Expression of House Fly CYP6A1 and NADPH–Cytochrome P450 Reductase in *Escherichia coli* and Reconstitution of an Insecticide-Metabolizing P450 System†

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ABSTRACT: The house fly (*Musca domestica*) cytochrome P450 gene *CYP6A1* was expressed in *Escherichia coli*. The native protein was produced at a level of 0.25–0.34 μmol/L (15–20 mg/L) of culture with approximately 50% of the P450 being associated with the membrane fraction. The CYP6A1 protein was characterized spectrally and purified by a combination of hydrophobic interaction and hydroxyapatite chromatography. The house fly NADPH–cytochrome P450 reductase gene was also expressed in *E. coli*. Expression of a cytoplasmically directed reductase resulted in a protein that reduced cytochrome *c* but did not support P450 monooxygenase reactions. However, a periplasmically directed reductase was found to support monooxygenase reactions with CYP6A1 in a reconstituted system. The reconstituted system was effective in the epoxidation of the cyclodiene insecticides aldrin and heptachlor, with turnover rates of 12 and 34 min^{-1}, respectively. The enzyme showed little detectable activity in the *O*-dealkylation and *N*-dealkylation of various compounds that are metabolized by house fly microsomes. Incubation with polyclonal antisera raised against purified CYP6A1 inhibited the microsomal epoxidation of heptachlor by 65%. Under the same conditions, the metabolism of 7-methoxy-4-methylcoumarin was inhibited only slightly. The results suggest that CYP6A1 is a major cyclodiene epoxidase in the house fly and that multiple P450 forms are responsible for the elevated monooxygenase activities in insecticide-resistant flies.

Microsomal cytochrome P450 monooxygenases of insects are in many respects similar to the vertebrate enzymes. They are found in many insect tissues throughout development, are present in multiple forms, and are involved in the detoxification and activation of numerous xenobiotics (Hodgson, 1985). To date only six insect P450 sequences are available (Feyereisen, 1993), and they are all classified in the CYP4 and CYP6 families (Nelson et al., 1993). It has proven very difficult to obtain from the house fly (*Musca domestica*) (Fisher & Mayer, 1984; Moldenke et al., 1984; Ronis et al., 1988) or other species a homogeneous P450 of a single form suitable for reconstitution with NADPH–cytochrome P450 reductase. Several areas of research have been hampered by this difficulty. First, P450 enzymes have been implicated in many cases of insect resistance to insecticides, but the molecular mechanisms of resistance are poorly understood (Mullin & Scott, 1992). Second, P450 enzymes involved in molting hormone and juvenile hormone synthesis (Feyereisen, 1993) have been proposed as targets for selective inhibitors, but these enzymes need to be thoroughly characterized to allow the design of such potential insect control agents. Third, the detoxification of plant chemicals by inducible insect P450 has provided exquisite examples of adaptations of animals to naturally occurring chemicals (Cohen et al., 1992), but little is known of the evolution and substrate specificity of these enzymes. The cloning and heterologous expression of a P450 cDNA are thus needed to study the function of individual insect P450 forms. We present here our results on the expression of the house fly cytochrome P450 gene *CYP6A1* and of the house fly NADPH–cytochrome P450 reductase in *Escherichia coli*. The *CYP6A1* gene (Feyereisen et al., 1989) is inducible by phenobarbital (Cariño et al., 1992) and is overexpressed in several insecticide-resistant strains of the house fly (Cariño et al., 1992, 1994). High levels of constitutive expression suggest a possible role in metabolic insecticide resistance, but direct evidence for oxidative metabolism of insecticidal substrates by the enzyme CYP6A1 has been lacking. The metabolism of cyclodiene insecticides can now be demonstrated by *in vitro* reconstitution of CYP6A1 with house fly NADPH–cytochrome P450 reductase.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Bethesda Research Laboratories and Pharmacia. Avian reverse transcriptase was obtained from Pharmacia, and *Taq* polymerase was from Perkin-Elmer. The expression vector pSE380 was from Invitrogen, and pBluescript was from Stratagene. The substrates aldrin and heptachlor were from ChemService. Methoxyresorufin was from Molecular Probes Inc. 7-Methoxy-4-methylcoumarin, 7-methoxycoumarin, and 7-ethoxycoumarin were from Aldrich. $NADP^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were from Sigma.

Construction of Expression Vectors. Plasmid pSE380 was used for the expression of *CYP6A1* and the NADPH–cytochrome P450 reductase gene. This plasmid contains the inducible *trc* promoter fused with a ribosome binding site and a polylinker having an *Nco*I site at the end adjacent to the promoter region. An *Nco*I site was inserted at the 5′ end of the P450 coding region using PCR[1] mutagenesis. Cloning of this product into the plasmid *Nco*I site produced a precise fusion of the P450 coding region with *E. coli* control elements (Barnes et al., 1991).

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[1] Abbreviations: PCR, polymerase chain reaction; IPTG, isopropyl β-D-thiogalactopyranoside; MOPS, 4-morpholinepropanesulfonic acid; DTT, dithiothreitol; PMSF, *p*-phenylmethanesulfonyl fluoride; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

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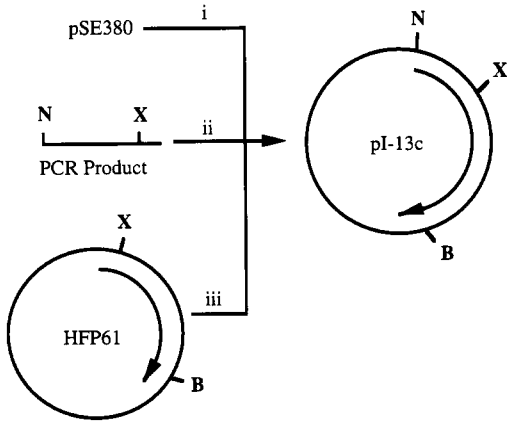

FIGURE 1: Construction of the CYP6A1 expression vector pI-13c. Digests: (i) *Nco*I–*Bam*HI, (ii) *Nco*I–*Xho*I, and (iii) *Xho*I–*Bam*HI. Abbreviations: N = *Nco*I: X = *Xho*I; B = *Bam*HI.

PCR mutagenesis was performed on the 5′ end of a *CYP6A1* genomic clone using the oligonucleotide primer CCGTCGACGCCATGGCTTTCGGTTCATTTC and the T3 sequencing primer. Creation of the *Nco*I site resulted in a change in the second coded amino acid residue in the wild-type sequence from aspartic acid to alanine. A partial cDNA of *CYP6A1* (HFP61) encoding all but the N-terminal 33 amino acids of the protein was used to remove a 60-bp intron found in the genomic sequence from the protein coding region (M. B. Cohen, J. F. Koener, and R. Feyereisen, unpublished data). The *Xho*I–*Bam*HI fragment of HFP61 was ligated with *Nco*I–*Xho*I-digested PCR product and *Nco*I–*Bam*HI-digested pSE380 to yield the expression vector pI-13c (Figure 1). Sequencing of the PCR-amplified portion of the vector showed no changes in the wild-type sequence outside of those introduced by the PCR primer.

A partial cDNA (HFP621) was available for the NADPH–cytochrome P450 reductase which lacked the coding sequence for the N-terminal 147 amino acid residues (Koener et al., 1993). RNA sequencing had established the putative cDNA sequence for this region, and this was verified by sequencing of a genomic clone (Koener et al., 1993). The presence of two introns in the genomic clone between the translational start and the 5′ end of the available cDNA made the genomic clone unsuitable as a template for PCR mutagenesis. A cDNA for the 5′ region of the gene was therefore obtained by reverse transcription PCR using an internal primer to prime cDNA synthesis from messenger RNA obtained from the abdomens of phenobarbital-induced female Rutgers house flies. A mutagenic oligonucleotide (CCGGTCGACGCCATGGCTGCGGAACACGTCGAAG) containing an *Nco*I site was added to the heteroduplex, and PCR was performed with the primer CACAAACAGATGGCC (internal reductase sequence). The *Nco*I–*Dra*I-digested PCR product was then ligated with the *Dra*I–*Sst*I fragment of the reductase cDNA (HFR621) and *Nco*I–*Sst*I-digested pSE380, giving the expression plasmid pI-32 (Figure 2). The *E. coli* strain XL-1 Blue was transformed with this plasmid, and the resulting strain was used for expression experiments.

A second reductase expression vector was constructed in the secretion vector pUHHG (J. G. Utermohlen, E. M. Hersh, and W. J. Grimes, unpublished data). This plasmid contains the *tac* promoter, the pelB signal sequence from *Erwinia chrysanthemi*, and an ampicillin resistance element. An oligonucleotide (CATCCCGGGAATGGCTGCGGAACAC) was used to insert an *Sma*I site directly upstream of the translational start in pI-35 by PCR (reverse primer CACAAACAGATGGCC as above). This site was situated to

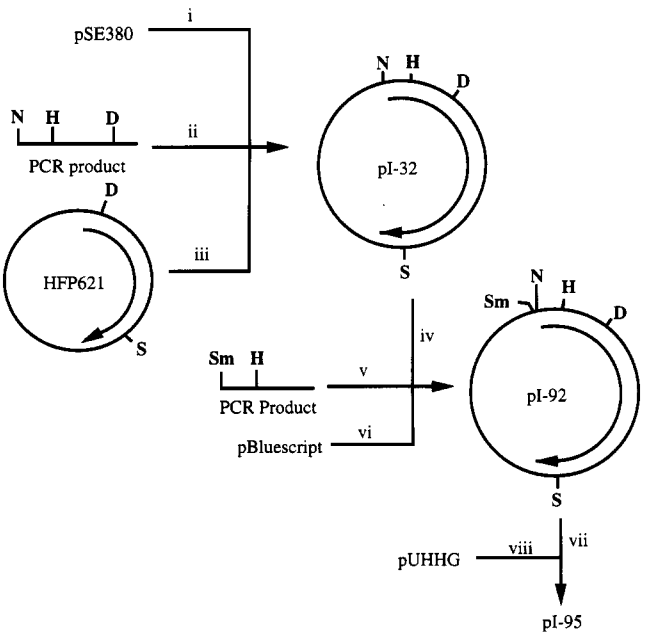

FIGURE 2: Construction of NADPH–cytochrome P450 reductase expression vectors. The cytoplasmic expression vector pI-32 was constructed in pSE380, whereas the periplasmic expression vector pI-95 was constructed in pUHHG. Digests: (i) *Nco*I–*Sst*I, (ii) *Nco*I–*Dra*I, (iii) *Dra*I–*Sst*I, (iv) *Hin*dIII–*Nco*I, (v) *Sma*I–*Hin*dIII, (vi) *Sma*I–*Sst*I, (vii) *Sma*I–*Sst*I, and (viii) *Sma*I–*Sst*I. Abbreviations: N = *Nco*I; H = *Hin*dIII; D = *Dra*I; S = *Sst*I; Sm = *Sma*I.

give an in-frame fusion of the reductase sequence with the pelB signal sequence (Figure 2). The PCR product was digested with *Sma*I and *Hin*dIII and ligated with the *Hin*dIII–*Sst*I fragment of pI-32 and *Sma*I–*Sst*I-digested pBluescript to give pI-92. The *Sma*I–*Sst*I insert from pI-92 was then excised and ligated with *Sma*I–*Sst*I-digested pUHHG, giving the expression vector pI-95 (Figure 2). This plasmid was transformed into *E. coli* strain DH5α*lac* I^q. Sequencing of the PCR-amplified portion of pI-95 revealed an apparent replication error resulting in the substitution of aspartic acid for lysine at position 40, which is thought to be in the membrane insertion domain of the reductase structure (Koener et al., 1993). This mutation in the N-terminal portion of the protein did not appear to influence the activity of the enzyme (see below).

Purification of CYP6A1 from E. coli(pI-13c). Erlenmeyer flasks containing 150 mL of Terrific Broth (with ampicillin at 50 μg/mL) were inoculated with 15 mL of an overnight culture of *E. coli*(pI-13c). The flasks were shaken at 250 rpm and 37 °C until the A_{550} of the culture was 0.5. IPTG was then added to a concentration of 1 mM, and the flasks were shaken at 150 rpm and 28 °C for 48 h (Barnes et al., 1991). The cells were pelleted by centrifugation at 3500*g*, washed with 100 mM MOPS, pH 7.3, and pelleted a second time. The cell pellet was suspended in 10 mL of 100 mM MOPS, pH 7.3, 1.0 mM EDTA, 0.2 mM DTT, and 10% glycerol (buffer A) and sonicated using four 15-s pulses on a Branson 450 sonifier. PMSF was then added to 0.2 mM, and cell debris was removed by centrifugation at 10000*g* for 10 min. The supernatant was centrifuged at 100000*g* for 1 h to pellet the membrane fraction, and the 100000*g* pellet was resuspended in 3 mL of buffer A.

Cytochrome P450 was detected spectrophotometrically (as described below) in both the soluble and membrane bound fractions, so total cell lysates were used for purification of CYP6A1. CHAPS was added to the total lysate to a concentration of 0.5%, and the suspension was stirred at 4 °C for 4 h. The solubilized lysate was centrifuged at 100000*g*,

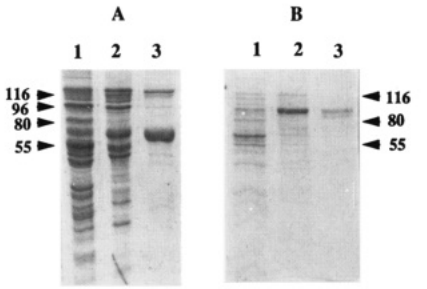

FIGURE 3: Purification of CYP6A1 and NADPH–cytochrome P450 reductase from *E. coli* expression strains. Panel A: CYP6A1 purification from *E. coli*(pI-13c): SDS–PAGE of solubilized cell lysate (lane 1), (ω-aminooctyl)agarose eluent (lane 2), and pooled P450-containing fractions from a hydroxyapatite column (lane 3). Panel B: NADPH–cytochrome P450 reductase purification from *E. coli*(pI-95): SDS–PAGE of the 100000*g* pellet (lane 1), CHAPS-solubilized protein from the 100000*g* pellet (lane 2), and pooled NADPH–cytochrome *c* reductase fractions from a TSK-Phenyl column (lane 3). Both gels were stained with Coomassie blue, and arrows indicate the sizes of molecular weight markers.

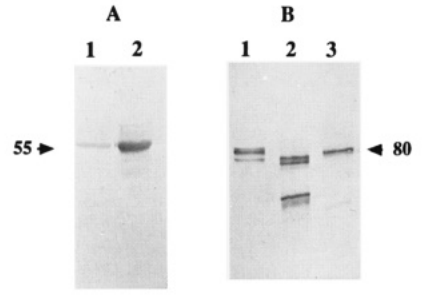

FIGURE 4: Immunoblots of CYP6A1 and NADPH–cytochrome P450 reductase from phenobarbital-induced house fly microsomes and from the expression strains *E. coli*(pI-13c), *E. coli*(pI-32), and *E. coli*(pI-95). Panel A: Detection of CYP6A1 after SDS–PAGE and western blotting of a microsomal preparation from phenobarbital-induced Rutgers house flies (lane 1) and a cell lysate from expression strain pI-13c (lane 2). Panel B: Detection of cytochrome P450 reductase after SDS–PAGE and western blotting of purified reductase from *E. coli*(pI-95) (lane 1), cell lysate from *E. coli*(pI-32) (lane 2), and a microsomal preparation from phenobarbital-induced Rutgers house flies (lane 3).

and the supernatant was applied to a column of (ω-aminooctyl)-agarose. The column was washed with 500 mL of 100 mM KH_2PO_4, pH 7.3, 0.2 mM DTT, and 10% glycerol (buffer B). The cytochrome P450 was then eluted with 150 mL of buffer B containing 0.2% Emulgen 911 and 0.4% sodium cholate. The P450-containing eluate was dialyzed against two changes of 10 mM NaH_2PO_4, pH 7.3, 10% glycerol, and 0.2 mM DTT and applied to a TSK HA-1000 hydroxyapatite HPLC column. This column was eluted with a gradient from 10 to 100 mM NaH_2PO_4, pH 7.3, containing 0.2 mM DTT over 30 min. The fractions showing significant absorbance at 416 nm were pooled. This procedure yielded detergent-free CYP6A1 estimated to be 80–95% pure (Figure 3A).

Purification of NADPH–Cytochrome P450 Reductase. The strain *E. coli*(pI-95) was grown and induced in a manner identical to that used for *E. coli*(pI-13c). After a 2-day induction, cultures were harvested and lysed as described above. Differential centrifugation was performed as described above to separate the cytoplasmic and membrane fractions. NADPH–cytochrome *c* reductase assays indicated that activity was present in both fractions, but western immunoblots showed that a full-length polypeptide was present only in the membrane fraction (Figure 4B). Membrane proteins were solubilized by adding CHAPS to 0.5% and stirring at 4 °C for 4 h. After centrifugation at 100000*g*, the solubilized membrane fraction was diluted 2-fold in buffer B, applied to a TSK-Phenyl column equilibrated with buffer B, and eluted with a gradient to 100% buffer B containing 0.2% Emulgen 911 and 0.4% sodium cholate (Wheelock & Scott, 1989). Fractions having cytochrome *c* reductase activity were pooled. Analysis by SDS–PAGE revealed a band with a molecular weight of about 80 000 representing the full-length reductase and a less prominent lower molecular weight species (Figure 3B).

Enzyme Assays. All cytochrome P450 assays were performed in 500 μL of 100 mM MOPS, pH 7.3, containing 1.2 mM $NADP^+$, 100 mM glucose 6-phosphate, 0.12 unit of glucose 6-phosphate dehydrogenase, 1.6 units of reductase (as the solubilized membrane fraction from the expression strain), and 0.015–0.065 nmol of CYP6A1. The final concentration of CHAPS in the assays was 3.25 mM, which is close to the optimal concentration of CHAPS for reconstitution of CYP2B4 activity (Wagner et al., 1984). The reaction mixtures were incubated for 5–15 min at 30 °C. The aldrin and heptachlor assays were stopped by vortexing with 1.5 mL of isooctane. Products of aldrin and heptachlor assays were analyzed by gas–liquid chromatography at 210 °C on a 5% SP2401 column (Supelco) with electron-capture detection. Products were identified by retention time comparison with authentic standards. 7-Methoxy-4-methylcoumarin, 7-methoxycoumarin, 7-ethoxycoumarin, and methoxyresorufin *O*-dealkylation were measured by fluorescence after the reactions were stopped with perchloric acid as described previously (Feyereisen & Vincent, 1984). *N*-Demethylation of *p*-chloro-*N*-methylaniline was assayed as described by Snyder et al. (1993), and benzphetamine *N*-dealkylation was measured as an adaptation of the aminopyrine assay described by Tate et al. (1982).

The reduction of cytochrome *c* by NADPH–cytochrome P450 reductase was measured as previously described (Feyereisen & Vincent, 1984). For the screening of chromatographic fractions, reactions were run in 200-μL volumes in microtiter plate wells and read at 550 nm with a Molecular Devices Thermomax kinetic plate reader. For kinetic studies reactions were run in 1-mL cuvettes at 30 °C, and the reaction progress was measured at 550 nm with a Perkin-Elmer Lambda 19 spectrophotometer.

Spectral Studies. Cytochrome P450 content was measured by the method of Omura and Sato (1964) on a Perkin-Elmer Lambda 19 spectrophotometer. Type I difference spectra were obtained from a 1.0-mL suspension of *E. coli*(pI-13c) membrane fraction in 100 mM MOPS, pH 7.3, at a P450 concentration of 0.5 μM. Ligands were added to the cuvette in ethylene glycol monomethyl ether. Spectra were recorded at 25 °C between 380 and 500 nm. Data were analyzed by linear regression of substrate concentration vs the ratio of the substrate concentration to the proportion of the maximal spectral shift from 420 to 390 nm, and all regressions exhibited r^2 values of at least 0.994.

Immunoinhibition Experiments. Antisera to purified native CYP6A1 were raised in New Zealand White rabbits by standard procedures. The IgG fraction was purified by bulk adsorption of sera to DEAE-Sepharose after dialysis against 10 mM K_2HPO_4, pH 6.5. Immunoinhibition experiments were performed in 500-μL volumes using the reconstituted system described above. The IgG content of the reaction was held constant at 8.0 mg/reaction by varying the ratio of anti-CYP6A1 to preimmune serum. Microsomes were prepared from the abdomens of phenobarbital-treated female Rutgers house flies by previously described methods (Feyereisen & Vincent, 1984). All reaction components with the exception of substrate were combined, and antiserum was added. The mixtures were incubated for 10 min at room temperature, and substrate was added in 10 μL of ethylene glycol monomethyl

Table 1: Distribution of CYP6A1 in Cell Fractions of *E. coli*(pI-13c)

fraction	total protein (mg)	total P450 (mg)	specific content (nmol of P450/ mg of protein)
cytoplasmic[a]	93.6	1.25	0.24
membrane[b]	16.8	1.01	1.05

[a] 100000*g* supernatant. [b] 100000*g* pellet. Values are based on a 150-mL culture as described under Materials and Methods.

ether. The reaction mixtures were then incubated at 30 °C for 20 min and analyzed as described above.

Other Procedures. Total protein was measured using the bicinchoninic acid method or, when interfering detergents were present, by the Lowry procedure after protein precipitation. Sequencing was done by the chain-termination method of Sanger et al. (1977). For western immunoblots, proteins were transferred to nitrocellulose and incubated with anti-CYP6A1 (diluted 1:1000) or anti-reductase antibodies (Feyereisen & Vincent, 1984) for 1 h, and a goat anti-rabbit IgG–alkaline phosphatase conjugate was used for visualization of reactive bands.

RESULTS

Expression of CYP6A1. A CYP6A1 cDNA was constructed by PCR with a mutation in the second codon from GAT (Asp) to GCT (Ala). This cDNA, suitable for expression in *E. coli*, was cloned into the vector pSE380. *E. coli* cells were transformed, and cytochrome P450 was detected in the cell lysate after an induction period of 48 h at 28 °C by a characteristic reduced CO/reduced difference spectrum with a peak at 448 nm.

Fractionation of crude cell lysates by differential centrifugation indicated that native cytochrome P450 was present in both the soluble and membrane-bound fractions (Table 1). The total P450 content was 15.1 mg/L of culture, representing approximately 2% of the total cell protein (Table 1). Approximately 50% of the measured P450 was found in the 100000*g* supernatant, although the membrane fraction had a higher specific content of P450 (Table 1). The soluble P450 was not proteolytically degraded, as shown by western blot analysis. Moreover, fractionation in 10 instead of 100 mM MOPS buffer did not have any effect on the ratio of membrane-associated to soluble P450 (data not shown). With a reconstituted cytochrome P450–P450 reductase system that will be described in more detail below, both the soluble fraction and the suspended membranes showed the ability to epoxidize the cyclodiene insecticide aldrin.

Fractionation of the solubilized cell lysate by hydrophobic interaction and hydroxyapatite chromatography resulted in the purification of a protein with a reduced CO/reduced difference spectrum identical to that of the cell lysate. When analyzed by SDS–PAGE, the purified enzyme showed a M_r of 57 000 as predicted by the DNA sequence (Figure 3A). Western immunoblots probed with polyclonal antisera raised against purified CYP6A1 showed that CYP6A1 purified from the *E. coli* expression strain corresponded in mobility to a single immunoreactive band in microsomes from phenobarbital-induced house flies (Figure 4A). The specific content of cytochrome P450 (+P420) in purified preparations (3.5–5.75 nmol/mg) was somewhat below the expected value, indicating the presence of a high level of apoprotein, possibly due to a loss of the heme chromophore during purification or to a lack of complete incorporation of heme into the expressed protein. This low value was not caused by a low CO affinity of the reduced hemoprotein.

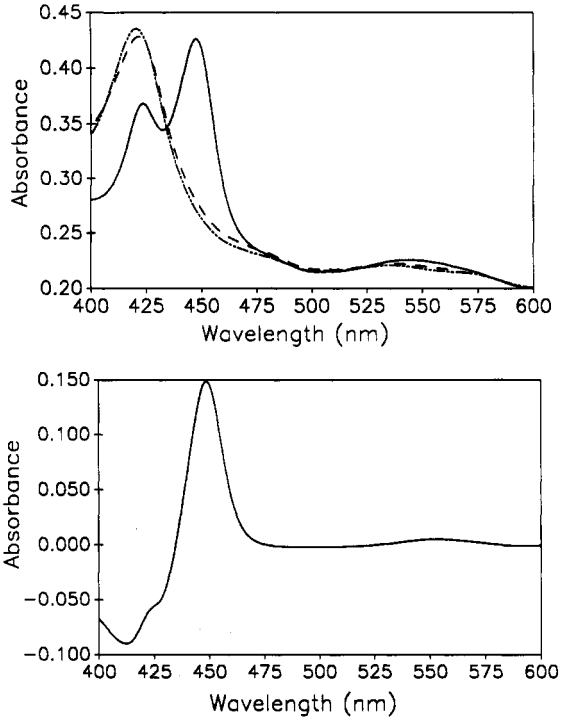

FIGURE 5: Panel A (top): Absolute spectra of oxidized (- · · -), reduced (- - -) and CO-bound reduced CYP6A1 protein (—). Panel B (bottom): Difference spectrum of the ferrous CO complex of CYP6A1 minus the ferrous enzyme. The spectra were taken with a hydroxyapatite-purified CYP6A1 preparation from *E. coli*(pI-13c) (see Materials and Methods) at 0.34 mg of protein/mL.

Spectral Characterization of CYP6A1. The absolute spectra of oxidized, reduced, and CO-bound reduced CYP6A1 protein were typical of P450 proteins (Figure 5). When the purified enzyme was reconstituted with NADPH–cytochrome P450 reductase and an NADPH-generating system, the maximum of the reduced CO/reduced difference spectrum was recorded at 448.0 nm. The ferrous CO complex was unstable, after both NADPH and dithionite reduction, being rapidly converted to cytochrome P420. The half-time of conversion in the solubilized cell lysate was 5.3 min at 25 °C and 19.9 min at 10 °C. The reduced enzyme appeared more stable than its CO complex. In contrast, oxidized P450 incubated at 25 °C for 10 min followed by rapid reduction and CO binding showed little conversion to P420 when compared to a control kept at 0 °C. Moreover, addition of 200 μM heptachlor increased the half-time for conversion of the reduced CO complex to 34.9 min at 25 °C, suggesting that ligand binding offset the destabilization caused by CO binding to the reduced enzyme.

The membrane-bound enzyme was essentially in the low-spin form as indicated by analysis of the second-derivative spectra (Guengerich, 1983). Membrane-bound CYP6A1 gave a typical type I binding spectrum with a number of potential substrates. The type I spectrum obtained with aldrin (Figure 6) was characterized by a peak at 390 nm and a trough at 424 nm. Spectral dissociation constants measured by titration of the membrane-bound enzyme with ligand indicated that aldrin and heptachlor had a high affinity for CYP6A1, whereas 7-methoxy-4-methylcoumarin and ecdysone bound weakly to the enzyme. Benzphetamine gave a normal type I spectrum with CYP6A1, and the spectral dissociation constant was similar to that observed with microsomes from phenobarbital-treated Rutgers fly abdomens (Table 2).

Expression of NADPH–Cytochrome P450 Reductase. An NADPH–cytochrome P450 reductase expression vector (pI-32) was first constructed using the same methodology as was

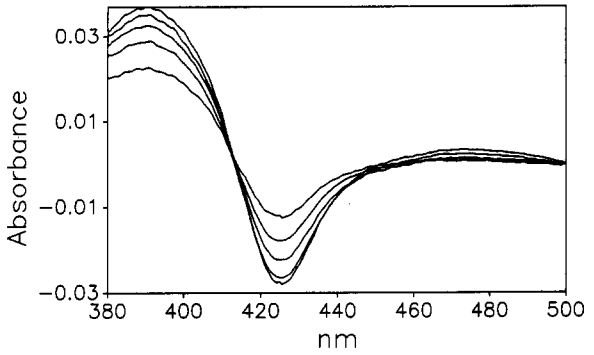

FIGURE 6: Aldrin type I difference spectra with CYP6A1. A suspension of the membrane fraction of *E. coli*(pI-13c) at 0.5 μM P450 was titrated with aldrin at final concentrations of 16, 36, 56, 76, and 116 μM.

Table 2: Spectral Dissociation Constants (K_s) for CYP6A1 Ligands[a]

ligand	K_s (μM)
aldrin	13.5
heptachlor	11.7
7-methoxy-4-methylcoumarin	485
ecdysone	155
benzphetamine	65.8
benzphetamine (microsomes)[b]	77.2

[a] All values were determined by linear regression (see Materials and Methods). [b] Microsomal fraction prepared from abdomens of phenobarbital-induced flies.

Table 3: Monooxygenase Reactions by the Reconstituted CYP6A1–NADPH–Cytochrome P450 Reductase System

substrate[a]	turnover (min^{-1})
aldrin	12.22 ± 0.98[b]
heptachlor	34.23 ± 7.09
7-methoxy-4-methylcoumarin	<0.05
7-ethoxycoumarin	<0.05
7-methoxycoumarin	<0.05
p-chloro-*N*-methylaniline	<0.10
methoxyresorufin	<0.05
benzphetamine	<0.10
ecdysone	<0.02

[a] Substrate concentrations were 0.1 mM for aldrin and heptachlor, 0.2 mM for the alkoxycoumarins, 1.2 mM for *p*-chloro-*N*-methylaniline, 0.04 mM for methoxyresorufin, 0.05 mM for benzphetamine, and 0.04 mM for ecdysone. [b] Mean and standard error of triplicate assays.

used for CYP6A1, i.e., with a second codon mutated from AGC (Ser) to GCT (Ala). After a 48-h induction, the crude lysate as well as the soluble and membrane fractions showed cytochrome *c* reductase activity (data not shown). Reconstitution of solubilized CYP6A1 and P450 reductase solubilized from the membrane fraction of *E. coli*(pI-32) with CHAPS failed to show any aldrin epoxidation activity. However, when purified rat cytochrome P450 reductase (kindly provided by Dr. J. A. Halpert, Department of Pharmacology and Toxicology, University of Arizona) was substituted for the solubilized *E. coli*(pI-32) membrane fraction, the formation of dieldrin was observed, suggesting that the cloned house fly reductase was not functional. An immunoblot containing microsomal protein from phenobarbital-induced house flies and the cell lysate from *E. coli*(pI-32) and probed with a polyclonal antibody raised against the purified house fly enzyme (Vincent & Feyereisen, 1984) showed that the largest product from the expression strain lysate was slightly smaller than the house fly enzyme but that the full-length product was absent (Figure 4B).

To reduce the likelihood of proteolytic degradation of the reductase, the expression vector pSE380 was replaced by a

Table 4: Apparent Kinetic Constants for Cytochrome *c* Reduction and NADPH Oxidation by NADPH–Cytochrome *c* Reductase and for Heptachlor and Aldrin Epoxidation by a Reconstituted CYP6A1–P450 Reductase System

A. Reductase

substrate	K_M (μM)	V_{max} [μmol min^{-1} (mg of protein)$^{-1}$]
cytochrome *c*[a]	9.03 ± 0.48	38.5 ± 0.9
NADPH[b]	4.34 ± 0.33	34.7 ± 0.7

B. CYP6A1

substrate	K_M (mM)	V_{max} [nmol $nmol^{-1}$ min^{-1}]
heptachlor	0.19 ± 0.07	74.0 ± 19.0
aldrin	0.07 ± 0.02	30.7 ± 7.3

[a] NADPH concentration constant at 300 μM. [b] Cytochrome *c* concentration constant at 60 μM.

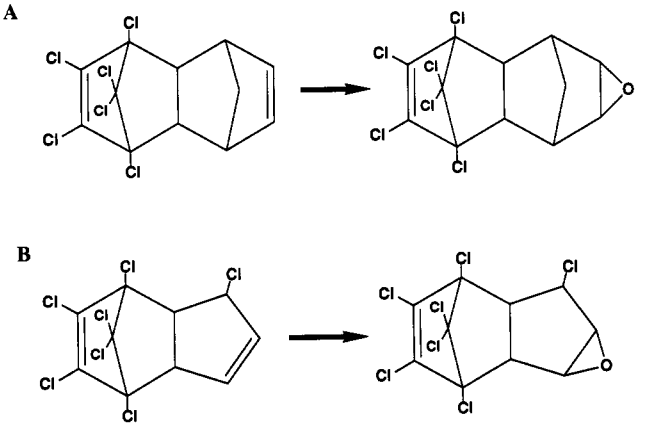

FIGURE 7: Epoxidation of aldrin to dieldrin (A) and heptachlor to heptachlor epoxide (B).

secretion vector (pUHHG) to give the plasmid pI-95. Fractionation of a lysate of *E. coli*(pI-95) into cytoplasmic and membrane fractions revealed that the cytochrome *c* reductase specific activity was 5-fold higher in the membrane fraction than in the soluble fraction. An immunoblot of the latter showed that it contained almost exclusively lower molecular weight products, including a very prominant form of M_r ca. 45 000. Hydrophobic interaction chromatography of the CHAPS-solubilized membrane fraction yielded a purified product that possessed cytochrome *c* reductase activity. Immunoblotting showed that it was a full-length protein product (M_r ca. 80 000) appearing as a doublet band (Figure 4B). The doublet nature was probably due to incomplete processing of the pelB signal peptide. Also present was a shorter product that corresponded approximately in length to the largest product of the cytoplasmic expression system (Figure 4B). Kinetic parameters (Table 4) for cytochrome *c* reductase activity of the purified protein were similar to those previously reported for house fly reductase purified from microsomes (Mayer & Durrant, 1979; Vincent & Terriere, 1985).

Reconstitution of the CYP6A1–House Fly Reductase System. The reconstituted CYP6A1–reductase system contained hydroxyapatite-purified CYP6A1 and the CHAPS-solubilized membrane fraction from the reductase expression strain *E. coli*(pI-95) at a molar ratio of P450 to reductase of ca. 1:30. The cyclodiene insecticides aldrin and heptachlor were epoxidized by this system to the products dieldrin and heptachlor epoxide, respectively (Figure 7), but none of the other potential substrates tested were converted to products at a significant rate under these conditions (Table 3). The *E. coli*(pI-13c) lysate was unable to support epoxidation without added reductase.

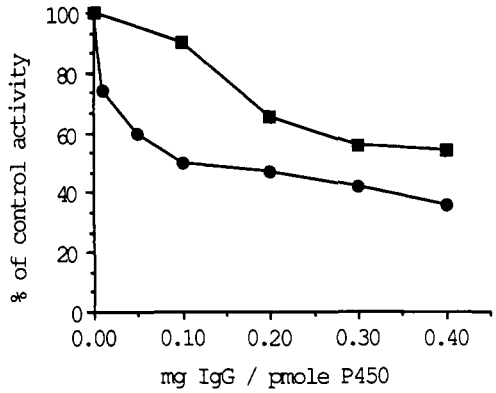

FIGURE 8: Immunoinhibition of microsomal monooxygenase activities with anti-CYP6A1. Preimmune serum and anti-CYP6A1 were added to reaction mixtures to give a total of 8.0 mg of IgG/reaction. Enzymatic activity was measured at increasing ratios of anti-CYP6A1 to preimmune serum and expressed as a percentage of the control activity, i.e., the activity with only preimmune serum. (●) Heptachlor epoxidation by induced microsomes; (■) 7-methoxy-4-methylcoumarin *O*-demethylation by control microsomes. Each point is the mean of triplicate assays.

Kinetic constants were estimated for the reconstituted system with aldrin and heptachlor. Because the K_M values were quite high for both substrates, solubility considerations limited data collection to substrate concentrations of less than $2K_M$ (Table 4). Estimates of V_{max} and K_M for both substrates showed that heptachlor is metabolized at a higher rate than aldrin but also has a K_M that is higher by about 2-fold. The similarity of the spectral dissociation constants for the two compounds (Table 1) suggests that the difference in catalytic rate is not due to a difference in binding affinity between the substrates, but may be due to a more favorable positioning of the target unsaturation of heptachlor in relation to the activated oxygen atom.

Immunoinhibition of Cyclodiene Epoxidase Activity in House Fly Microsomes. Immunoinhibition experiments were performed with microsomes from phenobarbital-induced female Rutgers house flies. More than 65% inhibition of heptachlor epoxidase activity was achieved with microsome preparations (Figure 8), although fairly large amounts of polyclonal antiserum were required to accomplish this (presumably due to a low titer of CYP6A1-specific antibodies). *O*-Demethylation of 7-methoxy-4-methylcoumarin (which is not a CYP6A1 substrate) was also inhibited substantially at high concentrations of antiserum. Half-maximal inhibition of heptachlor epoxidation was observed at a concentration less than one-tenth that causing inhibition of *O*-demethylation, suggesting that the antiserum is showing some specificity for CYP6A1.

DISCUSSION

The expression in *E. coli* of functional eukaryotic cytochrome P450 proteins (Barnes et al., 1991; Larsen et al., 1991; Fisher et al., 1992a; Wada & Waterman, 1992; Winters & Cederbaum, 1992; Hsu et al., 1993; Richardson et al., 1993; Palmer et al., 1993) and of a P450 reductase–P450 fusion protein (Fisher et al., 1992b) has recently been achieved. We have obtained high levels of expression of the house fly P450 CYP6A1 under the control of the regulatable promoter *trc* (Porter & Larson, 1991). For the expression of P450, only the second codon of CYP6A1 was mutated to Ala and a functional protein was obtained in both the soluble and membrane fractions. CYP2E1 expressed in *E. coli* with a translation enhancer sequence was also found in both fractions, with about 30% in the cytosol (Pernecki et al., 1993). Expression of a reductase protein functional in a reconstituted system required fusion of the reductase with the pelB signal peptide from *Erwinia chrysanthemi*. This signal directs secretion to the periplasm via the secB pathway. A similar system based on the ompA signal sequence has been described for the expression of rat cytochrome P450 reductase, resulting in an apparently full-length polypeptide possessing cytochrome *c* reductase activity (Shen et al., 1989) and capable of supporting the catalytic activity of CYP1A2 (Fisher et al., 1992a).

Reconstitution of the reductase–P450 system showed that CYP6A1 is a highly active epoxidase of the cyclodiene insecticides aldrin and heptachlor. The turnover rates observed with aldrin and heptachlor were higher than those reported for microsomes from Rutgers house fly abdomens, suggesting that CYP6A1 is the major cyclodiene-metabolizing P450 in the house fly. This conclusion is strengthened by the immunoinhibition results which showed that microsomal epoxidation of heptachlor is drastically inhibited by anti-CYP6A1 antibodies. Conversely, the catalytic rates for the other potential substrates tested were not significant, indicating that additional P450 forms are responsible for metabolism of these substrates *in vivo*. For instance, immunoinhibition studies have shown that P450Lpr is a house fly P450 form responsible for the majority of methoxyresorufin *O*-demethylation (Wheelock & Scott, 1992). There was a good agreement between the CYP6A1 spectral dissociation constants and the data from the reconstitution experiments. The substrates heptachlor and aldrin showed stronger binding affinities than benzphetamine, ecdysone, and 7-methoxy-4-methylcoumarin, which were not metabolized. Ecdysone is metabolized to the active steroid hormone 20-hydroxyecdysone by P450 enzymes in insect tissues, and hydroxylase activity has been detected in both microsomal and mitochondrial fractions (Weirich, 1989). CYP6A1 did not convert ^{3}H-labeled ecdysone to 20-hydroxyecdysone, showing that it does not contribute to the microsomal hydroxylase activity reported previously. Binding to P450 enzymes such as CYP6A1 may represent some of the low-affinity ecdysteroid binding observed in cellular binding studies.

The role of individual P450 forms in metabolic resistance to insecticides is largely unknown due to difficulties encountered in the isolation of active P450 enzymes from insect tissues (Hodgson, 1985). Several lines of evidence obtained in this and previous studies suggest that CYP6A1 is at least partially responsible for the elevated monooxygenase activities seen in microsomes from the (Diazinon-R) Rutgers strain of *Musca domestica* that is resistant to several classes of insecticides including cyclodienes. First, *CYP6A1* is expressed constitutively at high levels in the Rutgers strain and at low levels in the susceptible sbo strain (Cariño et al., 1994). This observation is consistent with earlier results showing that elevated cytochrome P450 levels and aldrin epoxidation levels in the Rutgers strain are correlated with insecticide resistance levels (Tate et al., 1974; Plapp et al., 1976). The absorption maximum of the reduced CO/reduced difference spectrum of CYP6A1 is also typical of the Rutgers strain, i.e., about 2 nm lower than that seen in most susceptible strains (Perry et al., 1971; Philpot & Hodgson, 1972). In contrast, methoxyresorufin and 7-methoxy-4-methylcoumarin are poor substrates for CYP6A1 and are not elevated in microsomal preparations from the Rutgers strain (Vincent et al., 1985). Second, *CYP6A1* transcription is strongly inducible by phenobarbital in both Rutgers and sbo strains (Cariño et al., 1992). It has previously been shown that phenobarbital treatment increases P450 levels in house fly larvae and adults

and increase the epoxidation of cyclodiene insecticides in particular (Yu & Terriere, 1973; Moldenke & Terriere, 1981; Feyereisen, 1983). Third, microsomes from resistant flies or phenobarbital-induced susceptible flies give a strong type I binding spectrum with benzphetamine, whereas microsomes from susceptible flies give a very weak or undetectable spectrum (Hodgson et al., 1974; Moldenke & Terriere, 1981). CYP6A1 obtained from the *E. coli* expression strain showed an easily detectable type I binding interaction with benzphetamine (Table 2). The type I binding interaction with benzphetamine was most closely linked to the diazinon resistance gene on chromosome II (Plapp et al., 1976), and the constitutive overexpression of *CYP6A1* is also linked to a locus on chromosome II (Cariño et al., 1994). The *CYP6A1* gene is located on chromosome V (M. B. Cohen, J. F. Koener, and R. Feyereisen, unpublished data), and the overexpression of the gene is thus controlled in *trans* by a chromosome II locus, probably in conjunction with other P450 genes and one or more glutathione *S*-transferases (Plapp, 1984). The mutation causing resistance in the Rutgers strain affects expression of *CYP6A1*, but *CYP6A1* alone does not explain the full resistance phenotype.

The expression and reconstitution methods described here will allow us to study the substrate specificity of individual forms of insect P450 and the role of P450 enzymes in the metabolism of insecticidal compounds. P450 monooxygenase reactions are known to be important in resistance to a variety of organic insecticides including organophosphorus compounds, chlorinated hydrocarbons, carbamates, and pyrethroids. They are also fundamentally involved in the activation reactions that convert less toxic thio derivatives of organophosphorus insecticides to the highly active oxon or sulfoxide forms. Resistance to these compounds must involve a shift in the balance of activation and detoxification reactions catalyzed by different P450 forms, most likely through a change in the pattern of P450 gene expression. As more insect P450 genes become available for study, these problems of regulation and substrate specificity will become experimentally approachable, with implications for the development of new insecticide synergists and the management of insecticide resistance. In addition, the ability to express insect P450 in a heterologous system and to reconstitute monooxygenase activities will also facilitate the study of the P450 enzymes responsible for hormone and pheromone metabolism in insects.

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