Biotransformations using prokaryotic P450 monooxygenases

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Summary

Recent studies on microbial cytochrome P450 enzymes cover several new areas. Advances have been made in structure-function analysis. New non-enzymatic/electrochemical systems for the replacement of NAD(P)H have been developed. The properties of some enzymes have been re-engineered by site-directed mutagenesis or by methods of directed evolution. New P450s have been functionally expressed and characterized. A combination of these approaches is believed to facilitate the use of isolated P450 monooxygenases in biocatalysis.

Abbreviations

P450cam = CYP101	cytochrome P450 enzyme from *Pseudomonas putida*
P450 BM-3 = CYP102	cytochrome P450 enzyme from *Bacillus megaterium*
PAH	polycyclic aromatic hydrocarbons
error-prone PCR	error-prone polymerase chain reaction

Introduction

While enzymes are widely used in industry for regio- or stereospecific hydrolysis, for chiral ester synthesis and isomerization reactions, biological oxidation has yet to be utilized on an industrial scale. Bacterial biotransformations based on the action of dioxygenases are currently in an advanced state of academic research [**1]. , e.g. the dihydroxylation of benzoic acid with *Alcaligenes eutrophus* [2]. Processes used in industry such as the oxidation of sorbit to sorbitol in vitamin C synthesis are usually based on dehydrogenations, and there are very few examples of direct microbial oxidations such as the 11β-hydroxylation of Reichstein S (Figure 1a), the formation of dicarboxylic acids from alkanes (Figure 1b) or the *p*-hydroxylation of phenyllactic

acid (Figure 1c), all carried out by fungal fermentations and P450 monooxygenase enzymes.

Recently, several groups have started to investigate these cytochrome P450 monooxygenases (also called CYP) for use in biotransformations employing the isolated enzymes. These are a potentially very useful class of catalysts for oxidation as they are able to introduce oxygen at non-activated carbon-hydrogen bonds to yield sterically and optically-pure compounds. In addition, they present one of the largest superfamilies of enzyme proteins known: genes coding for diverse P450 monooxygenases have been found in virtually all living organisms - in bacteria, algae, plants as well as in humans [*3,*4], and the number of CYP encoding sequences now amounts to over 660 (http://www.icgeb.trieste.it/~p450srv/new/p450.html). The reactions catalyzed by CYPs are quite diverse and range from the participation in the biosynthesis of hormones in animals and secondary metabolites in plants to the biodegradation of xenobiotic compounds. Mammalian CYPs, located in the liver microsomes, have been widely studied in the context of drug activation and the liver's response to toxic chemicals. However, there are considerable challenges in any application of isolated P450 enzymes in biocatalytic reactions. They are intrinsically not very active and exhibit poor stability in an isolated form. They require associated proteins such as ferredoxin and FMN-reductases for electron transfer, these complexes often being membrane-associated. Furthermore, as NAD(P)H, the cofactor of CYP-catalyzed reactions, is consumed in the reaction, a technical process is required to supply or regenerate this expensive chemical continuously. As a result, the search for alternative co-factors, or for NAD(P)H-regeneration, has met with great interest.

In spite of these difficulties, the development of efficient CYP biocatalysts has been initiated in several groups [*5]. Their work includes:

- a better understanding of structural features relating to the mechanism
- inexpensive electron transfer systems
- rapid, sensitive and automated high throughput screening methods
- the generation of more selective mutant enzymes.

In this review, we summarize the studies on the aspects on prokaryotic CYP enzymes mentioned above, most of which were published in 2001-2002.

Structure and function

Rational design of an enzyme requires a solid structural basis and an understanding of its catalytic mechanism. The structures of CYPs determined by X-ray crystallography at high-resolution have provided some of this information [6-13]. In spite of quite low sequence identity of < 20 %, all eight enzyme structures resolved so far demonstrate quite close structural similarity (as example the structure of P450 BM-3, Figure 2). Their tertiary structure is composed of both α-helixes and β-sheets, with significant dominance of the α-helical regions. Only two complexes of CYPs with their natural substrates – CYP101 [14] and CYP102 [7] - have been determined by X-ray analysis. As a result, rational analysis of enzyme-substrate interactions remains difficult. Moreover, activated intermediates in the reaction pathway are not covalently bound, thus excluding the use of pseudosubstrates or inhibitor complexes for the interpretation of substrate binding states. Seven of the eight CYPs whose structure was determined by X-ray crystallography are water soluble proteins, which were overexpressed and crystallized; two more structures (of CYP175 from *Thermus thermophilus* (J.K. Yano, F. Blasco, R.D. Schmid, T.L. Poulos, Abstract P105, 12th International Conference on Cytochrome P450, 11-15 September 2001, La Grande Motte, France) and of CYP154C1 from *Streptomyces coelicolor* (L.M. Podust, M.R. Waterman, Abstract CO1_3, International Conference "Genomics, Proteomics and Bioinformatics for Medicine", 22-30 June 2002, Moscow, Russia)) have been solved and will provide additional valuable information. Eukaryotic CYPs are membrane-associated proteins and much more difficult to crystallize; only one X-ray structure of the membrane-bound mammalian CYP2C5 has been partially determined so far [12], as it was not possible to derive complete structural information from the crystals. As a result, comparative molecular modeling is widely used as an alternative method to investigate structure-based features of mammalian and human cytochrome monooxygenases. In these studies, the structures of known prokaryotic CYPs have often served as a template for building structures of mammalian CYPs. An interesting recent example is the homology modeling of the active sites of two mammalian cytochromes, CYP11B1 and CYP11B2, on the basis of protein structures of P450 BM-3 from *Bacillus megaterium* and P450terp from *Pseudomonas* sp. [15].

The analysis of 3D structures has allowed the elucidation of the role of some residues in catalysis or electron transfer. Thus, using the P450 BM-3 crystal structure and

constructing mutants, Ost and coworkers could prove a critical role for F393 in providing electrons to the heme iron [16,17]. High-resolution crystal structures of P450cam in complex with ruthenium sensitizer-linked substrates revealed a “transient open state” of this enzyme [18]. It has been suggested that this “open state” conformation allows the substrate to access the active site. Indirect evidence suggested similar open/close conformations of other P450s [9,19]. From the comparison of P450cam bound to different substrate analogues, the authors concluded that the F- and G-helices undergo large motions during substrate binding. This fact, also confirmed for P450 BM-3 [20], was inferred in the wide substrate diversity observed for most microsomal CYPs in eukaryotic organisms.

Crystallography also helped to identify with certainty the positions and mechanistic role of solvent molecules. An excellent recent example is the crystal structure of a complex between P450 BM-3 and N-palmitoylglycine at a resolution of 0.165 nm. It revealed previously unrecognized features of the reorganization of the active site during substrate binding, and the pivotal role of one water molecule in this reorganization [21]. Crystals of fatty-acid hydroxylase CYP152A1from *Bacillus subtilis* either without substrate or in the presence of myristic acid were obtained [22]. Both crystal forms diffracted to 0.25 nm resolution and preliminary X-ray data were recorded using synchrotron radiation. CYP152A1 is an unusual peroxygenase which catalyses the hydroxylation of fatty acids by introducing an oxygen atom from H_2O_2 at the C_α- or $C_ß$-carbon. Using lysine scanning mutagenesis of amino acids in the putative distal helix, residues with a putative mechanistic role were determined [23]. Further mutagenesis revealed that P243, which is located close to the heme iron, plays an important role in the hydrophobic interaction with the substrate, whereas R224 is a critical residue for substrate binding and H_2O_2-specific catalysis.

Artificial electron-transfer systems

CYPs are hydroxylating enzymes, in which the addition of one atom of molecular oxygen activated by heme iron represents the essential step in the oxidation of a substrate. The second oxygen atom is reduced to water, using electron transfer from NAD(P)H via a flavoprotein or a ferredoxin. In a non-productive side-path (“peroxide shunt”), hydrogen peroxide may be employed as a source of both electrons and oxygen.

The mechanism of binding and electron transfer between the redox partners has been thoroughly investigated for P450cam [24,25] and P450 BM-3 [26] and extrapolated to other CYPs. Artificial fusion proteins between such redox partners have been studied for a number of years. An artificial electron transfer system has been constructed from CYP119 of *Sulfolobus sulfataricus*, putidaredoxin and putidaredoxin reductase [**27]. Site-directed mutagenesis improved the interaction of CYP119 with putidaredoxin and led to enhanced electron transfer. Gilardi and colleagues [**28] have assembled an artificial redox chain in a chimeric fusion protein, using the heme domain of P450 BM-3 and flavodoxin from *Desulfovibrio vulgaris*, connected by a natural loop of P450 BM-3 reductase; the working electrode was a glassy carbon disc with platinum wire (Figure 3a). A computer model of this complex assisted in the design of the two cofactors for very close proximity, resulting in a better current, thus indicating a tight contact of this construct between the heme domain and the electrode surface. Direct electrochemistry of the P450s on the electrodes, however, is generally difficult due to the deeply-buried cofactor and instability of the enzyme upon interaction with the electrode surface. Recent improvements include the modification of the electrode surface and the use of attached mediators, which assist in the catalytic cycle. Thus, Lei with colleagues showed by cyclovoltammetry that CYP101, immobilized on a glassy carbon electrode modified with sodium montmorillonite, exhibits reversible redox behavior [29]. The electrochemical reduction of oxygen might replace NADPH as a very expensive electron donor. Artificial electron donors have been investigated for this purpose. Thus, cobalt sepulchrate, described earlier as a mediator for flavin reduction, [30] has been successfully used in combination with zinc dust in a continuous reactor based on CYP102 (Figure 3b) [*31].

An alternative non-enzymatic approach for cytochrome P450 reduction is based on the high photosensitivity of flavin nucleotides [**32], which are covalently attached to CYPs. It was demonstrated that riboflavin can be used as a light-induced electron donor for the transfer of electrons to the heme domain of mammalian P4502B4: irradiation of cytochrome P450 leads to the photoreduction of riboflavin, followed by the reduction of the heme iron.

Assays

Many CYP enzymes involved in the oxidation of hydrocarbons have no or very few functional groups, such as alkanes, terpenes, sterols or fatty acids. For this reason, assays are usually performed using GC or HPLC, which cannot be applied in a high-throughput screening (HTS) format required for the modern genetic techniques of enzyme improvement, e. g. for error-prone PCR. The development of CYP HTS assays has thus been the focus of two recent studies. Schwaneberg et al. [33] have explored the use of the surrogate substrates *p*-nitrophenoxycarboxylic acids (pNCA) of different chain lengths, using protein engineering to enhance sensitivity. The product of the reaction – *p*-nitrophenolate - is easily monitored at 410nm. [34]. Through suitable permeabilization of the recombinant *Escherichia coli* cells producing CYP, these authors were able to measure the oxidation of pNCAs directly in whole-cell microtiter plates. This assay allowed for the screening of thousands of samples per day and reduced cofactor expenses more than 10-fold. Another new and more general method for NAD(P)H-linked oxidoreductase activity assays [35] is based on the detection of the alkali product of NADPH-oxidation. It was also applied to partially lysed *Escherichia coli* cells and proved to be a good alternative to the traditional monitoring of NADPH-consumption at 340 nm.

Engineering new properties into prokaryotic CYPs

Most studies have addressed altered selectivity. Rational protein design has been used for this purpose, as well as procedures of directed evolution such as error-prone PCR or gene shuffling. From an applied point of view, it is surprising that no report was found of enhancing activity or stability, though the operational and storage stability of isolated CYPs is certainly a major bottleneck for the technical application of this group of enzymes. A significant amount of work has been put into altering the substrate or stereospecificity of CYP102 [28,36,37,*38] and CYP101 [**39,40,41], whose structures are available at high resolution.

CYP102. Several groups have addressed the question of chain-length specificity of this enzyme. In a procedure termed “rational evolution” by the authors (protein design combined with directed evolution), substrate specificity was shifted from C-12 fatty acids to C-10 and C-8 in the fatty acid pseudosubstrates 10- and 8-*p*-nitrophenoxycarboxylic acid [42]. Three different mutants (A74G/F87V/L188Q; R47F/A74G/F87V/L188Q and V26T/R47F /A74G/F87V/L188Q) showed the same behavior, though in different ratios. The same mutants also showed activity towards

the free C-8 and C-10 fatty acids [37]. Arnold and colleagues have studied directed evolution for the conversion of CYP102 into an efficient octane hydroxylase [43]. They succeeded in generating mutants which accepted n-octane. The screening for alkane oxidation activity was based on a substrate analogue which generated, after terminal hydroxylation, aldehyde and yellow *p*-nitrophenolate (Figure 4). After two rounds of error-prone PCR, the screening of 3000 clones led to some variants that were approximately 6 times more active towards n-octane than the wild type. The same group continued to evolve the heme domain of this enzyme for an enhanced "peroxide shunt" pathway efficiency [44]. After five rounds of directed evolution, the authors obtained a variant that consumed hydrogen peroxide 100 times more effectively than the wild type enzyme. Appel et al. have shown that a triple mutant (A74G/F87V/L188Q), but not the wild-type enzyme, not only can hydroxylate indole [45], but also a wide range of other substrates, all of which bear little or no resemblance to fatty acids, the natural substrate. Thus, naphthalene (a polycyclic aromatic hydrocarbon) (Figure 5a), n-octane (an n-alkane) (Figure 5b), and 8-methylquinoline (a heteroarene) (Figure 5c) were oxidized. These unusual activities come close to the wide substrate specificity of some eukaryotic CYPs (e. g. those of mammalian liver) and might be used for chemical syntheses as well as for the industrial degradation of environmental contaminants.

A target reaction in the latter area was the oxidation of polycyclic aromatic hydrocarbons (PAHs) [46,47]. Carmichael and Wong [**48] found that two hydrophobic substitutions CYP102 (R47L/Y51F) increased the activity of this enzyme against PAHs such as phenanthrene (Figure 6a), fluoranthene and pyrene up to 40-fold. If these mutations were combined with mutation Ala264Gly, PAH oxidation increased another 5-fold, with simultaneous enhancement of NADPH oxidation and coupling efficiency between NADPH oxidation and substrate hydroxylation. All of the above mentioned residues are located in the substrate binding pocket of the enzyme. The substitution of Met354 and Leu437, however, both lying within the active site, led to the reduction of PAH oxidation activity. Li and others [49] have described another mutant (A74G/F87V/L188Q) which exerts high activity for the oxidation of other PAHs such as naphthalene, fluorene (Figure 6b), acenaphthene, acenaphthalene and 9-methylanthracene. The authors suggest that Ala74 and Leu188 enhance hydrophobic interactions with these substrates while mutants Arg47 and Tyr51 act as anchors for the carboxylic group of substrates. Using

the alkali NAD(P)H assay, Gilardi and colleagues [28] identified the best random mutants of a P450 BM-3 library which had been generated by error-prone PCR. A region between residues 3 and 320 was chosen for mutagenesis as it contains helices B', F and G, which are strongly involved in substrate binding and catalysis. Lauric acid, a mixture of n-octanoic acid and n-dodecane, a mixture of polyaromatic hydrocarbons or a pesticide mixture were used as substrates. Several mutants were obtained which exhibited enhanced NADPH consumption. When the positions of the pertinent mutations were analyzed, the change in function was not obvious from the structural changes.

CYP101. A few recent investigations have addressed altered regio- or stereoselectivity of P450cam for biotransformations. Thus, mutants have been obtained by site-directed mutagenesis which exhibited enhanced activity towards diphenylmethane (Y96F/I395G), styrene or ethylbenzene (Y96F/V247L) [39]. French et al. [40] have shown that wild type CYP101 can catalyze the formation of 2-ethylhenoxic acid from 2-ethylhexanol, yielding (R)-2-ethylhexanoic acid 3,5 times faster than the (S)-enantiomer (Figure 7). Using rational protein, two active site mutations (F87W/Y96W) showed improved stereoselectivity, leading almost exclusively to the desired product (R)-2-ethylhexanoic acid [41]. Using error-prone PCR, Arnold's group created P450cam variants showing improved activity for naphthalene hydroxylation in the absence of the cofactor NADPH, again using the "peroxide shunt" pathway [50].

Conclusions

Several groups have recently turned their interest to exploring the potential of isolated bacterial CYP enzymes in biocatalysis. While key questions such as operational stability have not yet been adequately addressed, protein engineering, based on rational design or directed evolution, has recently made significant process. Inexpensive methods of cofactor regeneration are also the focus of several research groups. It can be safely assumed that the large potential of CYP enzymes for selective hydroxylation of non-activated carbon-hydrogen bonds will further stimulate biotechnologists to adapt this important enzyme class to the needs of technology.

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Related Articles, Links

Laboratory evolution of a soluble, self-sufficient, highly active alkane hydroxylase.

Glieder A, Farinas ET, Arnold FH.

Keywords

Cytochrome P450, structure-function relationships, electron-transfer, protein engineering, biotransformation, environmental contaminants

"Teaser"

Enzymatic hydroxylations using oxygen are an emerging field in biocatalysis. In view of regio- and stereoselective hydroxylations at non-activated carbon atoms, they often compare favorable to cumbersome chemical procedures. P450-Monooxygenases are very useful enzymes for this type of reaction, but various bottlenecks such as stability and cofactor regeneration remain to be solved.

Legends to figures.

Figure 1. Three examples of direct microbial oxidation: (a) 11ß-hyroxylation of Reichstein S; (b) the formation of dicarboxylic acids from alkanes (which involves two additional steps catalyzed by dehydrogenases); (c) the *p*-hydroxylation of L-(-)-3-phenyllactic acid (Figure 1c).

Figure 2. Structure model of CYP102, based on X-ray data. The structure of the reductase domain has not been completely solved, in fact it is larger than the heme domain. Heme domain in bright blue, heme in red, FMN-binding domain in yellow, FMN in green.

Figure 3. Schematic representation of two artificial electron transfer systems:

(a) artificial fusion protein, consisting of the heme domain of CYP102 and flavodoxine from *Desulfovibrio vulgaris*, connected by one loop of P450 BM-3 reductase as a linker [**28];

(b) artificial electron transfer pathway from zinc dust to P450 BM-3 heme domain using cobalt sepulchrate as a mediator [30,*31].

Figure 4. Screening for n-octane oxidation, based on a substrate analogue – 8-p-nitrophenyl octane [43]. After terminal hydroxylation, aldehyde – 1-octanal and yellow *p*-nitrophenolate are generated.

Figure 5. P450 BM-3 mutants demonstrate a wide substrate specificity compared to the wild type enzyme [*38].

Figure 6. Oxidation of polyaromatic hydrocarbons by wild type and mutants of CYP102 as an example of bioremediation using monooxygenases:

(a) oxidation of phenanthrene [**39];

(b) oxidation of fluorine [36].

Figure 7. Wild type CYP101catalyzes the formation of (R)-2-ethylhexanoic acid from (1R)- camphor 3,5 times faster than the (S)- 2-ethylhexanoic acid [40].