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Modifications in the N-terminus of an insect cytochrome P450 enhance production of catalytically active protein in baculovirus-Sf9 cell expression systems

Wenfu Mao¹, May R. Berenbaum^{1,3}, and Mary A. Schuler^{2,3,4,*}

¹Department of Entomology, University of Illinois, Urbana, IL 61801

²Department of Cell and Developmental Biology, University of Illinois, Urbana, IL 61801

³Department of Plant Biology, University of Illinois, Urbana, IL 61801

⁴Department of Biochemistry, University of Illinois, Urbana, IL 61801

Abstract

Although baculovirus vectors are powerful tools for the heterologous expression of proteins in insect cell cultures, some insect and plant microsomal P450 proteins are not effectively expressed in this system. Hypothesizing that their expression failures might result from collisions between their N-terminal sequences and adjacent cytosolic sequences, we compared and mutated the N-terminus of *Papilio multicaudatus* CYP6B33, which is inappropriately folded in Sf9 cells, to sequences present in its *Papilio polyxenes* CYP6B1 counterpart, which is efficiently expressed and appropriately folded. Molecular modeling of the three differences in the linker separating the signal anchor domain (SAD) and the cytosolic domain identified Val32 in CYP6B33 as a residue potentially important for folding and/or positioning of the cytosolic domain. Mutation of Val32 to Ala32 in the CYP6B33 linker (CYP6B33 V32A mutant) or replacement of the CYP6B33 SAD with that of CYP6B1 (CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ mutant) allowed for significant P450 expression, indicating that complex interactions involving both the signal anchor and membrane linker affect folding and activity of P450s in this heterologous expression system.

Keywords

cytochrome P450 monooxygenases (P450s); heterologous expression of insect P450s; molecular modeling; allelochemical metabolism; *Papilio*

INTRODUCTION

Microsomal cytochrome P450 monooxygenases (P450s) are integral membrane proteins in the endoplasmic reticulum (ER) membrane. Consuming an oxygen and two electrons supplied by NADPH-dependent cytochrome P450 reductases, P450s catalyze the oxidative metabolism of a wide range of organic compounds through such diverse reactions as C-H bond/N-hydroxylations, O-, N- or S-dealkylations, S-oxidations and epoxidations (Mansuy, 1998) in

*Corresponding author: Dr. Mary A. Schuler, Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, 1201 W. Gregory Dr., 161 Edward R. Madigan Laboratory (ERML), Urbana, IL 61801 USA, 217-333-8784, 217-244-1336 (fax), maryschu@uiuc.edu.

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a regio- and stereoselective manner. Many examples now exist of P450 enzymes engaged in biosynthetic reactions in diverse groups of organisms, while others play important roles in the detoxification of exogenous compounds including drugs, pesticides, herbicides and naturally occurring toxins (Ranson et al., 2002; Werck-Reichhart et al., 2002; Nelson et al., 2004; Feyereisen, 2005; Kelly et al., 2005; Guengerich, 2006; Claudianos et al., 2006; Schuler et al., 2006).

As genome sequencing projects have progressed, increasing numbers of P450 genes have been identified in wide array of organisms (<http://drnelson.utmem.edu/CytochromeP450.html>). Because of their high degree of diversity and wide range of potential substrates, heterologous expression of their active proteins has become one crucial component in their functional and structural characterizations. Successful heterologous expression of a particular P450 protein provides virtually unlimited quantities of that protein for synthesis of pharmaceuticals (Andersen et al., 1993; van Beilen et al., 2003, 2005; Bischoff et al., 2005; Jennewin et al., 2005) and for X-ray structure determinations (Hasemann et al., 1995; Johnson and Stout, 2005; Poulos and Mehareenna, 2007). Among the prokaryotic and eukaryotic systems used to express catalytically active microsomal P450s (Gonzalez and Korzekwa, 1995; Guengerich et al., 1997; Duan and Schuler, 2006), *Escherichia coli* is the most commonly used host organism for inexpensive production of recombinant proteins. Effective expression of eukaryotic microsomal P450s in this bacterium nearly always requires multiple modifications in the region coding for their N-terminal membrane anchor domain. Among the most frequent modifications are replacement of this hydrophobic region with a modified version of the bovine CYP17A1 signal sequence (Barnes et al., 1991), replacement of the second codon with an alanine codon that is the preferred codon for high expression of T7 phage proteins in *E. coli* (Looman et al., 1987; Gold, 1990) and enhancement of the AT content in the first few codons to minimize the potential of forming RNA secondary structures (Guo et al., 1994). Sometimes more drastic deletions of the entire signal sequence have been used for expression at the high levels needed for crystal structure determinations (Barnes, 1996; Guengerich et al., 1997).

Because these deletions and codon modifications are not needed for the expression of microsomal P450s in baculovirus-infected insect cells, increasing numbers of plant, insect and mammalian P450s are being expressed in this eukaryotic system (Gonzalez and Korzekwa, 1995; Kutchan, 1996; Duan et al., 2004; Feyereisen, 2005; Wang-Buhler et al., 2005; Duan and Schuler, 2006). But, despite its efficient expression and folding of many different types of P450s, there remain a number of microsomal P450 proteins that have been expressed only in P420 form (an incorrectly configured form of P450) or not at all. Challenged with one such problematic insect P450 (*Papilio multicaudatus* CYP6B33 described in Mao et al. (2007)), we investigated a number of strategies for improving baculovirus expression of P450 proteins using, as a point of comparison, the N-terminus of *Papilio polyxenes* CYP6B1, a closely related P450 expressed at high levels in Sf9 cells. Molecular modeling and one modest replacement (V32A) in the CYP6B33 linker domain have allowed us to demonstrate that this P450 mediates the epoxidation of xanthotoxin, an allelochemical encountered routinely in the hostplants of *P. multicaudatus*.

EXPERIMENTAL PROCEDURES

Reagent

Xanthotoxin and NADPH were obtained from Sigma (St Louis, MO) and HPLC solvents from Fisher Scientific L.L.C. (Pittsburgh, PA). PfuTurbo DNA polymerase was from Stratagene (La Jolla, CA) and DpnI was from New England Biolabs (Ipswich, MA). All other restriction enzymes, Sf9 cells, SF-900 serum-free medium, fetal bovine serum (FBS), pFastBac1 vector and DH10BAC competent cells were from Invitrogen (Carlsbad, CA) and penicillin/

streptomycin was from Bio-Whittaker (Walkersville, MD). The pGEM-Teasy vector was from Promega (Madison, WI).

Molecular modeling of the V32A mutant

Using the CYP6B33 molecular model described in detail in Mao et al. (2007), the V32A replacement model was generated by introducing this substitution and subjecting the model to another round of energy minimization.

Construction of expression vectors

The CYP6B33 cDNA described in Mao et al. (2007) was inserted into the pFastBac1 vector using a PCR strategy with primers directed against the N- and C-termini of the CYP6B33 coding sequence. The forward primer, PMCBamHI (sequences for primers are in Table 1), contains the translation start codon and a BamHI site on its 5' end; the reverse primer, PMCXbaI, contains the stop codon and an XbaI site on its 5' end. The PCR amplified CYP6B33 cDNA product was digested with BamHI and XbaI and ligated into BamHI-XbaI cut pFastBac1 vector.

The chimeric CYP6B1₁₋₃₂/CYP6B33₃₃₋₅₀₀ cDNA was constructed by amplifying the CYP6B1₁₋₃₂ and CYP6B33₃₃₋₅₀₀ fragments from the CYP6B1 and CYP6B33 cDNAs (Cohen et al., 1992; Mao et al., 2007) using PCR strategies with PfuTurbo DNA polymerase and the CYP6B1BamHI(NF)/CYP6B1HindIII(NR) and CYP6B33HindIII(NF)/PMCXbaI primer pairs. The amplified fragments were digested with HindIII, ligated together and inserted into the pGEM-Teasy vector using the terminal adenosine present on the ligated PCR product. The chimeric cDNA was transferred from the pGEM-Teasy vector into the pFastBac1 vector by digesting with BamHI and XbaI and, finally, the HindIII site between the CYP6B1₁₋₃₂ and CYP6B33₃₃₋₅₀₀ sequences was deleted using single-stranded DNA site-directed mutagenesis methods with the CYP6B33(-HindIII) primer. The CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ chimera was constructed using the PCR gene fusion method of Yon and Fried (1989) with the CYP6B1 and CYP6B33 cDNAs amplified with the CYP6B1BamHI(NF), CYP6B1linker and PMCXbaI primers. The chimeric gene was digested with BamHI and either XbaI or HindIII and ligated into the pFastBac1 vector cut with the corresponding restriction enzymes.

The single CYP6B33 Val32Ala and double Val31Ala/Val32Ala mutants were constructed by single-stranded DNA site-directed mutagenesis (Makarova et al., 2000) using the pFastBac1 plasmid containing CYP6AB33 and the V32A and V31A/V32A primers, respectively. The chimeric CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A mutant was constructed using the pFastBac1 plasmid containing CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ and the V32A primer. All of the final expression constructs were confirmed by sequencing each full-length coding region with vector and internal primers.

Expression of recombinant baculoviruses in Sf9 cells

All recombinant virus expressions in Sf9 cells were performed as described by GibcoBRL/Life Technology (Carlsbad, CA). For each construct, a plate (100×20 mm) was seeded with 8×10⁶ of Sf9 cells in SF-900 serum-free medium supplemented with 8–10% fetal bovine serum (FBS), 50 µg/ml streptomycin sulfate and 50 units/ml penicillin and infected with varying MOI of recombinant P450 virus or co-infected with a constant MOI of recombinant P450 virus and varying MOI of recombinant house fly P450 reductase virus. Hemin was added to a final concentration of 2 µg/ml 24 h after the initial infection and the insect cell cultures were harvested 72 h after the initial infection by centrifuging at 1000×g for 5 min. The pelleted cells were washed twice with ice-cold 0.1 M phosphate buffer (PB; pH 7.8), resuspended in one-tenth of the original volume of ice-cold cell lysate buffer [0.1 M PB (pH 7.8), 1.1 mM EDTA, 0.1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 5 µg/ml (w/v) leupeptin, 20%

glycerol] and lysed by sonication on ice for 3×15 s. After the cell lysates were cleared by centrifugation at 3,200×g for 5 min, their supernatants were stored at −80°C for future use. Concentrations of expressed CYP6B33 protein were measured by CO difference spectra (Omura and Sato, 1964).

Analysis of P450 reductase interactions with P450 mutants

For analysis of the catalytic activities of the P450 mutants that monitor for their ability to interact with P450 reductase, Sf9 cells were co-infected with a constant MOI of recombinant CYP6B33 V32A virus (MOI of 1) or CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A virus (MOI of 7.2) (MOI values that yield the same amount of P450 protein as determined by CO difference spectroscopy) and house fly P450 reductase at three different MOIs (corresponding to 0, 0.05 and 0.1). Although P450 reductase levels were not directly measured in the final extracts derived from Sf9 cells expressing both P450 and P450 reductase, the process of optimizing expression for these proteins routinely involves a first series expressing each recombinant P450 virus at varying MOI to optimize the amount of P450 produced in its correctly folded form (with the least P420 produced). Subsequent to this (and as detailed in Wen et al. (2003)), a second series expressing the recombinant P450 reductase virus at varying MOI monitors substrate turnover to demonstrate that P450 reductase levels are non-limiting catalytic activities. For this particular study, some of this optimization can be seen in Fig. 4 where increasing the MOI of P450 reductase virus from 0.05 to 0.1 only slightly increases the protein activities while maintaining significant differences in the optimum activity attained for each protein.

With extracts prepared at the different MOI for P450 reductase, reaction mixtures were set up with a fixed amount of P450 (15 pmol as defined by CO difference spectra), 2 µl 5 mM furanocoumarin stock solution, 25 µl NADPH (5 mg/ml in PB, pH 7.8) or 25 µl PB (as a no NADPH control) and a suitable volume of 0.1 M PB (pH 7.8) to a final volume of 250 µl. After incubation at 30°C for 30 min in a shaking waterbath, reactions were terminated by adding 50 µl 2 M HCl and 5 µl of 5 mM angelicin were added as an internal control for extraction efficiency. Unmetabolized furanocoumarins were extracted with one 250 µl aliquot of ethyl acetate and analyzed by normal-phase HPLC as previously described in Mao et al. (2006). All assays were replicated three times.

Purification and characterization of the xanthotoxin metabolite

Multiple reaction mixtures containing heterologously expressed CYP6B33 V32A protein were set up as described using CYP6B33 protein co-expressed with house fly P450 reductase at a MOI ratio of 1:0.05, incubated at 30°C for 30 min and extracted with ethyl acetate. After drying of the organic phase in a Speedvac centrifuge, the residue was resuspended in 50 µl methanol and the xanthotoxin metabolite was fractionated using a reverse-phase HPLC (4 µm C-18 column, Waters Novapak, 4.7×150 mm) and gradient elution with water (solvent A) and 100% acetonitrile (solvent B) at a flow rate of 1 ml/min and the following parameters: 100% A/0% B for 5 min, 100% A/0% B to 0% A/100% B over a 60 min time course, 100% B/0% A for 5 min, and 100% B/0% A to 100% A/0% B over a 5 min time course. UV absorbance was monitored at 325 nm. MS/MS spectrometric analysis of the purified metabolite was performed at the Mass Spectrometry Service Facility at the University of Illinois.

RESULTS

Sequence comparisons

Based on our hypothesis that the failure of *P. multicaudatus* CYP6B33 to CYP6B37 expressions using baculovirus vectors resulted from inappropriate collisions with adjacent P450 domains and/or nearby ER-localized lipid and protein molecules or that appropriate N-

terminal amino acids are needed for proper P450 folding, we compared the N-terminal sequences of these CYP6B proteins with the CYP6B1 protein from the black swallowtail *P. polyxenes*. As shown in Fig. 1, downstream of their variable N-terminal signal anchor domains, the CYP6B33 to CYP6B37 proteins share a high degree of amino acid identity (10/13) with CYP6B1 in the short α -helix separating the signal anchor domain (SAD, residues 1–20) from the proline-rich region (PKP residues 34–36 in CYP6B33 to CYP6B37; PKPTP residues 34–38 in CYP6B1). However, in this group of CYP6B subfamily proteins, the less structured valine-rich region immediately forward of the proline-rich region (NVVG residues 30–34 in CYP6B33; NVAG residues 30–34 in CYP6B1) contains one amino acid variation (Val32Ala) in addition to the two found in the short α -helix (Asn24Asp, Asp28Lys). Comparison with other members of the CYP6B subfamily cloned from a range of lepidopteran insects indicate that, within this Val/Pro-rich region, variations are limited to Asn24Asp, Asp28Lys, Val32Ala, Thr37Ile and Val38Pro (with the first residue designating that found in CYP6B33).

Heterologous expression of signal anchor and linker mutants

Because of the different degrees of sequence variation in the signal anchor and linker domains, two chimeric constructs, CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ and CYP6B1₁₋₃₂/CYP6B33₃₃₋₅₀₀, were expressed in Sf9 cells at an MOI of 1 to determine whether CYP6B1 SAD and downstream α -helical sequences affect CYP6B33 expression. Monitored by carbon monoxide (CO) difference analysis that differentiates correctly folded P450 protein from incorrectly folded P420 protein (Omura and Sato, 1964), the CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ protein was expressed at a concentration of 0.22 nmol P450/ml culture and the CYP6B1₁₋₃₂/CYP6B33₃₃₋₅₀₀ protein was expressed at a concentration of 0.14 nmol P450/ml culture (Fig. 3). In contrast to the wildtype CYP6B33 protein that repeatedly forms only P420 in Sf9 cells, the recombinant CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ and CYP6B1₁₋₃₂/CYP6B33₃₃₋₅₀₀ proteins generate P450 and P420 at optical ratios of 0.80 and 0.50, respectively, in this expression system.

To determine whether any of the amino acid variations in the CYP6B33 linker domain affect the folding of recombinant CYP6B33 during its synthesis in Sf9 cells, a molecular model for CYP6B33 was developed as outlined in Mao et al. (2007) using, as the main template, the crystal structure of human CYP3A4 that lacks its SAD (residues 1 to 27) but retains its linker domain (residues 28 to 38) preceding the Pro-rich region (Yano et al., 2004). In this, Val32 is the one variable amino acid in the linker domain that has potential to interfere with proper P450 folding, if the integrity of this microsomal protein is affected by collisions with nearby molecules in the Sf9 cell system. As shown in Fig. 2B, the side chain of Val32 is predicted to be <3 Å from Phe64, Lys68 and Val70 in the loop between the first structural elements of this P450 (A-helix and β 1-sheet). Mutagenesis of Val32 to Ala32 in the full-length CYP6B33 protein (designated as the CYP6B33 V32A mutant) and the chimeric CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ protein (designated as the CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A mutant) allowed for high level expression, 0.48 nmol P450/ml culture and 0.67 nmol P450/ml culture, respectively, with no apparent P420 being formed. Additional mutagenesis of the invariant Val31 to Ala31 (designated as the CYP6B33 V32A/V31A mutant) potentially decreasing the side chain volume in this region yielded no detectable P450.

Analysis of P450 interactions with P450 reductase

Based on the knowledge that the SAD deletions and/or replacements did not change mammalian P450 catalytic activities expressed in *E. coli* and reconstituted with P450 reductase (Gonzalez and Korzekwa, 1995), we checked whether SAD replacement could affect the interaction of CYP6B33 with the P450 reductase that is its electron transfer partner essential for catalytic activity. Co-expression of the CYP6B33 V32A mutant and CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A chimeric proteins with varying MOI of the recombinant P450 reductase virus indicates clear differences in their responses to increasing levels of P450 reductase (Fig.

4). In reactions containing only the endogenous P450 reductase in Sf9 cells (not supplemented with house fly P450 NADPH reductase), the CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A chimeric protein displays a higher activity toward xanthotoxin than the CYP6B33 V32A mutant protein. Supplementations with house fly P450 reductase at MOI values of 0.05 and 0.1 increase CYP6B1₁₋₂₀/CYP6B33 V32A₂₁₋₅₀₀ activities toward xanthotoxin from 0.72 to 3.90 pmol/min/pmol P450 (5.4-fold increase) and CYP6B33 V32A activities from 0.11 to 8.05 pmol/min/pmol P450 (73-fold increase). The fact that the unsupplemented activity of CYP6B1₁₋₂₀/CYP6B33 V32A₂₁₋₅₀₀ is higher than that obtained CYP6B33 V32A indicates that the different SAD sequences cause the CYP6B33 protein to interact more effectively with the endogenous P450 reductase when the first twenty amino acids of CYP6B1 are present. The fact that the higher level of P450 reductase virus (MOI of 0.1) used in the process of optimizing co-expression of both P450 and P450 reductase (see Experimental Procedures) produces only slightly higher catalytic activities than the lower level of P450 reductase virus (MOI of 0.05) indicates that these supplemented activities are obtained under non-limiting P450 reductase levels and characteristic of strong interactions with the heterologous house fly P450 reductase. Together with the fact that the CYP6B33 V32A mutant produces only a P450 maximum in CO difference analyses, the high activities of this single site mutant indicate the extent to which the V32A mutation can enhance the folding and activity of the CYP6B33 protein.

Metabolites of the CYP633 V32A mutant

With the wildtype CYP6B33 protein expressed at levels too low to detect any metabolism, the high activity of the CYP6B33 V32A mutant co-expressed with house fly P450 reductase (MOI ratio of 1:0.05) allowed us to identify its products derived from xanthotoxin. Reverse-phase HPLC analyses identified at least three metabolites produced by the CYP6B33 V32A mutant in the presence of NADPH (Fig. 5A). MS/MS analyses of these indicated that the two main metabolites, 6-(7-hydroxy-8-methoxycoumaryl)-acetic acid (peak 1) and 6-(7-hydroxy-8-methoxycoumaryl)-hydroxyethanol (peak 3) (Fig. 5B), share a common 2',3'-epoxide intermediate. As detailed in Mao et al. (2007), further analyses of the metabolic capacity of the CYP6B33 V32A mutant have indicated that it also transforms a range of linear and angular furanocoumarins, including angelicin, bergapten, imperatorin, isopimpinellin and sphondin.

DISCUSSION

Together, our experiments suggest that expression problems encountered using the insect baculovirus system for production of CYP6B33 to CYP6B37 proteins result from protein folding problems and/or interference with adjacent macromolecules in the endoplasmic reticulum of Sf9 cells. The successful expression of CYP6B33 using either a single-site linker replacement or a chimeric SAD replacement indicates that both of these N-terminal domains have unappreciated roles in defining the folding and activity of P450s expressed in this, and possibly other, heterologous systems.

Using the human CYP3A4 crystal structure lacking only its SAD, we have modeled the structure of the CYP6B33 linker region to be a short α -helix containing several charged side chains and a Val/Pro-rich linker providing a "basement" structure below the soluble cytosolic domain. The four positively charged amino acids, Arg21, Lys27, Arg29 and Lys34, have potential to block the transfer of this P450 across the ER membrane as well as fix the cytosolic domain of this enzyme on the membrane through interactions with the negatively charged phospholipids. Our mutagenesis indicates that, rather than being a randomly organized spacer region, the Val/Pro-rich linker contains highly conserved residues (NVVGPKPTV with underlines designating absolute conservations in CYP6B proteins) that are involved in maintaining a special structural foundation for this subfamily of P450 enzymes. Among the constituents in this region of CYP6B33, the three valines contain tertiary β -carbons that

increase the stiffness of the polypeptide backbone and the two prolines assume rigid, dihedral conformations that bend and kink the backbone. The predominance of these residues contrasts with the high frequencies of small, flexible amino acids (Ser, Gly, Ala and Thr) found in the linker regions extending between functional domains in a variety of other proteins (Argos, 1990). The structural constraints imposed by these hydrophobic and rotationally less flexible valine and proline residues in different expression systems may explain why a single proline-to-alanine switch or deletion of multiple prolines in this region of mammalian CYP2C2, CYP2C11 and CYP17 (Chen et al., 1997, 1998; Kusano et al., 2001a, b) and more drastic deletion of seven residues (22-28) in the Gly-rich linker of CYP2C2 or replacement with seven valines (Chen et al., 1997) have all resulted in P450 misfolding in COS-1 cells. Comparisons with our predicted structure for the CYP6B33 linker suggest that the Gly-rich CYP2C2 linker has different constraints on its ability to facilitate P450 folding. This flexible linker, which is not capable of forming any α -helical structure, can form appreciable amounts of active P450 even with replacements with three prolines or seven alanines, but any shortening of it, blocks assembly of active CYP2C2 and produces increasing amounts of P420 (Chen et al., 1997).

Several studies have now indicated that the N-terminal hydrophobic segments of microsomal P450s function as ER membrane insertion sequences that are recognized by the signal recognition particle (SRP) as microsomal P450s are cotranslationally inserted into the ER membrane (Sakaguchi et al., 1984, 1987). Deletion of these hydrophobic segments and optimization of some N-terminal codons to reflect *E. coli* translation preferences have allowed for the expression of the bovine CYP17A1 protein at 16 mg/liter in bacterial cells (Barnes et al., 1991). Similar modifications and replacements of the N-terminal regions of other microsomal P450s have also enhanced their expression, with expression levels varying from 20 nmol/liter to 700 nmol/liter depending on the P450 (Fisher et al., 1992; Palmer et al., 1993; Richardson et al., 1993; Sandhu et al., 1993; 1994). In this study, even without codon optimizations for Sf9 cells, replacement of the SAD of CYP6B33 with that of CYP6B1 allows for expression and assembly of P450 in insect cells at a moderate level (0.22 nmol P450/ml culture). Our further examination of the metabolic capacity of the CYP6B33 V32A mutant protein indicates that this single substitution alters folding so significantly that inactive CYP6B33 is converted to a functionally active form capable of mediating the detoxification of xanthotoxin as well as other linear and angular furanocoumarins (Mao et al., 2007). NMR structural determinations on the SAD of mammalian CYP8A1 (prostaglandin I₂ synthase) have suggested that this signal anchor assumes a helix-turn-helix structure embedding its initial helical residues (3-11) within the phospholipid bilayer (Ruan et al., 2002). Contrasting with the continuous long α -helix that is typically viewed as the membrane-spanning anchor for P450s, the atypical broken helix of CYP8A1 defines interactions with its electron transfer partner, cyclooxygenase (Ruan et al., 2005). While replacement of the CYP8A1 SAD with other anchor sequences reduces its activity toward prostaglandin and contrasts with the enhancement of CYP6B33 activity conferred by swapping with a CYP6B1 SAD, both of these studies provide evidence that signal anchor domains moderate interactions between the multiple components involved in the P450 reaction cycle.

Comparisons of the linker sequences in other insect P450s expressed in baculovirus systems suggest that the structural constraints of this Val/Pro-rich linker are not limited to these CYP6B proteins. As highlighted in Fig. 6, amino acid sequence conservation extends to representative proteins in the CYP6 family members in *Drosophila melanogaster* (fruit fly), *Anopheles gambiae* (malaria mosquito), *Helicoverpa zea* (corn earworm) and *Papilio* species described in this study as well as the highly divergent *H. zea* CYP321A1 that metabolizes a range of insecticides (Sasabe et al., 2004). With this last P450 sharing only 30% overall identity with the CYP6 proteins, this high degree of conservation in the linker sequence is especially striking. Comparisons among linkers in representative mammalian CYP1, CYP2 and CYP3 family members show some degree of identity within the individual P450 subfamilies and length

variation between P450 families. The extent to which these other linker sequences affect coupling with P450 reductases in other heterologous expression systems remains to be determined.

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Figure 1.

Alignment of N-terminal amino acids in *P. polyxenes* CYP6B1 with *P. multicaudatus* CYP6B33 to CYP6B37.

The N-terminal amino acids of CYP6B subfamily members are shown with positive amino acids displayed in grey and prolines displayed in turquoise. Subdomains are designated at the top as SAD (membrane-embedded signal anchor domain), helix (helical region in linker beyond the SAD) and Val/Pro-rich linker preceding the first α -helix in the cytosolic domain.

Figure 2.

Molecular model of CYP6B33.

(A) The molecular model of CYP6B33 developed using the crystal structure of CYP3A4 (Yano et al., 2004) as the main template and CYP2C8 (Schoch et al., 2004) as the FG region is shown with its predicted interactions with the ER membrane surface. Positively charged amino acids (Arg21, Tyr23, Lys27, Arg29, Lys35) are shown along the protein backbone. (B) Val32 in CYP6B33 (shown in red stick format) is superimposed with Ala32 in CYP6B1 (shown in green stick format). Residues within 3 Å of Val32 are shown in elemental ball-and-stick format and include Phe64, Lys68 and Val70.

Figure 3.

P450 levels expressed in Sf9 cells.

Sf9 cells transfected with recombinant CYP6B viruses at a constant MOI of 1 were monitored for their P450 content using CO difference analyses (Omura and Sato, 1964). The P450 yields on the abscissa reflect the P450 contents of each preparation relative to the ml of Sf9 culture media collected from five plate transfection. The ratios of P450/P420 in the column on the right reflect the amounts of each form not adjusted for the amount of culture media collected. These ratios range from 0.50 for CYP6B1₁₋₃₂/CYP6B33₃₃₋₅₀₀ 0.80 for CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ to 7.4 for CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A; the wildtype CYP6B33 and CYP6B33 V31A/V32A proteins generated only P420 and the CYP6B33 V32A protein generated only P450.

Figure 4.

Xanthotoxin metabolic activities of mutant CYP6B33 proteins.

Sf9 cells cotransfected with recombinant CYP6B1₁₋₂₀/CYP6B33₂₁₋₅₀₀ V32A chimeric virus (grey bars) or CYP6B33 V32A mutant virus (black bars) at a constant MOI of 7.2 or 1.0, respectively, and recombinant house fly P450 reductase virus at a MOI of either 0.05 or 0.1 were monitored for xanthotoxin metabolism; the activities of these proteins in reactions not supplemented with recombinant house fly P450 reductase (MOI of 0 for P450 reductase virus) represent the metabolic capacities of these P450 proteins coupled with the endogenous P450 reductase levels in Sf9 cells.

Figure 5.

Characterization of xanthotoxin metabolites generated by CYP6B33 V32A mutant.

(A) Reverse-phase HPLC analysis of xanthotoxin metabolized by the CYP6B33 V32A mutant protein co-expressed with house fly P450 reductase at an MOI ratio of 1: 0.5. The upper panel shows metabolites generated in the presence of NADPH and the lower panel shows control reactions lacking NADPH.

(B) MS/MS analyses of peak 1 (left panel) and peak 3 (right panel) shown in panel A.

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CYP6B1v2      MLYLLALVTVLAGLLHYFT--RTFDYWKKNVAGPKVFFFGNLKDSVLRKKP
CYP6B33       MLTILTVTATLFAILYLYFT--RNFNYWKDRNVVGPKEPTVFFGNIMESVIRKKH
CYP6G1        MVLTEVLFVVVAALVALYT-WFQ--RNHSYWQRKGIPYIPEPTIIGNTKVVFKMENS
CYP6A8        MALTYILFQVAVALLAILTYIYH--RKLTYFKRRGIPFVAPHLIRGNMEELQKTKNI
CYP6B4        MLTIFIVTATLFAILYFYFT--RNFNYWKDRNVVGPKEPTVFFGNIMESVIRKKH
CYP6B3        MLYVITLVTVLAGLLHYFT--RNFYWKKNVAGPKVFFFGNLKDSALRRKP
CYP6B8        MWVLYLPAVLSVLIVTLYLYFT--RTFNYWKKNVRGPEPTVFFGNLKDSTLRKKN
CYP6a2        MFVLIYLLIAISSLLAYLYH--RNFNYWNRGVPHDAPHPLYGNMVG--FRKNR
CYP6Z2        MFVYTLALVAIVFLVLR--YIYSHWERHGLPHLKPEIPYGNIRTVAEKKES
CYP6Z1        MilyTIGLIVAFVFLALK--YVYSYWDROGLPNLRPEIPYGNLRILAQKKES
CYP321A1      MLQLPLILLGLVLLLTWYLIGRHNNENYWKARGVKFYSKNKVIGPSWDYLFTKGA
               .           :           : : : : :           *           .

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Figure 6.

Linker sequence conservations in insect P450s.

Linker sequences are compared for *Drosophila melanogaster* CYP6A2 (BT015971), CYP6A8 (NM_079025) and CYP6G1 (NM_136899), *Anopheles gambiae* CYP6Z1 (AF487535) and CYP6Z2 (AF487780), *Papilio polyxenes* CYP6B1v2 (M83117) and CYP6B3 (U25819), *P. glaucus* CYP6B4 (U47059), *Helicoverpa zea* CYP6B8 (AF102263) and CYP321A1 (AY113689). Aqua highlights indicate prolines and yellow highlights indicate amino acids conserved between *H. zea* CYP321A1 and various CYP6B proteins.

Table 1

Primers used in construction of recombinant virus

Primers	Sequences
PMCBamHI	5'-GGCGGATCCGATGTTAACGATATTAACAG-3'
PMCXbal	5'-CGCTCTAGACGTAATCTTTAATTGTTAG-3'
V32A	5'-TACTGGAAAAGATAGAAATGTCGCTGGACCTAAACCGACCGTATTC-3'
V31A/V32A	5'-AACTACTGGAAAAGATAGAAATGCCGCTGGACCTAAACCGACCGTATTC-3'
CYP6B1BamHI(NF)	5'-GGCGGATCCGATGTGTATCTTTTAGCTCTTG-3'
CYP6B1HindIII(NR)	5'-GGCAAGCTTAGCGACATTCTTTTCTTCC-3'
CYP6B33HindIII(NF)	5'-GGCAAGCTTGGACCTAAACCGACCGTATTC-3'
CYP6B33(-HindIII)	5'-TGGAAGAAAAGAAATGTCGCTGGACCTAAACCGACCGTATTC-3
RFPHindIII	5'-GGCAAGCTTTTACTTGTACAGCTCGTCC-3'
RFPHindIII	5'-GGCAAGCTTTTATGATCTAGAGTCGCGGC-3'
CYP6B1linker	5'-GTTGAAATTTCTAGTAAAGTAGTAATGTAAAAGGCCGGC-3'
GFPlinker	5'-TATATCTTTACTTTACTAGAATGGTGAGCAAGGGCGAG-3'
RFPlinker	5'-CATTACTACTTTACCAGGATGGTGAGCGGCCTGCTG-3'