Proc Indian Natn Sci Acad, **70, A,** No. 2, March 2004, pp. 283-291

STRUCTURE AND MECHANISM OF FUNCTION OF CYTOCHROME P450

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(Received 07 April 2003 ; Accepted 30 April 2003)

The article reviews briefly structural and functional properties of cytochrome P450s. In particular, the role of protein structural fluctuations and substrate dynamics in the catalytic function of P450s is critically examined.

Key Words : Cytochrome P450; Monooxygenases; Hydroxylation Reaction; Drug Metabolism; Catalytic Cycle; Peroxidase Shunt; Protein Dynamics and Substrate Mobility; Time Resolved Fluorescence Spectroscopy

Introduction

Cytochromes P450 (P450s) form a ubiquitous and very large superfamily of hemeprotein monooxygenases that metabolize physiologically important compounds vital to life of most organisms from protists to plants to mammals[1]. They function not only to catabolize compounds as energy source, detoxify xenobiotics compounds including drugs, procarcinogens and carcinogens, but also catalyze synthesis of steroid hormones, cholestrol, bile acids and arachidonate metabolites, and degradation of endogenous compounds including fatty acids and steroids. The reactions catalyzed by P450s include both aliphatic and aromatic hydroxylations, N-, O- and S-dealkylations via an initial hydroxylated intermediate, as well as the oxidation of heteroatoms[2]. In some cases, the oxidative metabolism of the foreign chemicals produces products that are reactive toxins and mutagens[3]. The reactions usually require an external source of reducing equivalents like NADH (nicotinamide adenine dinucleotide) and auxiliary proteins (e.g. putidaredoxin, putidaredoxin reductase in $P450_{cam}$) to transfer the electrons to P450 (Fig. 1). Over 1000 P450 genes (CYP) have been identified to date, which make up P450 gene superfamily[1,2]. The P450 gene superfamily can be divided into four classes based on the mode of delivery of the electrons from NADH to P450. In *Class I*, there are two electron transfer proteins: a FAD (flavin adenine dinucleotide) containing reductase and an iron sulfur protein. Bacterial P450s (including *Pseudomonas putida* $P450_{cam}$) and P450s found in mitochondria of eukaryotes are members of this class. Members of *Class II* require only one electron transfer partner, a reductase that contains both FAD and FMN (flavin mononucleotide). These enzymes are largely xenobiotic metabolizers or steroidogenic, and are found in the endoplasmic reticulum of eukaryotes and in mammals. This includes *Bacillus megaterium* $P450_{BM3}$, a catalytically self-sufficient fatty acid omega-hydroxylase/epoxidase. The *Class III* P450s, which have been identified to date, do not require an electron transfer partner, and act on peroxy fatty acids. Examples of members of this class are thromboxane synthase and allene oxide synthase. Finally, *Class IV* P450s do not also require electron transfer partners, and instead directly bind and transfer electrons from reduced pyridine nucleotide to the heme iron. An example of this class of P450s is denitrifying fungi *Fusarium oxysporum* P450*nor,* nitric oxide reductase. Another subdivision of P450 superfamily has been done on the basis of sequence identity. P450 superfamily has been subdivided into over 70 families, enzymes with less than 40% identity being classified into different families with less than 15% identity between families.

P450 substrates are predominantly hydrophobic compounds with products generally made more water-soluble by monooxygenation. Such oxygenation reactions can be utilized for industrial, therapeutic and environmental applications[2]. P450 gene family has been the focus of drug metabolism research. The enzymes encoded by P450 genes are responsible for metabolizing most drugs used today, including many for treating psychiatric, neurological and cardiovascular diseases[2]. Recently, P450-based

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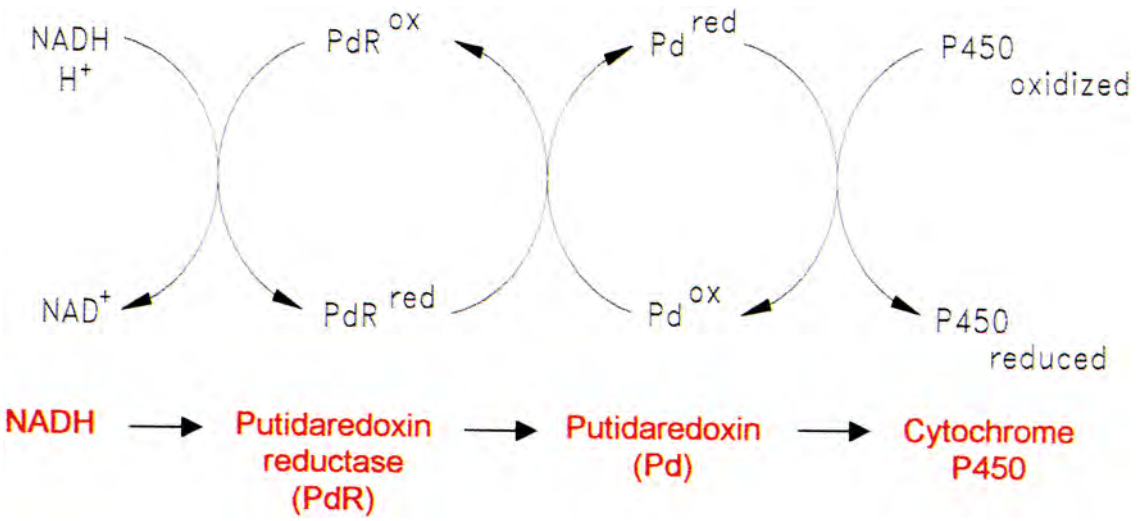


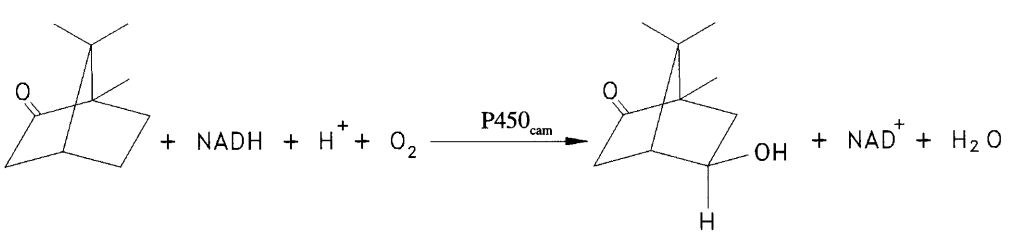
Fig. 1 The electron transfer pathway from NADH to cytochrome P450 in camphor 5-monooxygenase (CM).

cancer therapy has been developed to sensitize tumor cells to cancer chemotherapeutic prodrugs (e.g. cyclophosphamide, ifosfamide)[4]. Bacterial $P450_{BM3}$ has been converted into efficient epoxygenase, that catalyses the NADH-dependent epoxidation of arachidonic acid to epoxyeicosatrienoic acid (EET) that regulates Na^+, K^+ and Ca^{2+} in flux in kidneys[5]. Also, genetically engineered P450s, mainly $P450_{BM3}$ and $P450_{cam}$ variants are utilized in the breakdown many environmentally toxic compounds such as polycyclic aromatic hydrocarbons and fluorocarbons[6].

Structure

In understanding diverse functions and properties of P450 enzymes, knowledge of their structure and catalytic mechanism has been crucial. The journey towards understanding P450 structure at the atomic level began in 1985 with the publication of the first P450 structure, *Class I - Pseudomonas putida* $P450_{cam}$ (CYP101: 5-exo-hydroxylation of camphor) [7]. It took several years before the structure of another P450 became available, that of *Class II* - $P450_{BMP}$ and P450 domain of $P450_{BM3}$[8]. Subsequently the crystal structures of several other P450s were reported. These include *Pseudomonad* P450terp[9], *Sacchaaopolyspora erythraea* P450eryF[10], *Fusarium oxysporum* P450nor[11],P450sca (CYP105A3) [12], rabbit P450 2C5[13,14], *Mycobacterium tuberculosis* P450 14 alpha-sterol demethylase (CYP51) [15], thermophilic Archaeon *Sulfolobus Solfactaricus* P450 (CYP 119) [16], and different states of $P450_{cam}$, $P450_{BMP}$ and P450eryF (e.g. with and without substrate and mutated forms)[17]. The structures of some of these P450s are shown in Fig. 2 for comparison. All these structures offer a template for modelling other mammalian P450s, which help in drug binding studies and in the prediction of drug-drug interactions[2]. The available structures for P450s show that the overall P450 structural fold is preserved during evolution from bacteria through mammals[2, 7-25], even though the sequence identity may be less than 20% among them[26] . P450s show variable regions and diversity in the primary, secondary, and tertiary sequences as well. Such variability in structure is likely to be associated with recognition and binding of structurally diverse substrates, redox partners and targeting cellular location of the protein[27].

Among all cytochrome P450s, soil bacterium cytochrome P450cam, grown on bicyclic terpene



camphor 5-exo-hydroxycamphor

camphor, has served as the best model for the understanding of structural and biochemical properties of the enzymes. P450cam catalyzes the stereo and regio specific 5-exo hydroxylation of camphor:

The crystal structure of P450cam with and without substrate has been solved, and it has provided a paradigm for our understanding of other P450s. The structure of the camphor bound enzyme is shown in Fig. 3. The heme group in the camphor-bound enzyme contains iron (III) ion which is five-coordinated high spin, the fifth coordination site being occupied by a cystein. However in the camphor-free state, a water molecule is coordinated to the heme iron at the sixth position making it low-spin. This change in coordination geometry on binding of substrate in catalytically important as discussed below.

Catalytic Cycle

P450s catalyze stereospecific hydroxylation of nonactivated hydrocarbons at physiological temperature- a reaction when uncatalyzed requires extremely high temperatures to proceed even for non-specific action. In absence of dioxygen and reducing cofactors, the substrate can be hydroxylated by P450 by addition of peroxide through a "peroxide shunt" pathway[2] (Fig. 4) . The oxygen insertion/hydroxylation

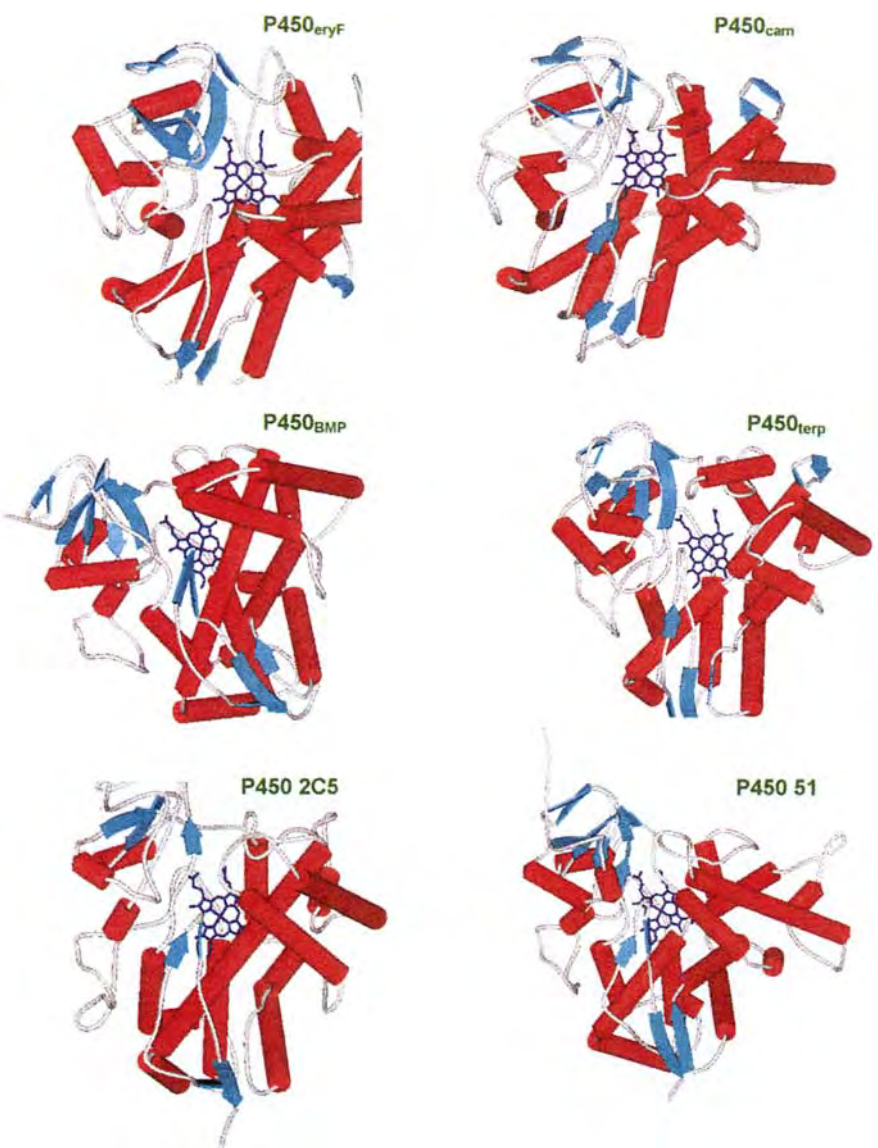


Fig. 2 Comparison of six structurally determined P450s-*Sacchaaopolyspora erythraea* $P450_{eryF}$, *Bacillus Megaterium* $P450_{BMP}$, *Pseudomonas putida* $P450_{cam}$, *Pseudomonad* $P450_{terp}$, rabbit P450 2C5, *Mycobacterium tuberculosis* P450 51. Heme ring is shown in blue, alpha helices with red cylinders, ribbons of beta-sheets as light blue arrows, and randomly coiled amino acids are shown as grey rope.

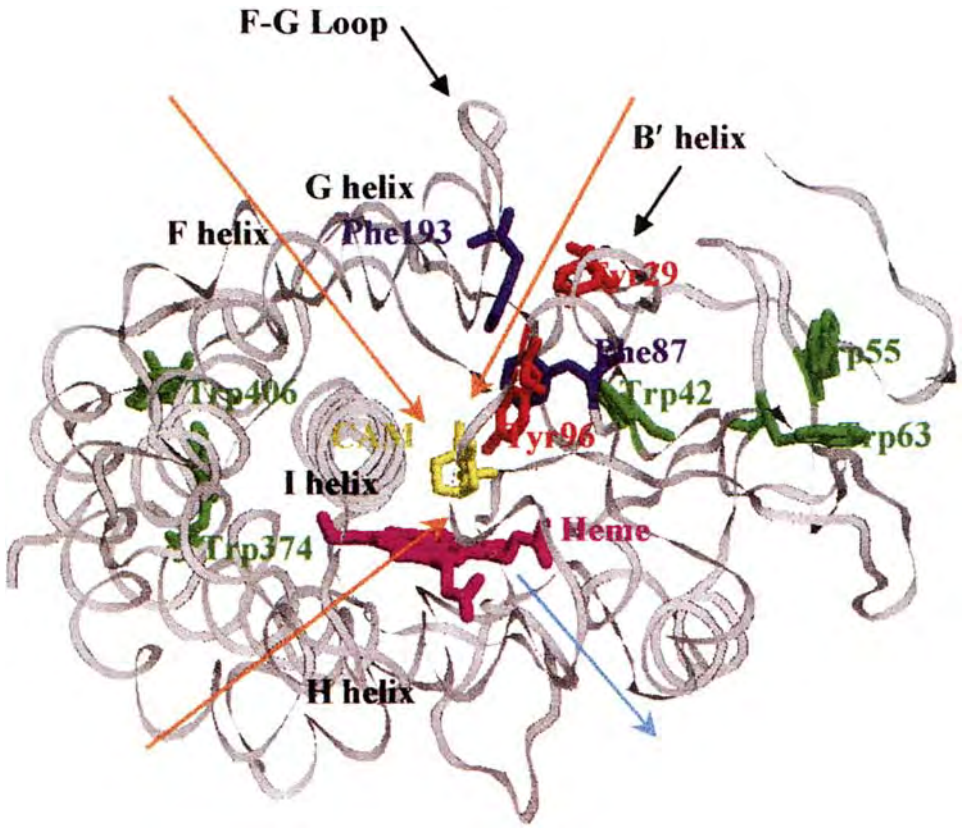


Fig. 3 Schematic structure of camphor-bound cytochrome $P450_{cam}$ generated from its crystal structure (PDB code: 2CPP)[3]. The location of tryptophan is shown in green, camphor (CAM) in yellow and the three possible entry channels as orange coloured arrows. The product exit channel for $P450_{cam}$ was proposed[3] to be along the water channel shown as light blue arrow.

reaction catalyzed by P450s, is thermodynamically favourable but is a high kinetic barrier reaction. The mechanism by which P450s are able to activate oxygen/peroxide to carry out this difficult chemistry has been subject of several studies. Much of this research has focused on a structurally and biochemically characterized *Class I* water-soluble bacterial camphor 5-*exo* hydroxylase, $P450_{cam}$ from *Pseudomonas putida*. However, many details in the reaction cycle of this enzyme such as nature of the activated oxygen species, mechanism of activation of the bound oxygen molecule and the roles of specific residues in the mechanism are only recently being unraveled[28a]. The catalytic cycle of P450 for hydroxylation of substrate starts with the resting state of enzyme being an Fe (III) protein in a low-spin state with cysteine and water as fifth and sixth axial ligands (**I**)[2] (Fig. 4) . The substrate binds to the active site cavity close to Fe centre but is not directly ligated to Fe. The binding of substrate to $P450_{cam}$ (**II**) results in expulsion of water, coordinated to heme iron, leading to high-spin ferric heme substrate bound enzyme. Binding of substrate induces changes in redox potential (from –300mV to –170mV) and conformation that triggers interaction of P450 with its redox partners. The increase in redox potential allows Fe to be reduced and serves as an important control to ensure that oxygen is not activated without substrate bound (reduced oxygen forms hydrogen peroxide which is hazardous to the cell). The addition of first electron reduces Fe (III) to Fe (II) (**III**). Oxygen binds to the Fe (II) species, which is accompanied by reduction of oxygen to superoxide, O_2^-, and oxidation of Fe (II) to Fe (III), the overall charge remaining 2+ (oxy P450 (**IV**)). P450s are named for their unusually red-shifted Soret absorption maximum at 450nm when reduced in the presence of CO (**V**). Introduction of another electron reducing equivalent to oxy P450 (**IV**) generates oxygen in the peroxide oxidation state, and two protons cleave off the distal oxygen atom as water, resulting in an active oxidant (**VII**). The nature of the active oxidant in the catalytic cycle of P450 is uncertain. This active oxidant is suggested to initiate H atom abstraction from

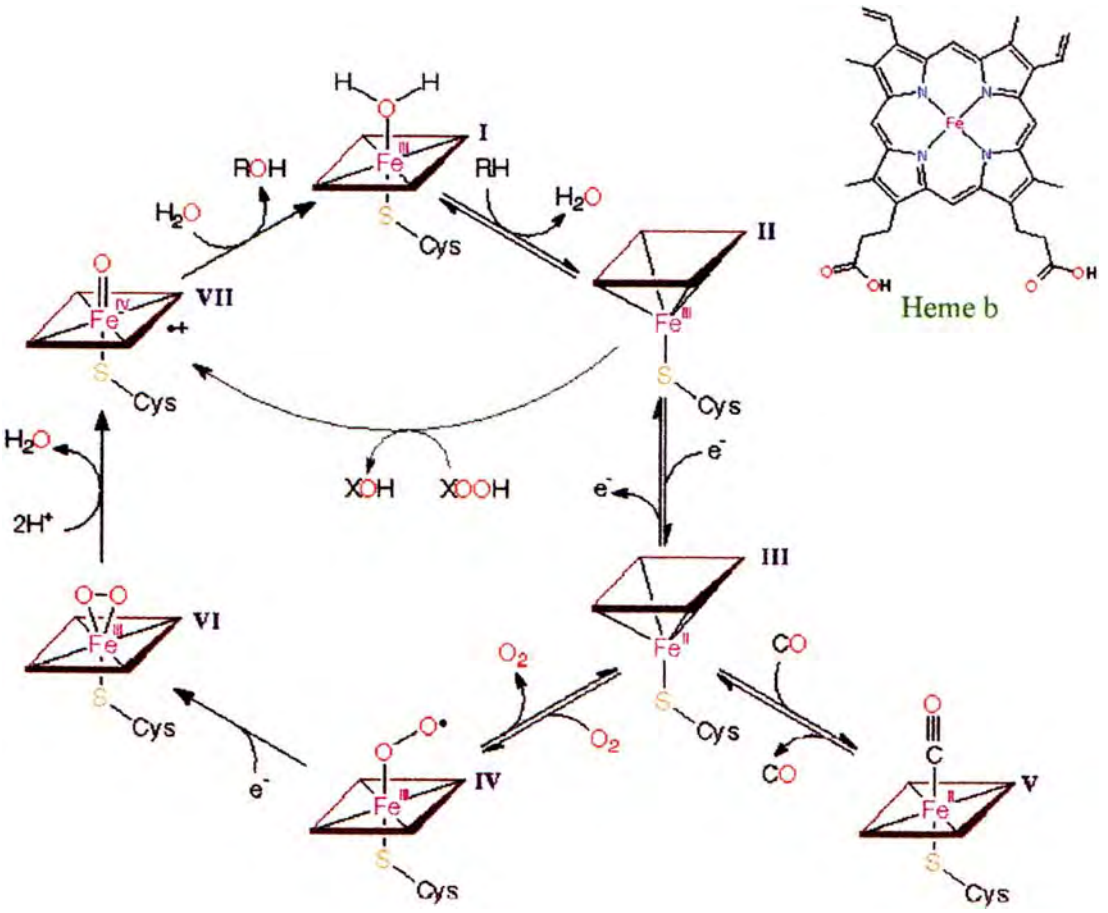


Fig. 4 The proposed reaction pathway for substract hydroxylation by P450. The catalytic cycle of P450 involves substrate (RH) binding close to the heme b prosthetic group, converting the six-coordinate, low-spin met from (I) of the enzyme to five coordinate, high-spin from (II). The ferrous form of the enzyme (III) rapidly binds dioxygen to give the six coordinate ferric-oxy intermediate (IV). Heterolytic cleavage in VI gives rise to an oxidising species, the proposed, oxyferryl intermediate, (VII) that leads to insertion of the iron-bound oxygen into the substrate to produce hydroxylated product (ROH). In absence of dioxygen and reducing factors, addition of peroxide (XOOH) hydroxylates the substrate (RH) through the "peroxide shunt" pathway via intermediate (VII).

the substrate, and oxygen radical rebound leading to hydroxylated product, and the enzyme finally returns to its low-spin resting state (**I**) after product release. It has been proposed that oxy P450 (**IV**) returns to the low-spin resting state (**I**) extremely fast with a rate constant over 10^6 M^{-1} s^{-1}. Based on spectroscopic studies of various P450s and model compounds[28b], and from analogy with other heme proteins such as peroxidases[29], the oxyferryl species has gained widespread acceptance as active oxidant/oxygenating agent in P450 reaction cycle[28a,,b]. This intermediate, referred to as compound I, has been well characterized for peroxidases but has not yet been characterized for any native P450s. Direct observation of the 'compound I-like' intermediate in the $P450_{cam}$ reaction cycle was first reported for reaction of m-chloro-peroxybenzoic acid with substrate-free $P450_{cam}$[30]. The reactions catalysed by P450s such as aldehyde oxidation and N-oxygenation, also point to an analogous intermediate during their reaction cycle[31]. In fact it is widely assumed that the majority of oxidative chemical transformations effected by P450s occur via the obligatory formation of the oxyferryl intermediate. Apart from oxyferryl intermediate, it has been proposed in some cases that ferric-peroxo intermediate (which is formed prior to oxyferryl species) is directly responsible for the terminal step in the demethylation of androgens to estrogens by the enzyme P450aromatse[32]. It has been shown that xenobiotic aldehyde deformylation and substrate hydroxylation by

P450 2B4 utilize different active oxidant in catalysis, with deformylation occurring, most likely, via a nucleophilic attack of a ferric peroxo species at the electropositive carbonyl carbon of the substrate, forming a transient peroxo hemiacetal[33].

Protein and Substrate Dynamics

Although classical enzymology together with structural biology has provided profound insights into the chemical mechanisms of many enzymes[34], protein dynamics and their relation to catalytic function has only recently received attention. Dynamics of enzymes during catalysis have been detected with methods such as fluorescent resonance energy transfer, atomic force microscopy, stopped-flow fluorescence, nuclear magnetic resonance (NMR) spectroscopy which report on global motions of the enzyme or dynamics of particular molecular sites[35]. Increasingly, the role of dynamics of protein and substrate in the catalytic activity of P450 enzymes and substrate specificity/product control is being recognized and considered very crucial. Such information is helpful in deciding whether a specific compound will be a substrate for a given P450 enzyme. Such information is also vital for design and construction of P450 enzymes with tailored substrate specificities, and for the prediction of potential toxic interactions. The capability of P450s in catalyzing enormous range of substrates in performing physiologically important and diverse processes and in metabolizing all drugs currently in use is rather striking[36]. Despite broad substrate diversity, all P450s have significant structural constraints on their activity: P450s must control water access to the active centre to avoid the conversion of activated dioxygen to superoxide or peroxide. Thus, the binding sites of P450 isozymes must be structurally diverse, yet it must conserve a mechanism of catalysis and solvent exclusion. An unanswered question is how one enzyme family whose chemistry requires significant structural constraint metabolizes thousands of substrates. Structural flexibility/dynamics and substrate mobility in P450s are therefore expected to play important roles in controlling substrate-binding orientation during catalysis, and in accommodating diverse range of substrates within heme pocket. Based on sequence comparisons between some members of human P450s and $P450_{cam}$, the regions that are believed to show variability and flexibility with substrate binding are helices A, B, B´, F, and G (marked in Fig. 3) and their adjacent loops[37]. The mutation of amino acid residues within these regions has been shown to confer new substrate specificities onto P450s or alter its stereo- and regio-selectivity[38-40]. The crystal structures of $P450_{terp}$ and P450 2C5 (Fig 2) show disorder/flexibility in the F-G region[41]. Using ruthenium-linker substrates-bound $P450_{cam}$ it has been shown that large changes in peripheral enzyme structure (F and G helices) are coupled to conformational changes in the active-site residues (I helix) and the F/G helix region flexibility[42]. Such structural flexibility in P450s is thought to be the reason for its observed substrate diversity. Random expulsion molecular dynamics and classical molecular dynamics simulation studies on P450cam, $P450_{BM3}$, and P450eryF show that even though the substrate access to the active site is common in all these proteins, the mechanism of ligand entrance/exit is different and apparently adapted to the physicochemical properties of the substrate leading to different substrate specificity in them[43]. Thus, the physicochemical properties and steric characteristics of the P450 active sites may not be the only determinant of substrate specificity; the properties of access channel may play a key role, which may dynamically modulate substrate binding. In addition to protein dynamics, substrate mobility in the active-site region of the enzyme has also been recognized to play an important role in positioning the substrate within the active-site and determining regio-specificity of substrate hydroxylation[44,45]. The involvement of the mobility of substrate in P450 catalytic function has been proposed based on kinetic analysis of the hydroxylation deuterium kinetic isotope effects[46], deuterium magic angle spinning[47], high pressure[48] and FTIR studies[49] on CO bound P450s . Substrate docking algorithms and molecular dynamics simulation studies suggest a degree of plasticity in $P450_{cam}$ active site and substrate motions, to control substrate hydroxylation and for accommodating diverse range of substrates within active-site[50-53].

A combination of steady state and time-resolved fluorescence studies on substrate-free and substrate-bound $P450_{cam}$ complexes has been useful to elucidate the role of protein and substrate dynamics in regulation of substrate hydroxylation, diversity and product control in this enzyme[44,45]. Steady state and pico-second time resolved intrinsic tryptophan fluorescence experiments on P450cam have demonstrated the existence of structural fluctuations involving substrate access into the active-site region via a channel next to the flexible F-G helix-loop segment[44] (Fig.3). This has led to the identification of the substrate access channel, and have also shown the presence of substrate-dependent dynamic fluctuations in $P450_{cam}$. Larger dynamic fluctuations are exhibited by substrates with altered

protein-substrate interactions in the active-site region by norcamphor relative to physiological substrate, camphor. The observed substrate-dependent dynamic fluctuations (even for closely related substrates) indicate existence of variable and flexible structural states in $P450_{cam}$. The structural heterogeneity of the local active-site and substrate dependent structural flexibility with energetically low-lying structural states can be important in regulating binding orientations for substrate during catalysis and accommodating diverse range of substrates. The orientations of tryptophan relative to heme for Trp 42, obtained from time-resolved tryptophan fluorescence measurements, show variation with type of substrate bound to $P450_{cam}$, indicating that regions distant from heme active-site are affected by local active-site protein-substrate interactions. The fluorescence spectroscopy approach appears to very suitable for studying substrate access in other enzymes as well, where the crystal structure does not unambiguously pinpoint the route of substrate entry. This method may be particularly useful in cases like microsomal P450 2C5, where use of site-directed mutagenesis for the identification of residues lining the access channel is complicated due to lack of definitive phenotypes expected for site-specific mutants, and the inability of the modelling studies to predict the movement[54].

Even though the importance of substrate mobility in P450 had been recognized for sometime, direct observation of substrate motion and possible correlation of substrate mobility to product control in P450 has been possible only very recently. Substrate dynamics in the active-site region of $P450_{cam}$ has been monitored by time-resolved substrate anisotropy measurements utilizing the fluorescence properties of camphor and its related compounds (norcamphor, adamantone and fenchone)[45] . Time-resolved fluorescence anisotropy directly monitors the reorientation of the emission transition dipole moment of the fluorophore and hence best suited for the investigation of its local molecular dynamics near the active site. The anisotropy decay of substrates bound to $P450_{cam}$ indicates that mobility of substrates is modulated by physicochemical and steric factors/protein-substrate interactions of local active-site structure, and provides an understanding of factors controlling observed hydroxylated products for substrate bound $P450_{cam}$ complexes. Using the X-ray structural data, the disposition of different amino acid residues in the active site region of camphor, adamantone and norcamphor bound $P450_{cam}$ is shown in Fig. 5. The

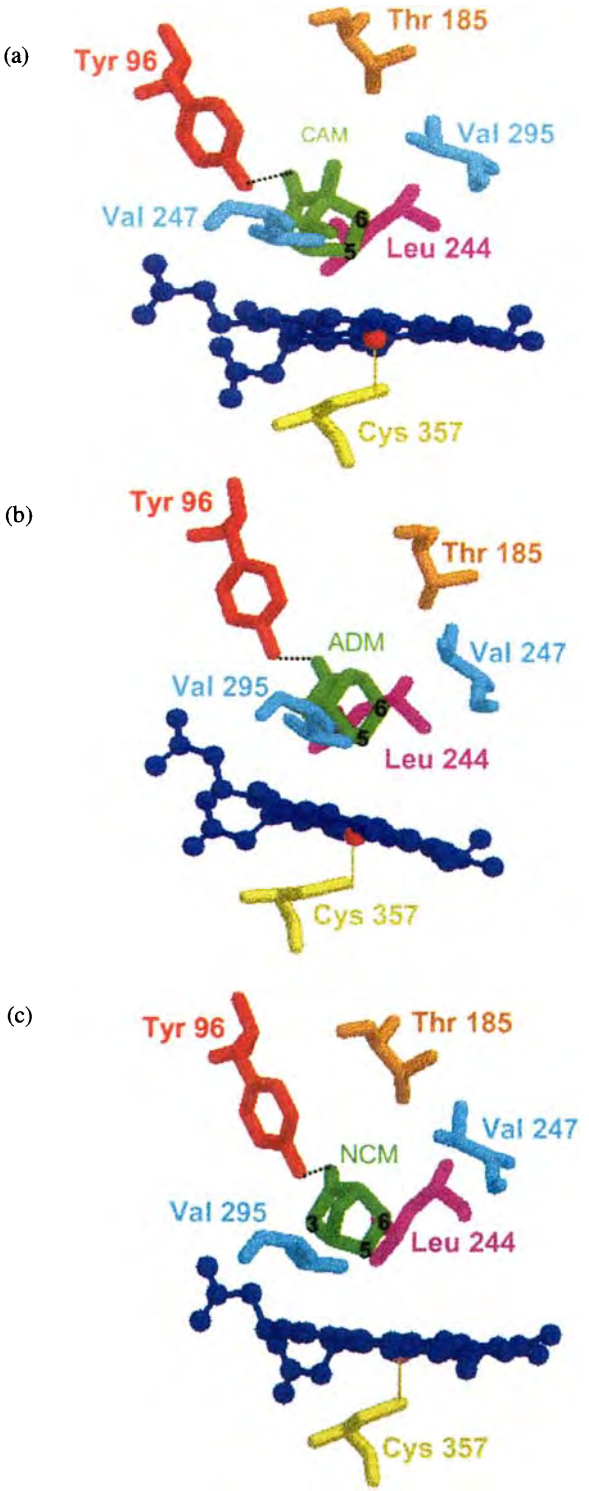


Fig. 5 Schematic representation of the active site structure of (a) camphor (CAM, PDB code: 2CPP); (b) adamantone (ADM, PDB code: 5CPP); (c) norcamphor (NCM, PDB code: 7CPP)-bound cytochrome $P450_{cam}$ generated from their crystal structure. The location of the hydrogen bond between substrate and Tyr 96 is shown as black dotted line

steric constraints/ freedom of the movement of these substrates in the active site region of the enzyme are consistent with the measurements of rotational correlation times using time resolved fluorescence anisotropy[45] . Time-resolved fluorescence decays for substrates in $P450_{cam}$ also suggest structural heterogeneity in active site of $P450_{cam}$, which along with substrate mobility can play an important role in regulating orientations for substrate binding during catalysis and for accommodating diverse range of substrates in $P450_{cam}$ heme pocket (see Fig. 5).

Clinical Implications

The evolutionary history of the P450 superfamily suggests that the ancestral P450 genes arose prior to the divergence of *Bacteria*, *Archaea* and *Eukara.* It is believed that modern P450s originated from an ancestral gene which existed about three and half billion years ago. At present there are over 2000 P450 genomic and cDNA sequences which are known. P450s not only play important roles in metabolizing modern xenobiotics (i.e. drugs, plant-derived or fungal-derived secondary metabolites consumed with food, and thousands of environmental pollutants) but are also involved in other critical life processes (*e.g.*, electrolyte balance, cell division, ligand activation, mating) as well[55]. Mammalian P450s act on many endogenous substrates, introducing oxidative, peroxidative, and reductive changes into small molecules of widely different chemical structures. A number of modern P450s are known to catalyze reactions essential to cell growth, development, ligand-modulated transcriptional regulation, neuroendocrine processes and the induction of apoptosis. A particularly elegant example of this is the maintenance of calcium homeostasis through P450 mediated-activation of the vitamin D receptor. P450s also appear to play an important role in the metabolism of endogenous substrates in early development. Transcriptional expression of P450 CYP1A1, for example, is detectable in the ovum just 12 hours after fertilization[56] and transcription factors that regulate the expression and hence the steroidogeneic activities in any P450s[57,58] are induced relatively early in development. It has been reported that the activity of human P450 CYP2C9 is actually 50 times greater in the buccal mucosa than it is in the liver where the bulk of drug metabolizing activities are concentrated. Similarly, although the expression of CYP2D6 has been associated with the metabolism of more than 40 different pharmaceutical compounds in the liver, this P450 isoform is also constitutively expressed at significant levels in the brain where it is reasonable to conjecture that it serves a different purpose[59,60]. This is based on the expectation that the blood brain barrier prevents the overwhelming majority of pharmaceutical agents from ever entering the central nervous system thereby making the need for "drug metabolizing" activity less evident.

As more and more P450 gene products are analyzed, it seems highly likely that their roles in diverse biological systems will expand. In the near future, many more studies are anticipated that will show associations between P450s and myriad genetic diseases, environmental toxic effects, and other complex physiological disorders.

Acknowledgement

We are very thankful to R Murugan for his assistance in preparing the manuscript.

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