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Insects Cytochrome P450 Enzymes: Evolution, Functions and Methods of Analysis

Abdullah M. Alzahrani

Department of Biology, King Faisal University, Hofouf, Saudi Arabia

Abstract: Cytochrome P450s, also known as monooxygenases or mixed-functionoxidases (MFO), comprise a heme-thiolate superfamily of enzymes that catalyze various types of reactions and act upon various types of substrates. Having been found in all organisms, this gene family has evolved more than 3 billion years ago from a single gene which has gone subsequent gene-duplication events leading to the expansion of the P450s as diverse as they are today. In insects, they play an integral role in the metabolism of endogenous as well as exogenous compounds; they catalyze the metabolism of juvenile hormones, steroids, fatty acids, as well as the detoxification reactions of insecticide and plant allelochemicals. Thousands of insects P450 isoforms, both mitochondrial and microsomal, have been identified in the past few years. Our understanding of the specific function (s) of each isoforms is currently limited due to the difficulty of isolating membrane-free P450 enzymes. However, there has been an ever increasing effort to replicate and/or advance the current methods of studies, i.e. heterologous expression and subsequent crystallization studies, carried out mostly on human P450s.

Key words: Insects cytochrome P450 · Allelochemical metabolism · Heterologous expression · Plant alkaloids · Insecticide resistance

INTRODUCTION

Cytochrome P450s comprise a large, ancient superfamily of heme-thiolate proteins. The cytochrome P450 enzymes, sometimes referred to as MFO (mixed-function oxidases) or monooxygenases, have been found in every major domain of living organisms, *Bacteria*, *Archaea* and *Eukarya* [1]. This remarkably diverse ancient (evolved 3.5 billion years ago) class of isoenzymes catalyze very important and diverse oxidation reactions of both exogenous as well as endogenous steroid substrates [2, 3] hence the name mixed function oxidases. They are known to catalyze at least twelve different types of oxidation reactions on thousands of substrates [4]. Furthermore, P450s play a critical role in metabolizing endogenous metabolites including steroids, fatty acids and prostaglandins and a wide range of xenobiotics including pharmaceuticals, carcinogens, environmental pollutants, pesticides and plant allelochemicals [5, 6]. In this regards, it is widely believed that the P450s defense against foreign chemicals is comparable to the role of the immune system against foreign pathogen. P450s are, also, involved in sterol biosynthesis [7] and vitamin D homeostasis [2]. It appears that bacterial and mitochondrial P450s are more selective than microsomal P450s with the respect to the substrates they oxidize. Recently, however, some hepatic (mammalian) P450s have been found in mitochondria which display broader substrate specificity.

Almost all P450 enzymes isolated in eukaryotic organisms thus far are membrane-bound with the exception of nitric oxide reductase, which is a member of family CYP55 [1]. All prokaryotic P450s, however, are soluble [7]. In addition, most of the eukaryotic P450 enzymes are microsomal (e.g. located in the endoplasmic reticulum). However, several are known to be mitochondrial (embedded in the mitochondrial membrane).

Discovery and Nomenclature: The ability of mammalian tissues to metabolize non-polar xenobiotics was well-recognized as early as the 1950s. In an effort to identify the enzymes responsible for catalyzing the detoxification reaction of these compounds, G.R. Williams and M. Klingenberg, working at the Johnson Foundation, University of Pennsylvania, treated rat liver microsomes with a reducing agent (sodium dithionite)

Corresponding Author: Abdullah M. Alzahrani, Department of Biology, King Faisal University, Hofouf, P.O. Box 1758, 31982, Saudi Arabia

in the presence of CO gas. As a result of the formation of a CO-hemethiolate complex, they recorded a strong absorption band at 450 nm on a spectrophotometer [7]. In 1962, Omura and Sato, who were working at the same foundation, purified the enzyme responsible for the spectral absorbance peak at 450 nm and named it cytochrome P450. They also discovered that the P450 protein exhibits a spectral peak at 420 nm (cytochrome P420) when it is irreversibly denatured [8, 9]. The enzyme discovered by Omura and Sato was subsequently characterized and found to be a hepatic microsomal hydroxylase.

Since the discovery of P450, additional P450 proteins have been reported for almost all phyla including mammals, reptiles, amphibians, insects and other arthropods and bacteria. In the past few years, the number of identified P450s has increased almost exponentially. More than 750 sequences of P450 were known by mid-1998 and this number has already exceeded 11000 identified cytochrome P450 genes divided into 977 different families and 2519 subfamilies.

To distinguish among all of these proteins, a standardized nomenclature system was implemented. In the current system, cytochrome P450 genes or cDNA sequences are named with the italicized root CYP followed by an Arabic numeral for the gene family, a letter for the subfamily and another Arabic numeral for the gene number. For example, *CYP6G1* refers to a cytochrome P450, family 6, subfamily G and gene number 1. Proteins and mRNA are named in the same way but without italicizing the CYP designation [10]. Cytochrome P450s in the same gene family generally exhibit greater than 40% amino acid sequence identity while greater than 55% amino acid sequence identity indicates that two genes belong in the same subfamily [10, 11]. There are exceptions, however, (e.g., (*CYP2D* and *CYP11* and certain sequences within family 6) where genes with lower levels of sequence identity are still placed in the same family.

Initially, blocks of two digit numbers were assigned for different organisms. Thus, CYP families 1-49 were reserved for animals, families 51-69 for lower eukaryotes, families 71-99 for plants and family 101 and above for bacteria. However, because of the overwhelming number of newly discovered P450 gene families, new blocks have been added such that CYP1-CYP49 and CYP301-CYP499 have been reserved for animals, CYP71-CYP99 and CYP701-CYP999 for plants, CYP51-CYP69 and CYP501-CYP699 for lower eukaryotes, CYP101-CYP299 for bacteria [12]. A web page at the University of Tennessee, Memphis is maintained by David Nelson (http://drnelson.utmem.edu/nelsonhompage.html) on behalf of the P450 Nomenclature committee. This site contains the most recent P450 sequence information.

P450 Structure: Generally, P450s enzymes range between 40 to 50 kDa in size and are around 500-600 amino acids in length. A P450 enzyme is composed of the α-helices domain (A'-A-B-B'-C-D-E-F-F'-G'-G-H-I-J-K'-K"-K-L) and the β-sheets domain with the active site buried inside the folded protein. The active site includes a haem-prosthetic group which contains an iron atom that is associated with six ligands. Four of those ligands are nitrogenous which are included in the porphyrin ring around the iron atom and the fifth is a negatively charged sulphur atom as part of a conserved cysteine amino acid among all P450s this cysteine is part of the so called heme-binding decapeptide FXXGXXXCXG (where X refers to un-conserved amino acid). The sulphur, referred to by chemist as thiolate anions, is the axial ligand of the iron atom and hence the name haem-thiolate proteins. Unlike most hemoproteins such as myoglobins, P450s has a unique spectroscopic feature which is the reason behind their discovery and names. When the iron atom is reduced (shifts from low to high spin state) in the presence of carbon monoxide (CO), P450s absorb light at the 450-nm wavelength which led to naming them as such [9]. There are other hemoproteins such as chloroperoxidase, nitric oxide synthase, thromboxane synthase, who have thiolate ligand and therefore share the spectroscopic character of P450 proteins.

Almost all eukaryotic P450s are membrane-bound enzymes found mostly in the endoplasmic reticulum or in the mitochondrial membrane. The membrane-embedded nature of eukaryotic P450s, their interaction with lipids, hydrophilic substrates and the hydrophobic domains of P450-reductases have made the detergent-free purification of catalytically functional P450 enzymes extremely difficult. For those reasons, only a few eukaryotic P450 enzymes have been recently crystalized to, CYP55A1 from the fungus *Fusarium oxyporum*, a modified form of CYP2C5 from rabbit and CYP2B4 [13-15], human CYP2A6, a modified human CYP2C9, CYP2C8, CYP3A4, CYP2D6 [16-20]. However, the structures of nine soluble bacterial P450 enzymes have been determined by X-ray crystallography including $P450_{cam}$ (CYP101) from *Pseudomonas puitda*), $P450_{terp}$ (CYP108) from *Pseudomonas* spp, the heme-containing domain of

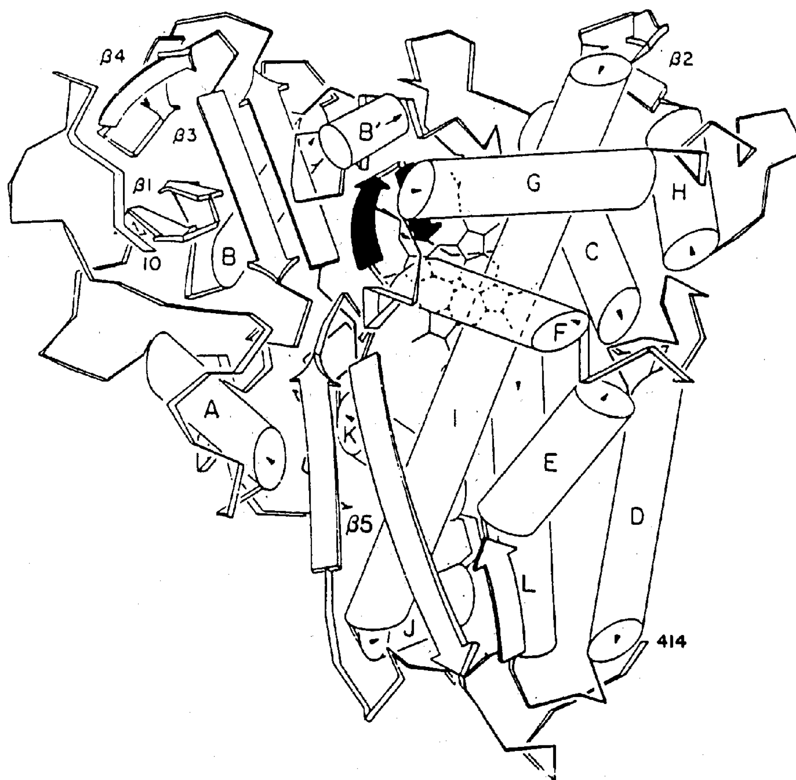

Fig. 1: Crystal structure of cytochrome $P450_{cam}$ showing the overall dimensions of the protein and location of the active site in the interior of the protein [7]

P450BM-3 (CYP102) from *Bacillus megaterium,* $P450_{eryF}$ (CYP 107A1), CYP111A1, CYP119A1, CYP121A1, CYP152 and CYP175A1 [1, 7, 21]. The structure of several other prokaryotic cytosolic as well as eukaryotic membrane-bound P450s has been predicted based on the DNA sequences similarity between the genes encoding these enzymes and the genes encoding the enzymes whose crystal structure has been resolved. Along with some biochemical and physiological experiments, the fact that P450BM-3 and the microsomal P450 enzymes are reduced by cytochrome P450 reductase supports the existence of some structural similarity between all members of cytochrome P450 superfamily. Furthermore, P450BM-3 is more similar to the mammalian family 4 enzymes (25-30%) than to $P450_{cam}$, or $P450_{terp}$ (15-20%) [7].

Based on the three dimensional structure of $P450_{cam}$, which has been solved to 1.63Å resolution [22] two decades ago the geometry of $P450_{cam}$can be described as a triangular prism with a depth of about 60 Å and a width of 30 Å [23]. Structurally, the protein consists of 12 α-helices, β-sheets and β-turns (Figure 1). The heme prosthetic group is buried between the I helix (on the distal side) and the L helix (on the proximal side). The heme prosthetic group is linked by hydrogen bonds to the absolutely conserved, heme-ligating Cys-357 (at the beginning of the L helix) and the basic residues Arg-299 and His-355 [4, 7]. The hydrophobic residues in the amino terminus of microsomal P450 enzymes are partially responsible for binding the enzyme to the endoplasmic reticulum or the mitochondrial membrane. This N-terminal region has been removed in almost all crystallized P450s along with mutating some key residues on the surface in order to obtain a soluble P450 that can be crystallized and be used for structural and functional studies. This engineering work on P450s was first used to resolve the structure of CYP2C5 by the group of Jhonson [24].

Despite the huge diversity of P450s, there are some highly conserved regions shared by all P450s across all phylla. For instance, the central and carboxy terminal of the I helix, the heme decapeptide (which includes a cysteine residue that is absolutely conserved in all P450s) and four out of five strands of β1-sheet (1-1, 1-2, 1-3, 1-4). In addition, helices C, E, K, as well as the meander region

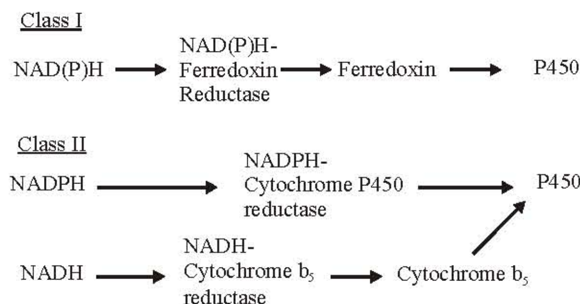

Fig. 2: Classification of cytochrome P450 monooxygenases. Class I enzymes are found in the eukaryotic mitochondria and in most bacteria and require NAD(P)H-ferredoxin reductase and ferredoxin. Class II enzymes are bound to the endoplasmic reticulum and interact directly with NADPH-P450 reductase (redrawn from [29]).

are also conserved. On the contrary, there are some variable regions such as helices B, D, J', G, H and β5. The conserved regions form the hydrophobic core of the enzyme while the variable regions represent the surface of the enzyme.

The beginning of the L helix contains the heme-binding site which is also known as cys-pocket (referring to the absolutely conserved fifth ligand cysteine residue). There are three other residues that are highly conserved: 2 glycine residues and a phenylalanine residue. The first glycine residue forms the β-hairpin while the second facilitate the transition between the cys-pocket and the L-helix. The phenylalanine residue plays a pivotal role in shielding the active site to ensure a hydrophobic environment around the active site. Another conserved sequence in the c-helix is WXXXR which further stabilizes the heme through the interaction of tryptophan and arginine in this sequence with the propionate side chain of the heme [25].

The meander region is another conserved was named as such because it seemed at the beginning as irrelevant to the structure of P450s. This region is about 20 residues at the N-terminal of the cys-pocket and contains the ERR-triad. The glutamine and the first arginine are from the K helix while the second arginine is from the meander region. The last arginine could be histidine or asparagines in some P450s.

The central and carboxy terminus of the I helix (which participates in the formation of the oxygen binding pocket) are very highly conserved in all P450 enzymes [25]. A highly conserved threonine residue in this helix is believed to be the source of the catalytic protons as solvents are not allowed around the iron-bound oxygen to prevent the production of hydrogen peroxide. Additionally, upon the binding of the substrate, this threonine residue is also believed to causes a distortion in the I-helix which is then served as a pocket for binding the molecular oxygen [22]. Mutagenesis studies, however, has shown that this residue is not critical to the formation of the oxygen binding pocket [26].

Because of the high degree of conservation at both the I-helix and the heme-decapeptide at the L-helix, they are the best targets for degenerate PCR primers that are capable of amplifying a broad diversity of cytochrome P450 genes.

Evolution of Cytochrome P450s: The P450 superfamily is believed to have evolved approximately three and a half billion years ago from a single gene [27]. The P450s may have assumed the role of protecting early life forms from oxygen toxicity, when oxygen start to accumulate in the atmosphere about 2.8 billion years ago. This is supported by the increase/decrease of oxidative stress when uncoupling/coupling the oxygen reduction of substrates by P450 [28]. The P450 superfamily can be divided into two main classes of enzymes based on the mechanisms by which they receive their reducing equivalents from NADPH. The first class consists of enzymes that are found in bacteria and eukaryotic mitochondria. They receive electrons via two accessory proteins. Members of the second class, *i.e.*, the microsomal P450s, receive electrons from NADPH-cytochrome P450 oxidoreductase (Figure 2 [29]). Based on the common use of an iron-sulfur and a flavoprotein in the electron donor mechanism it has been postulated that the mitochondrial P450s and the prokaryotic P450s such as $P450_{cam}$ (CYP 101) share a common evolutionary ancestor and that the bacterial enzymes represent that more ancestral state. The microsomal P450s, in turn, could have arisen from a mitochondrial P450 following the transfer of DNA from the

mitochondrial to the nuclear genome. Conversely, they might have evolved from prokaryotic P450s via an unknown mechanism [11]. In terms of the evolution of gene family, it appears that the cholesterol side chain cleavage enzymes (CYP11) and cholesterol 27-hydroxylase (CYP27) are the result of a gene duplication event that happened 1.5 billion years ago. Ancient cytochrome P450s such as CYP11 and CYP27 may have evolved to help maintain membrane integrity of the earliest organisms by metabolizing steroids. Another expansion of the family is thought to have the happened about nine hundred million years ago. This resulted in rise of endogenous steroid-synthesizing P450s such as the mammalian CYP7 (cholesterol 7α-hydroxylase), CYP19 (involved in bile acid and estrogen synthesis) as well as CYP21 (steroid 21-hydroxylase). The fatty acid-metabolizing family, CYP4 family, probably evolved a little later. CYP21 is believed to have subsequently diverged to give rise to the mammalian CYP1 and CYP2 families which are involved in metabolizing foreign compound [11]. CYP3 and CYP6 families are closely related and are believed to have diverged before CYP1 and CYP2 families [30]. The latest round of large scale gene duplication events is believed to have happened in the past 400 million years and to involve numerous CYP2 family members [31]. This expansion, which may have included as many as 30 gene duplication events, is thought to have been the result of animal-plant warfare. As animals start ingest plant 1200 million years ago, plants responded by developing phytoalexins then animals had to look into their P450 arsenal to detoxify the phytoalexins [31]. Moreover, it has been hypothesized that xenobiotic-metabolizing P450s, e.g., members of CYP1 and CYP2 families, originally evolved for a different function such as controlling cell division, but then assumed a role in detoxification. This hypothesis is based on the observation that some ligands for nuclear hormone receptors are able to induce xenobiotics-metabolizing genes and the induced P450 enzymes are able to degrade these ligands [11].

The sequence characteristics of P450s can be explained, in part, by the "molecular drive" theory. Some P450s could have evolved by means of DNA turnover events including DNA polymerase slippage, gene duplication by unequal crossing over, gene conversion and transposition [32, 33]. Gene conversion, which results in 100% identity between parts (or all) of previously diverged genes, seems to be a factor in P450 evolution. In CYP2D, for example, four genes (CYP2D2, CYP2D3, CYP2D4 and CYP2D5) appear to have undergone many gene conversion events. Two flanking regions (including exon 1) of CYP2D3 exhibit signs of having undergone gene conversion with CYP2D5. In CYP2D2, exon 6, 8 and part of exon 9 display high similarity to the same regions of CYP2D5 [31]. Conversely, gene duplication and speciation gives rise to paralogous (results of gene duplication events) and orthologous (genes diverged as a result of speciation events) genes. The generation of additional P450s may provide organisms with the tools to detoxify xenobiotics in evolving plants. This, in turn, may enable them to exploit a new food source [11]. In fact, gene conversion is not unique to the P450 gene superfamily gene conversion has been reported in other gene families including the major histocompatibility, immunoglobulin and the eggshell chorion protein gene families.

The evolution of the P450 superfamily is thought to reflect the pressure associated with exposure to potentially, toxic dietary substrates [31]. It appears that this evolution has also occurred quickly since the unit evolutionary period (UEP = the time required for a protein to show 1% amino acid sequence change since a divergence event) of most P450s ranges from two to four million years compared to hundreds of millions of years for many other gene families [34]. The UEP can be determined by comparing sequence divergence with the time of species divergence, based on the fossil record for that species. Based on this calculation, birds and mammals split 300 million years ago, the mammalian radiation occurred 80 million years ago with mice and rats diverging only 17 million years ago. By comparing the sequences of putatively orthologous P450 proteins in these species, it appears that P450s did not diverged in a linear fashion. Therefore, it is difficult to accurately determine the time when two P450s diverged. Moreover, back mutations and exchange of genetic information between closely related P450 genes complicates the estimate of divergence time between P450s [11].

Insect Cytochrome P450s

Diversity: The Full genome sequence of *Aedes aegypti, Anopheles gambiae, Apis mellifera, Bombyx mori, Drosophila melanogaster* and *Drosophila pseudoobscura* species has been completed and full length of P450 isoforms belonging to the aforementioned species can be viewed at URL http://p450.sophia.inra.fr/. Insects Cytochrome P450 enzymes play a critical role in the metabolism of endogenous as well as exogenous compounds. They are specifically important in their postulated role in conferring insecticide resistance.

Table 1: Tally of Insects Cytochrome P450s

Family	Sequence/ Family	Sequence/ Family
4	50	456
6	91	506
9	36	219
12	11	45
15	4	11
18	2	11
28	5	13
31	1	1
48	1	2
49	1	11
301	3	16
302	1	13
303	1	10
304	5	16
305	3	18
306	1	12
307	2	20
308	1	2
309	1	4
310	1	2
311	1	2
312	1	2
313	3	11
314	1	13
315	1	10
316	1	2
317	1	3
318	1	2
321	4	14
324	1	3
325	37	94
329	2	3
332	1	7
333	3	18
334	4	5
336	4	4
337	2	4
338	1	1
339	1	1
340	12	19
341	5	12
345	4	6
346	2	5
347	1	4
348	1	1
349	1	3
350	4	4
351	4	11
352	1	2
353	1	1
354	1	5
357	2	3
358	1	1
359	1	1
365	1	1
366	1	1
367	2	4
369	1	1
379	1	3
59 Families	338 Subfamily	1675 Genes

In 1994, only 5 insect P450 sequences had been reported in the literature. These included CYP6A1, CYP6A2 from *Musca domestica* and *D. melanogaster* respectively [35], CYP6B1 from Papilio polyxenes, the black swallowtail butterfly [36], CYP4C1 from Blaberus discoidalis, the cockroach [37] and CYP4D1 from D. melanogaster [38]. By the summer of 1998, there were approximately 216 insect P450 sequences in six separate P450 families (i.e., families CYP4, CYP6, CYP9, CYP12, CYP18. and CYP 28). By the summer of 2009, there are 1675 known insect P450s (Table 1) according to the P450 nomenclature committee. The number of identified insect P450s exceeds the number of identified P450s in all other animals.

Biochemistry: The ability of insects to metabolize insecticides has attracted many researchers to look for P450s in insects. There is ample evidence that insects P450s are involved in the metabolism and detoxification of xenobiotics [39, 40]. Although a few studies characterizing the function of specific isoforms of P450 have been published (Table 2), the functions of the vast majority of insect P450s remain unknown. Some generalizations are, however, possible. Most, but perhaps not all, of the isoforms in the CYP6 and CYP28 families are thought to be involved in the metabolism of xenobiotic compounds. Based on similarity to mammalian CYP4 isoforms, insects CYP4 enzymes are thought to be primarily involved in the mobilization of energy and fatty acids oxidation, but some family 4 isoforms may also be involved in resistance to xenobiotics [41]. P450s belonging to family 12 are thought to be mitochondrial and perhaps steroidogenic [42]. The first insect P450 (CYP6A1) was identified in an insecticide resistant strain of housefly [43]. Although no definite correlation between high expression of specific P450s and feeding specialization, high P450 expression has been linked to the presence of high concentration of terpenoids.

Insect P450s are also involved in the metabolism of endogenous substrates. Microsomal P450s have been shown to play an important role in the synthesis of juvenile hormones [44, 45, 46]. The role of P450s in the metabolism of ecdysteroid is well established. The conversion of cholesterol to 7-dehydrocholestrol is suggested to be mediated by P450s [47]. Moreover, Insects P450s are also involved in catalyzing the synthesis of insects pheromone [48]. CYP 4C1 is suggested to be involved in fatty acid hydroxylation [49].

Table 2: Some major insects P450s and their hypothesized target substrate

Name	Substrate/ Activity	Organism
CYP4C7	Terpenoid hydroxylase/ suppression of juvenile hormone synthesis	*Diploptera punctata*
CYP4S4, CYP4L4	odorant-detecting	Mamestra brassicae
CYP4C1	Fatty acid hydroxylation (suppressed by Juvenile hormone)	Blaberus discoidalis
CYP4AB1, CYP4AB2	Unknown	*Solenopsis invicta*
CYP6A1	Aldrin, Heptachlor, Juveile hormones (I and III)	*Musca domestica*
CYP6A2	Aldrin, dieldrin and diazinon, aflatoxin B1	Drosophila melanogaster
CYP6B1	Bergapten, xanthotoxin, isopimpinellin	*Papilio polyxenes*
CYP6B2	Insecticide resistance	*Helicoverpa armigera*
CYP6B3	Furanocoumarin	Papilio polyxenes
CYP6B4	Isopimpinellin, imperatorin, bergapten, xanthotoxin and psoralen	*Papilio glaucus*
CYP4D10, CYP28A1, CYP28A2, CYP9B3	Isoquinoline alkaloids	Drosophila mettleri
CYP4G20, CYP9A13	Metabolism of odorant/taste	Mamestra brassicae
CYP6AT1 (antennae-rich) CYP4AW1 (antennae-specific)	Inactivation of pheromones	Phyllopertha diversa
CYP6D1	Methoxyresorufin, deltamethrin, cypermethrin	*Musca domestica*
CYP6AB1	Furanocoumarin	Depressaria pastinacella
CYP6A9	Barbital-induced	Drosophila melanogaster
CYP6H1	putative ecdysone 20-hydroxylase	Locusta migratoria
CYP6X1	Undetermined/associated with pyrethroid resistance	Lygus lineolaris
CYP28B1, CYP4G13	Unknown	*Musca domestica*
CYP6G1, CYP12D1	DDT-resistant	Drosophila melanogaster
CYP9A1	Insecticide resistance	Heliothis virescens
CYP12A1	Mitochondria (aldrin, amitraz, diazinon, heptachlor, progesterone, testosterone)	Musca domestica
CYP302A1	Ecdysone	Drosophila melanogaster
CYP321A1	Furanocoumarins/ cypermethrin	Helicoverpa zea

Methods for Studying Insect P450s: Most of our knowledge about structural and functional aspects of eukaryotic P450s is based on the three-dimensional structure of bacterial P450 enzymes. The multiplicity of P450 enzymes in organisms and the association of most eukaryotic P450s with the membranes has made the biochemical purification and the determination of the catalytic activity of individual P450 enzymes extremely difficult if not impossible in many cases.

However, there has been a number of different methods employed for studying cytochrome P450s at the gene level as well as the protein level. These methods include,but not limited to, sequencing the whole genome of an organism as has already been done with some insect species Another method is cloning novel P450 genes using degenerate primers designed to highly conserved regions such as the I helix and the heme decapeptide. If a gene specific primer is available, then the specific gene can be isolated by rapid amplification of cDNA Ends (3' RACE and/or 5' RACE). In addition, Identifying new genes or studying their expression profile can be achieved using a previously known P450 as a probe even if the probe or the subject of study is from a different species. Considerable information on structure and function can be obtained through *In vitro* metabolism studies and site directed mutagenesis using specific P450 cDNA which have been expressed in a heterologous system. Thus, expression systems may allow for the study of P450s identified by cloning strategies, the study of single uncontaminated P450 enzymes and the production of large quantity of a desired P450 for other biochemical and biophysical studies [50]. Heterologous expression of Insects P450s, with some N-terminal modification to produce soluble protein, has been extensively utilized in the past few years. The remaining of this paper will illustrate some of the features of the major systems used to express insects P450s.

Expression Systems for Insects P450s: As a result of rapidly advancing technology in the field of molecular biology, several expression systems have been developed in the last decade. Different P450 enzymes from different organisms have been expressed using one or more of these expression systems. Among the most widely utilized for P450s expression are the following systems.

Escherichia Coli Expression System: *E. coli* is by far the most wildly used expression system among all prokaryotic systems and eukaryotic systems as well. *E. coli* expression system is advantageous system for many reasons including rapid growth in a very short time (24-48 hours), they are easy to manipulate, less expensive to grow and they can be transformed with a broad variety of expression vectors. In addition, their genetics are well understood which led to the development of different strains with different features suitable for different expression needs. Perhaps the most advantageous features in *E. coli* systems are the production of high quantities of heterologous proteins and the ease with which active forms of the heterologously expressed protein can be purified from the inner surface of their cell membrane (if membrane-bound) or from their cytosol (if mitochondrial or bacterial) [51], or directly from the media in the case of secreted proteins. Up to 1.0 g/liter of heterologous protein can be obtained from *E. coli* [52]. In the case of toxic proteins such as cytochrome P450s, however, the production ranged from as low as 20 nmol/L up to 25-30 mg/L [53]. The diversity of plasmids with strong promoters and origins of replication as well as the different fermentation methods allow for such high protein production level.

Two fermentation methods can be utilized to achieve high concentration of heterologous protein in *E. coli* systems. In the first method, the expression of the heterologous gene is induced at late stage of fermentation to obtain the maximum protein production [52]. This is mainly achieved by placing the gene of interest behind a strong controllable promoter such as *tac* or its derivatives *trc* or *tic*. The two promoters are very strong promoters and are under the tight control of the *$lacI^q$* operon suppressor. A *lac* operon inducer such as isopropylthiogalacto-sidase (IPTG) can be added at late stage to induce the expression of the heterologous gene [7, 54]. This is a preferred method if the heterologous protein is toxic and therefore could disrupt metabolic pathway or catalyze undesirable reactions during the growth of the cells [7]. In the second method, the plasmid construct is designed to yield a reasonable amount of the expressed protein throughout the growth period and the culture is harvested at the maximum cell density. Since *E. coli* cells can grow to a very high density, the heterologously expressed protein can account for up to 10-20% of the total bacterial proteins, which is twice as much the concentration of the most abundant bacterial protein [52].

When utilizing *E. coli* expression systems, other factors that might affect the quantity and quality of the expressed protein should be considered. For example, *E. coli* cells strongly favor alanine as the second mRNA codon, which presumably increases the affinity of ribosomal binding [55]. In addition, the stability of mRNA in *E. coli* is highly affected by the nucleotide content upstream the Shine-Dalgarno region AT-rich sequences in this region destabilize the secondary structure of the mRNA as opposed to GC-rich sequences. Less stable mRNA secondary structure in this region facilitates higher ribosomal binding and subsequently increases the translational rate [56]. Furthermore, rare codon clusters can have a negative effect on the expression level in *E. coli* system. For instance, the rare arginine codons AGG and AGA occur at a frequency of 0.14% and 0.21%, respectively, in *E. coli* mRNA. It was reported that the translation of mRNA dropped significantly as the number of AGG codons increased from two to five especially if they occur at the N-terminal of the mRNA [57]. Another rare codon is the CUA codons. A cluster of nine CUA codons at the N-terminal was found to increase the disassociation of ribosomal complex in *E. coli* [57].

Usually, proteins that are not too small or too large and not too hydrophobic are readily expressed in *E. coli*. However, some complex mammalian proteins that require specific posttranslational modification or cell surface receptors that contain 40 disulfide bonds may not be expressed in *E. coli* [52]. This is attributed to the reducing environment in *E. coli* which does not permit cystine-rich proteins to form disulfide bonds required for proper folding. Secreting proteins to more oxidized extracellular environment overcomes this problem but this strategy does not work as efficiently if the protein is not naturally secreted [52].

One major drawback of *E. coli* system is the low expression level of membrane bound proteins. While expressed cytosolic protein may account for more than 20% of total bacterial protein, membrane-bound proteins typically account for less than 1% of the total bacterial membrane [51]. Another obstacle of expressing eukaryotic proteins in *E. coli* is that those proteins tend to be found in misfolded, insoluble and inactive forms.

Expression of microsomal P450s in prokaryotic cells has proved less efficient, presumably because they lack intracellular membranes into which hydrophobic P450 proteins sequester. Initially, *E. coli* were thought to lack P450 reductase which necessitated reconstructing the P450/P450-reductase chimera *in vitro* for biochemical studies. However, flavodoxin (FAD-containing) and

flavodoxin reductase (FMN-containing) proteins have been discovered and purified out of *E. coli* by Jenkins and Waterman [58]. Although less efficient than rat P450 reductase, these two proteins have been shown to support the reduction of bovine P450 CYP17 (17 α-hydroxylase).

Insect Cells Expression System: Heterologous expression of genes such as some P450s in prokaryotic-based systems has been associated with problems including an increase level of toxicity resulting from the expression of foreign proteins as well as the requirement for post-translational modifications which cannot be achieved in prokaryotic systems. Although some functional P450s have been successfully expressed in *E. coli*, other P450s have been deposited as insoluble inclusion bodies rendering them nonfunctional. Those types of problems can be avoided using mammalian cell systems. However, mammalian cells are very expensive and are difficult to maintain. The development of insect cell-based expression systems (i.e., Insect Select System™) has enabled the transient or stable expression of many foreign proteins (including some P450s) in significant quantities up to 500 mg/L [54].

There are many transformation systems based on dominant selection for use in mammalian cells whereas only a few have been used with insect cells [59,60]. The first system was the transformation of *Drosophila melanogaster* cells to be methotrexate resistant using a bacterial dihydrofolate reductase gene (DHFR) [61]. In Shotkoski and Fallon [62] reported the use of mosquito *Aedes albopictus* dihydrofolate reductase gene as a selectable marker in mosquito cells. In both cases, the DNA was randomly integrated into the genome as repetitive arrays or single copies and was usually lost in the absence of selective pressure. The introduction of the bacterial neomycin phosphotransferase gene into *D. melanogaster*, mosquitoes and SF9 cells has transformed those cell lines into being geneticin (G418) resistant. This selection system, however, is not usable in all the cases because of the occurrence of a high frequency of spontaneous resistance mutations as well as gene amplification induced by continued selection pressure [61]. A hygromycin resistance mosquito cell line was reported to be a more reliable and rapid selection system than the G418 system but the introduced plasmid was extensively amplified. Moreover, the plasmid was found to form long tandem arrays or as an extra chromosomal pseudo-chromosome in which cases the resistance gene was rapidly lost [61].

The InsectSelect™ System (Invitrogen, Carlsbad, CA) is another example of insect cell expression systems. This system allows constitutive stable or transient expression of a protein of interest in three different cell lines: SF9, SF21 and HighFive™ (Invitrogen, Carlsbad, CA). In this system, the gene of interest can be cloned into one of the many available shuttle vectors such as pIZ/V5-His expression vector. This vector contains the EM7 bacterial promoter as well as the strong *OpIE2* promoter upstream of the *Streptoalloteichus hindustanus ble* (*Sh ble*) gene. The *Sh ble* gene enables the selection *of E. coli* and insect cell transformants resistant to the antibiotic Zeocin™. Zeocin is a member of phleomycin antibiotic family from *Streptomyces verticillus* which can cause toxicity to many prokaryotes and eukaryotes. These types of antibiotics indiscriminately bind DNA and cause lethal, double-strands breaks [63]. Plasmids containing a *Sh ble* gene encode a 13.6 kDa protein which binds Zeocin in a stoichiometric manner to protect the DNA of the host cells [64]. The Zeocin selection system is advantageous over the other selection systems because of the low concentration required for selection, the possibility of using this selection scheme in other systems and the small size of the resistant gene (374 bp) [61].

Baculovirus Expression System: Many viral expression systems such as vaccinia virus and adenoviral expression system are available but, by far, the baculovirus expression system (BVES) is the most popular viral system. Dr. Max Summer at Texas A&M University (college station, Texas) and MicroGeneSys (Medrin, CT) are the pioneers in developing this system [65]. The BVES system is based on a group of viruses (over 500 species) that infect only insect cells. *Autographa californica nuclear polyhedrosis virus* (*Ac*NPV) is the most commonly utilized baculovirus for heterologous expression of gene products. It belongs to a subfamily of baculoviridae (Nuclear polyhedrosis virus) which has a large genome (180 Kb). During early stage of infection (1-3 days), these viruses hide in infected cells by budding off the cellular membrane, while towards the end of infection they are enclosed in protein envelopes called occlusion bodies. These envelopes are mainly composed of polyhedrin protein which protects the viral genome from nuclease attack upon lysis of infected cells. They can survive in the polyhedrin envelope until they encounter another cell. SF9, SF21 and High Five™ cell lines are all susceptible to *Ac*NPV. SF9 and SF21 were

both derived from ovarian tissues of the fall armyworm, *Spodoptera frugiperda* while High Five™ cells were derived from ovarian tissues of the cabbage looper, *Trichoplusia ni.*

This system allows one to produce high quantity of hetero-logous protein where many posttranslational modifications are correctly made in insect cells; e.g., phosphorylation, glycosylation, myristolation, palmitolation, acylation, proteolysis (Kidd and Emery, 1993) and amidation [67]. Although some modifications are not performed in insect cells, this system is very efficient and is reliable for producing proteins for therapeutic use [68]. For example, gp 160 (the product of the *env* gene of HIV), p24HIV vaccine and malaria vaccine are all produced in this system [65]. The level of production in BVES can reach up to 500 mg/L [54]. Many of the cell lines used with this system can grow at room temperature and do not require CO_2 or a humidified environment. In addition, some insect cells can grow in serum-free medium, which lowers the costs of experiments and facilitate the purification of heterologous proteins [54]. BVES, however, are transient expression systems because baculoviruses have a lytic life cycle. In contrast to *E. coli* and yeast, BVES systems are associated with some difficulties such as high cost, labor and intensive, time-consuming and cumbersome procedures and require personnel with advanced skills in growing and maintaining cell lines as well as skills in screening for recombinant plaques. Since baculoviruses have a large genome (80-220 KB), inserting the gene of interest requires a shuttle insertion plasmid. In addition, proteins entering secretory pathways associated with endoplasmic reticulum are often expressed in a heterogeneous form in BVES [69]. Heterologous proteins, expressed earlier in the infection, are more likely to undergo the proper posttranslational modifications relative to protein expressed in late in the infection. This indicates that the progression of the baculovirus infection disrupts the protein processing machinery in infected cells. To avoid such negative results, BVES vectors allowing early infection expression have been developed (i.e., the expression of the gene of interest is driven by the p10 promoter) have been developed [69].

Yeast Expression System: *Saccharomyces cerevisiae* are one of the most widely used expression vehicles for P450s. Among the advantages of yeast is that they posses endoplasmic reticulum (the normal environment for P450), NADPH-cytochrome P450 oxidoreductase and cytochrome b_5 (which increases P450 binding affinity to substrates by making the P450 a two electron acceptor) [70]. In addition to their low costs, large amounts of P450 can be synthesized (e.g., 8 nmol P450/liter) without worrying about proteolysis which has been an obstacle with other systems [71]. Moreover, yeast P450-reductase has been found to support the catalytic activity of several expressed human microsomal P450s to varying degrees.

Other Systems: In addition to the previous three systems, there are other expression systems that have been used although not as widely employed for P450s expression. Briefly, some of these systems are the following:

Vaccinia Virus is a very large plasmid (187,000 bp) which makes direct insertion of cDNA into it impossible. To construct a recombinant vaccinia virus, a cDNA must be cloned first into an insertion vector. When the insertion vector is mixed with wild-type vaccinia virus DNA, it integrates at a frequency of 0.1% carrying the inserted DNA with it. There are a number of different methods that can be used to construct a recombinant vaccinia virus [72].

The COS 1 cell line was developed by transforming african green monkey kidney cells (CV-1) with an origin-defective strain of simian virus 40 (SV40) DNA. The integration of the complete early region of SV40 allows COS cells to produce T antigen which is required for viral replication. COS 1 cells can be co-transfected with multiple cDNAs and still express functionally active P450 enzymes [73]. Moreover, the COS 1 cells express endogenous NADPH-P450 reductase, adrenodoxin and adrenodoxin-reductase which can interact with introduced P450s. The COS 1 cells system was originally established to express the steroidogenic microsomal P450, 17α-hydroxylase [73].

In addition to these expression systems, plant cells, V79 Chinese hamster cells and human B lymphoblastoid cells have also been used, but less frequently than more established expression systems.

CONCLUSION

Our understanding of P450s in general and insects P450s specifically has been greatly enhanced by the development of technology in whole genome sequencing as well as the advancement of the tools employed for isolating and studying single P450s. Although the number of isolated insects P450s is by far more than any other group, including mammals, the role of most of those

isolated P450 is either poorly understood or ambiguous at best. Our understanding and knowledge of insects P450s will be greatly enhanced if researchers in the future shift toward studying the structure and catalytic activity of insects P450s.

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