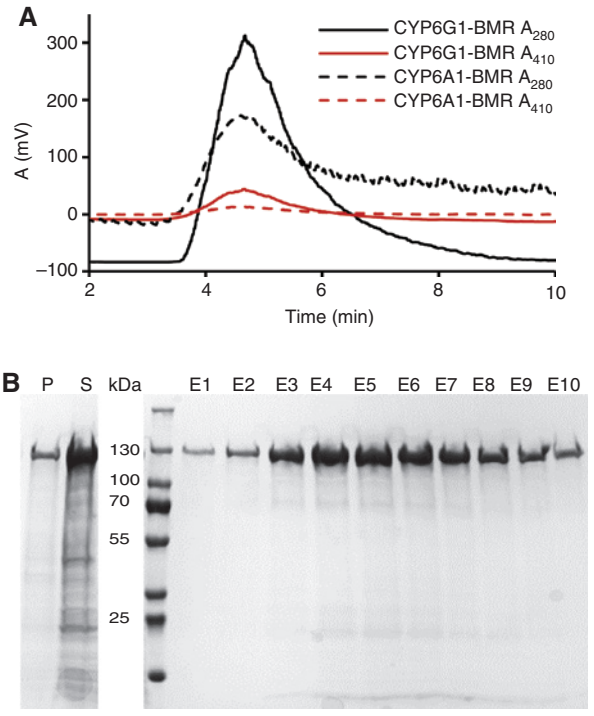

Figure 8: Purification of CYP6G1-BMR and CYP6A1-BMR. (A) IMAC elution profile of CYP6G1-BMR and CYP6A1-BMR monitored at 280 nm and 410 nm. (B) SDS-PAGE analysis of purified CYP6G1-BMR. P, pellet after sonication and centrifugation; S, supernatant after sonication and centrifugation; 1–10, elution fractions. The dominant band migrating at 120 kDa in lanes 1–10 corresponds to the CYP6G1-BMR fusion protein.

2.1.2 Expression and purification strategy

The constructs were introduced into *E. coli* for the production of soluble recombinant protein, which was encouraged by inducing expression at 28 °C. Buffers without detergents were used throughout purification, and all steps were carried out on ice or using cooled devices. The fusion proteins were captured by immobilized metal affinity chromatography (IMAC) and each eluted as a single peak in 250 mM imidazole buffer. The purity and integrity of the fusion proteins were then confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The IMAC elution profile and SDS-PAGE analysis of the fusion proteins are shown in Figure 8.

2.1.3 Activity of the fusion proteins

The activity of the reductase domain in the IMAC-purified fusion proteins was investigated by measuring NADPH turnover, which is an indirect indicator of catalytic activity and confirms correct protein folding. Reductase activity

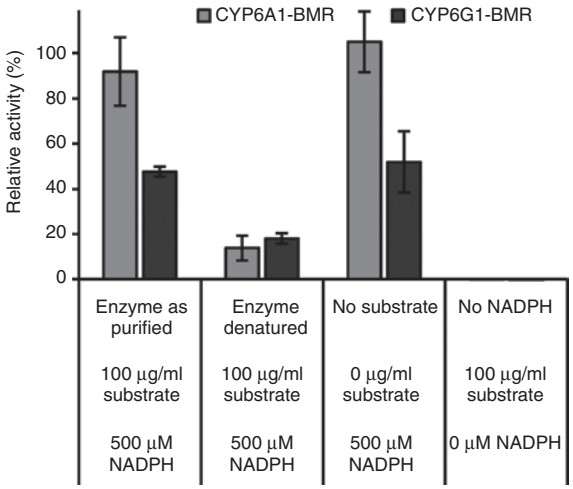

Figure 9: BMR activity assay. NADPH turnover of CYP6A1-BMR (162 µg/ml) and CYP6G1-BMR (78 µg/ml) with aldrin (CYP6A1-BMR) or imidacloprid (CYP6G1-BMR) as substrates.

was measured in the presence of aldrin, the substrate for CYP6A1 [26], and imidacloprid, the substrate for CYP6G1 [68], and in controls without substrate. The assays were performed for 12 h with measurements taken every minute. Denatured proteins were used as negative controls. Figure 9 shows that the relative activity of purified CYP6A1-BMR reached 100%, as determined by NADPH consumption, in the presence and absence of substrate. The denatured CYP6A1-BMR negative control only achieved a residual relative activity of <20%. Similar results were achieved with CYP6G1-BMR, although the activity was lower due to the lower enzyme concentration. Thus, we were able to confirm BMR activity for both insect P450 fusion proteins.

The activity of the P450 domain in the IMAC-purified fusion proteins was determined by measuring substrate turnover, which also provides evidence for the cooperation between the P450 and BMR domains. In the case of CYP6G1-BMR, we measured the conversion of the substrate imidacloprid to 4-hydroxyimidacloprid, 5-hydroxyimidacloprid, and 4,5-dihydroxyimidacloprid by ultra-high-performance liquid chromatography/mass spectrometry (UPLC-MS). In the case of CYP6A1-BMR, we measured the conversion of the substrate aldrin to dieldrin by gas chromatography-mass spectrometry (GC-MS). In both cases, we observed the loss of substrate during incubation, but the specific products were not detected.

2.1.4 Heme incorporation and substrate binding of the fusion proteins

To investigate the reason for the absence of P450 activity, we carried out further experiments to confirm heme

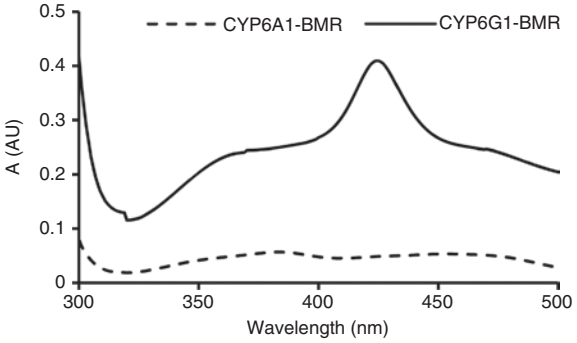

Figure 10: Absorbance spectra of purified BM3-CYP6A1 and BM3-CYP6G1 (300–500 nm). The peak at 420 nm indicates the presence of the heme group in the P450 domain of CYP6G1-BMR.

incorporation into the P450 domain. The absorbance spectrum of CYP6G1-BMR showed a peak at 420 nm confirming the inclusion of a heme group, whereas the absorbance spectrum of CYP6A1-BMR did not show a peak at 420 nm suggesting the loss of heme after IMAC purification (Figure 10). Accordingly, CYP6A1-BMR was not able to catalyze the conversion of aldrin into dieldrin due to the absence of a functional catalytic center after purification. A photometric heme substrate-binding test was then carried out to determine whether the substrate bound to the catalytic heme domain of the purified fusion enzyme. There was no evidence of heme-substrate interaction, which may indicate poor access to the substrate-binding site resulting in the absence of a catalytically productive binding mode [72].

3 Materials and methods

3.1 Gene synthesis

All genes were synthesized with a C-terminal His_6-tag (GHHHHHH) and were codon optimized for expression in *E. coli* K12 (MWG Eurofins, Ebersberg, Germany). The genes encoding the full-length BM3 and insect P450s were flanked by BsaI sites for restriction digestion and ligation into the vector pASK-IBA33plus (IBA, Göttingen, Germany). XbaI and SalI sites were introduced into the *BM3* gene to enable the removal of the endogenous P450 domain. The XbaI site was 41 bp upstream of the start codon, and the SalI site was inside the linker. Using this approach, three plasmids were generated: pASK-IBA33_BM3, pASK-IBA33_CYP6A1, and pASK-IBA33_CYP6G1.

3.2 Construction of the P450 fusion enzymes

The cloned insect P450 genes described above were amplified by PCR using the primers (MWG Eurofins, Ebersberg, Germany) listed in Table 3, adding XbaI and SalI sites at the 5′ and 3′ ends, respectively. The PCR products were double digested with XbaI and SalI (restriction enzymes from New England Biolabs, Frankfurt am Main, Germany) to create ~1500 bp P450 fragments, which were ligated into the 5113 bp vector backbone prepared by digesting pASK-IBA_BM3 with the same enzymes. This created the constructs pASK-IBA33_CYP6A1-BMR and pASK-IBA33_CYP6G1-BMR.

3.3 Expression

Terrific broth medium containing 100 µg/ml carbenicillin (Carl Roth, Karlsruhe, Germany), 0.5 mM 5-aminolevulinic acid (Sigma-Aldrich, Schnelldorf, Germany), 0.5 mM thiamine (Carl Roth, Karlsruhe, Germany), 0.25 µg/ml $FeCl_3$ (Sigma-Aldrich, Schnelldorf, Germany), 1 µM riboflavin (Sigma-Aldrich, Schnelldorf, Germany), and 160 mM D(+) glucose (Carl Roth, Karlsruhe, Germany) was inoculated with a fresh 16 h overnight lysogeny broth culture (Carl Roth, Karlsruhe, Germany) of *E. coli* (Invitrogen, Carlsbad, USA) carrying the vectors described above. The expression of genes carried on plasmid pASK-IBA33plus was induced

Table 3: Oligonucleotide primers used for the preparation of insect P450 fusion constructs.

Construct	Primer name	Sequence
pASK-IBA33_CYP6A1-BMR	CY6A1d20_fwd_XbaI	5′-GGTATCTAGAATGAGCCGTTGGAACTTTGGGTATTGGAAACGTCGTGGTATTCCG-3′
	CYP6A1_rev_linker+SalI	5′-CCGGTCGACGGACTAGGGATCCCTCCGAGGGGAATTTTCTTGCTTTTGATTTTCTTGCGAATTGC-3′
pASK-IBA33_CYP6G1-BMR	CYP6G1d20_fwd_XbaI	5′-GGTATCTAGAATGAGCCGCAATCACTCATACTGGCAGCGTAAAGGGATTCCGTACATTCCG-3′
	CYP6G1_rev_linker+SalI	5′-CCGGTCGACGGACTAGGGATCCCTCCGAGGGGAATTTTCTTGCTTTGAAGCGACGGAGCTGATTG-3′

with 200 µg/l anhydrotetracycline (IBA, Göttingen, Germany). Protein production was triggered by anhydrotetracycline once the OD_{600} reached 0.5. Cells were grown at 28 °C for 4 h and harvested by centrifugation before lysis.

3.4 IMAC

The cell pellet was resuspended in 40–60 ml lysis buffer [pH 7.5, 30 mM Tris-HCl (Carl Roth, Karlsruhe, Germany), 100 mM NaCl (Carl Roth, Karlsruhe, Germany), 20% glycerol (v/v) (Carl Roth, Karlsruhe, Germany)], and the lysed cells were centrifuged at 75,600 × *g* for 1 h at 10 °C. The supernatant was loaded on a freshly packed and conditioned 3 ml Ni-NTA (Macherey-Nagel, Weilmünster, Germany) column, connected to an SE04 system (ECOM, Prague, Czech Republic) maintained at 4 °C, at a flow rate of 1 ml/min. The column was washed with buffer A [30 mM Tris-HCl, 100 mM NaCl, 20% glycerol (v/v), 30 mM imidazole (Sigma-Aldrich, Schnelldorf, Germany), pH 7.5] at 3 ml/min until the baseline was reached. The enzyme was eluted with 250 mM imidazole at 3 ml/min.

3.5 Activity assay

Each reaction mixture contained 78 µg/ml (CYP6G1-BMR) or 162 µg/ml (CYP6A1-BMR) purified fusion enzyme, 0.5 mM NADPH (Sigma-Aldrich, Schnelldorf, Germany), 0.1 mM substrate, 25 mM Tris-HCl (pH 7.5), and 20% glycerol (v/v) in a total volume of 200 µl. As controls, samples were prepared without NADPH, without substrate and with heat-denatured enzyme. The oxidation of NADPH was monitored at 340 nm on an Eon microplate reader (Biotek, Bad Friedrichshall, Germany) with one measurement every minute for 12 h.

3.6 UPLC-MS

Each reaction mixture comprised 78 µg/ml purified CYP6G1-BMR, 100 µg/ml imidacloprid (Sigma-Aldrich, Schnelldorf, Germany), 0.5 mM NADPH and 25 mM Tris-HCl (pH 7.5). After incubation at room temperature for 25 h the samples were mixed 1:1 with acetonitrile and transported on ice to Chromicent (Berlin, Germany) for UPLC-MS analysis according to [73]. A Waters (Eschborn, Germany) UPLC instrument was equipped with an AQUITY HSS T3 column (100 × 2.1 mm internal diameter, 1.8 µm particle size) coupled to a Waters Xevo TQ-S micro mass spectrometer. Water with 0.1% formic acid (Sigma-Aldrich, Schnelldorf, Germany) was used as solvent A and acetonitrile (Sigma-Aldrich, Schnelldorf, Germany) as solvent B. The injection volume was 5 µl. Each separation took 17 min at a flow rate of 0.5 ml/min, starting at 95% solvent A (held for 3 min) and decreasing to 40% solvent A in 12 min (held for 2 min). MS was operated in positive electrospray ionization mode. The source temperature was set at 120 °C with nitrogen flow rates of 20 l/h for the cone gas and 1000 l/h for the desolvation gas. The desolvation temperature was 600 °C. Standard curves of imidacloprid and imidacloprid-olefin (Dr. Ehrenstorfer, Augsburg, Germany) (3.125 µg/ml, 6.25 µg/ml, 12.5 µg/ml, 25 µg/ml, 50 µg/ml and 100 µg/ml) were prepared in triplicate before the samples were processed. Imidacloprid and imidacloprid-olefin were quantified using the corresponding standard curve.

3.7 Photometric heme substrate-binding test

Samples for the photometric heme substrate-binding test were transferred to 1 ml cuvettes for analysis in a photometer (Specord 210, Analytik Jena AG, Jena, Germany). The baseline was determined using 400 µl of the appropriate buffer plus 40 µl dimethylsulfoxide (DMSO) (Carl Roth, Karlsruhe, Germany) as the substrate solvent. The first measurement from 300 to 500 nm was performed with 400 µl of an undiluted elution sample of CYP6G1-BMR, or denatured CYP6G1-BMR and 40 µl DMSO. After this measurement, 400 µl of the same samples were mixed with 40 µl 1 mg/ml imidacloprid in DMSO and the measurement was repeated. The results were compared to see if the peak shifted from 425 nm to another wavelength.

4 Conclusions and outlook

The enormous technological potential of insect P450 can only be realised if sufficient quantities of high-quality active enzymes are made available for molecular and biochemical characterisation. We applied the "Molecular Lego" strategy by constructing the insect P450-BMR fusion enzymes for expression as soluble proteins in *E. coli*, allowing their purification without detergents, but in vitro P450 activity could not be detected even though the BMR domain was functional in both candidates.

Several strategies remain available to improve the activity of our insect P450-BMR fusion proteins. The fusion proteins could be expressed in other heterologous

systems such as insect cells or yeast, which may provide a more appropriate molecular environment. Furthermore, we used vector pASK-IBA33plus because it has achieved the soluble expression of other insect enzymes in *E. coli* [74], but switching to the pCW ori+ system may generate better results because the latter is the best characterized vector for P450 expression [75]. The linker region could be modified by changing its length and/or composition to optimize the interaction between the BMR and P450 domains. The flexibility of the linker and the electron transfer rate between the BMR and P450 domains could also be improved by random or site-directed mutagenesis. In addition, the elimination of potential protease cleavage sites could enhance the stability of the fusion protein. Increasing the length of the N-terminus may improve enzyme activity because excessive truncation can inhibit heme incorporation and correct heme pocket folding [64]. The versatile RhF reductase domain may also prove useful because this has been previously successfully combined with P450s from other sources [76, 77]. X-ray diffraction data provide a more rational basis for the design of P450-CPR fusion proteins, but no crystal structures are yet available for the insect P450s [64]. Our results provide a solid foundation for future work on insect P450s, including their exploitation for industrial processes.

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