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Review

Use of directed evolution of mammalian cytochromes P450 for investigating the molecular basis of enzyme function and generating novel biocatalysts

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Abstract

Directed evolution has been successfully applied to the design of industrial biocatalysts for enhanced catalytic efficiency and stability, and for examining the molecular basis of enzyme function. Xenobiotic-metabolizing mammalian cytochromes P450 with their catalytic versatility and broad substrate specificity offer the possibility of widespread applications in industrial synthesis, medicine, and bioremediation. However, the requirement for NADPH-cytochrome P450 reductase, often cytochrome b_5, and an expensive cofactor, NADPH, complicates the design of mammalian P450 enzymes as biocatalysts. Recently, Guengerich and colleagues have successfully performed directed evolution of P450s 1A2 and 2A6 initially by using colony-based colorimetric and genotoxicity screening assays, respectively, followed by in vitro fluorescence-based activity screening assays. More recently, our laboratory has developed a fluorescence-based in vitro activity screening assay system for enhanced catalytic activity of P450s 2B1 and 3A4. The studies indicate an important role of amino acid residues outside of the active site, which would be difficult to target by other methods. The approach can now be expanded to design these as well as new P450s using more targeted substrates of environmental, industrial, and medical importance.

Keywords: Cytochrome P450; P450; Directed evolution; Random mutagenesis; Biocatalysts

Natural evolution is a spontaneous process that occurs during reproduction and survival of the whole organism, whereas directed evolution has a defined goal, and the key processes such as mutation, recombination, and screening or selection are controlled by the experimenter [1]. Directed evolution allows us to explore enzyme functions that were not required in the natural environment and for which the molecular basis is poorly understood [2]. This approach contrasts with the more conventional one, in which proteins are tamed 'rationally' using site-directed mutagenesis. The main requirements for successful directed evolution are: (1) the desired function must be physically and biologically feasible; (2) the libraries of mutants should be simple enough to screen for desired function and complex enough to contain rare, beneficial mutations; (3) there must be a rapid and cost-effective screen or selection that reflects the desired function. Directed evolution has the ability to tailor individual proteins (for enhanced catalytic efficiency, stability, and novel activities) as well as whole biosynthetic and biodegradation pathways for biotechnological applications [3,4].

In recent years, there has been increasing realization of the power of biocatalysts for the industrial synthesis of bulk chemicals, pharmaceuticals, agrochemicals, and food ingredients, especially when high stereoselectivity is required [3,5]. In addition, there is a greater demand of biocatalysts for detoxification of environmental contaminants and in cancer gene therapy [6–8]. Hydroxylation has been one of the most difficult enzymatic reactions to harness because of low stability and turnover and expensive cofactor

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requirements. However, exciting recent results with the bacterial enzymes P450 BM3 and P450cam have illustrated the potential of directed evolution for engineering new and efficient hydroxylation pathways and for enabling the utilization of artificial oxygen donors, such as H_2O_2, instead of molecular oxygen and NAD(P)H [9–12]. Xenobiotic-metabolizing mammalian cytochromes P450, with their generally broader substrate specificity than bacterial P450s [13–15], offer the possibility of even greater applications in industrial synthesis, medicine, and bioremediation.

In this review, first we will briefly describe directed evolution of bacterial P450s followed by the significance of designing xenobiotic-metabolizing mammalian P450s by directed evolution. Since it is not possible to provide a complete account of all the methods used for directed evolution, we will mainly introduce the methods and strategies used successfully in our laboratory. Alternative methods applied to biocatalyst evolution in other laboratories along with some computational approaches will be discussed briefly. The main focus of this review will be on directed evolution of mammalian P450s, which was successfully pursued in Dr. Fred Guengerich's and our laboratory.

Directed evolution of bacterial cytochromes P450

Unlike most other P450s, in P450 BM3 an FMN/FAD-containing reductase domain is directly linked to the C-terminus of the heme domain, yielding a self-sufficient fatty acid hydroxylase, which makes it a simple system for directed evolution [16]. Through directed evolution, a P450 BM3 mutant (139-3) was created that displayed up to a 2-fold increase in hydroxylation of fatty acids and up to 100-fold higher activities with unnatural alkane substrates compared with other monooxygenases known to metabolize the same compounds [9,10]. Interestingly, this evolved enzyme has 11 simultaneous mutations, only two of which are in the active site. A recent crystal structure of P450 BM3 mutant (139-3), however, showed no significant difference in the overall architecture compared with wild-type, and only the two active site mutations were found to be responsible for improved alkane hydroxylase activity [17]. More recently, directed evolution of P450 BM3 generated a series of variants active towards smaller alkanes. Mutants 53-5H and 35-E11, having at least 15 amino acid substitutions, supported thousands of turnovers of propane, and catalyzed the selective conversion of ethane to ethanol without over-oxidation [18].

In addition to increased activity and substrate diversity, the following studies have strengthened the potential of directed evolution for engineering P450 BM3 for industrial applications: (1) utilization of H_2O_2 to catalyze hydroxylation and epoxidation of fatty acids [11,12]; (2) enhanced thermal stability and T_{50} from 43 to 61 °C [19]; (3) improved catalytic activity by 10-fold in 2% THF and 6-fold in 25% DMSO [20]; (4) development of the gram scale production and purification as well as whole cell (WC) high-throughput screening assays for improved activity and enhanced catalytic tolerance to organic solvents [21]. However, limited substrate diversity is the major disadvantage in designing bacterial P450 enzymes for a variety of industrial purposes. In contrast, because of broad substrate specificity mammalian P450s may be more suitable for design as biocatalysts for industrial, environmental, and medical purposes [13–15].

Xenobiotic metabolizing mammalian cytochromes P450: significance

Mammalian cytochromes P450s comprise a superfamily of monooxygenases that are of considerable interest because they are the major catalysts involved in the oxidation of steroids, drugs, carcinogens, pesticides, and other xenobiotics [13–15]. Among the human enzymes, P450s 1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1, 3A4, and 3A5 are involved in the oxidation of >90% of environmental toxicants, drugs, and carcinogens. Human 3A4 alone accounts for oxidation of more than 50% of drugs and plays a significant role in metabolizing carcinogens [22]. 3A4 demonstrates homotropic cooperativity with a number of substrates as well as heterotropic cooperativity, which is characterized by increased oxidation of one substrate in the presence of an effector [23]. The homotropic and heterotropic cooperativity of 3A4 is critical for drug-drug and drug-food interactions. P450s from the 2B subfamily are involved in the metabolism of anti-cancer prodrugs such as cyclophosphamide (CPA) and ifosfamide (IFA), and environmental contaminants such as polychlorinated biphenyls (PCBs) [24–26]. P450s 1A2 and 2A6, which have been studied extensively by Guengerich and co-workers [27,28] and are a major subject of this review, are the major enzymes involved in the bioactivation of heterocyclic aromatic amine and indole oxidations, respectively. P450s from the 2C subfamily, which are also being considered for directed evolution, play a major role in drug metabolism (accounting for >30% of available drugs) [29].

Because of their pharmacological and environmental significance, P450 3A4 and P450s from the 2B subfamily have been the subject of a large number of structure–function studies involving X-ray crystallography, homology modeling, and site-directed mutagenesis of substrate recognition site (SRS) or active site residues [30–38]. The above mentioned subject and a number of other studies have revealed that substrate binding and oxidation are influenced not only by SRS or active site residues, but also by residues outside of the active site [30,32,33,35–42]. Non-SRS residues may influence reductase binding, substrate access or product exit, and conformational changes that occur in the catalytic cycle. Non-SRS residues may also be involved in global changes that affect residues in the substrate binding region. For example, comparison of the P450 2B4 X-ray crystal structures in the absence and in the presence of inhibitor, site-directed mutagenesis of 2B1 in B′ helix, F–G loop, and I helix regions, and molecular modeling studies suggests very dynamic substrate access channels [32,33,39–42], In addition, X-ray crystal structures of

P450 3A4 indicate a phenylalanine cleft that surrounds the active site and has been suggested to control the interaction with redox-partners such as NADPH-cytochrome P450 reductase (CPR) and cytochrome b_5 (b_5) [37,38].

Directed evolution approaches

Directed evolution requires the following initial set up of high-throughput screening methods and construction of mutant libraries, followed by screening/selection and characterizations of the mutants with desired properties.

Sensitive, reliable, and cost-effective screening or selection method

The most critical step in directed evolution is to find a simple, economical, and efficient screening system to measure enzyme activity directly on a multi-well microplate. Normally P450s require CPR and often b_5 in order to accept electrons from NADPH and activate dioxygen. To circumvent these steps, an alternate oxygen donor, H_2O_2, can be used. However, significant activity in the H_2O_2-supported reaction requires both the appropriate cytochrome P450 and substrate. Therefore, a suitable system was first developed for P450s 2B1 and 3A4 through screening a number of enzymes, substrates, and buffer systems ([43], unpublished observations). If an enzyme is not active with H_2O_2, an NADPH-supported assay can be employed using a co-expression system with P450 and CPR. Guengerich et al. [44] have developed bicistronic plasmids that co-express P450 and CPR, and are capable of oxidizing substrates in the presence of NADPH in vivo as well as in in vitro assay systems. Our laboratory has also constructed bicistronic plasmids for 3A4, termed as 3A4-CPR, which are capable of showing detectable activity with NADPH in a 96-well microplate using a whole cell assay (unpublished observations). The expression of 3A4 in 3A4-CPR construct is, however, reduced compared with the expression of 3A4 alone.

Another critical step in a high-throughput assay is to identify a suitable substrate that yields an oxidized product that can be monitored easily. Methods employed include: (1) in vitro colorimetric assays using substrates that yield phenolic compounds such as derivatives of *p*-nitrophenol [45]; (2) in vitro fluorescence assays using substrates that yield fluorogenic products such as coumarin, quinoline, and resorufin derivatives [43,46]; (3) colony-based colorimetric assays using substrates that yield colored products such as indole or its derivatives [47–49]; and (4) colony-based genotoxicity assays with capability of reversion in the presence of 2-amino-3,5-dimethylimidazo[4,5-*f*]quinoline (MeIQ) [50–52].

Random mutagenesis

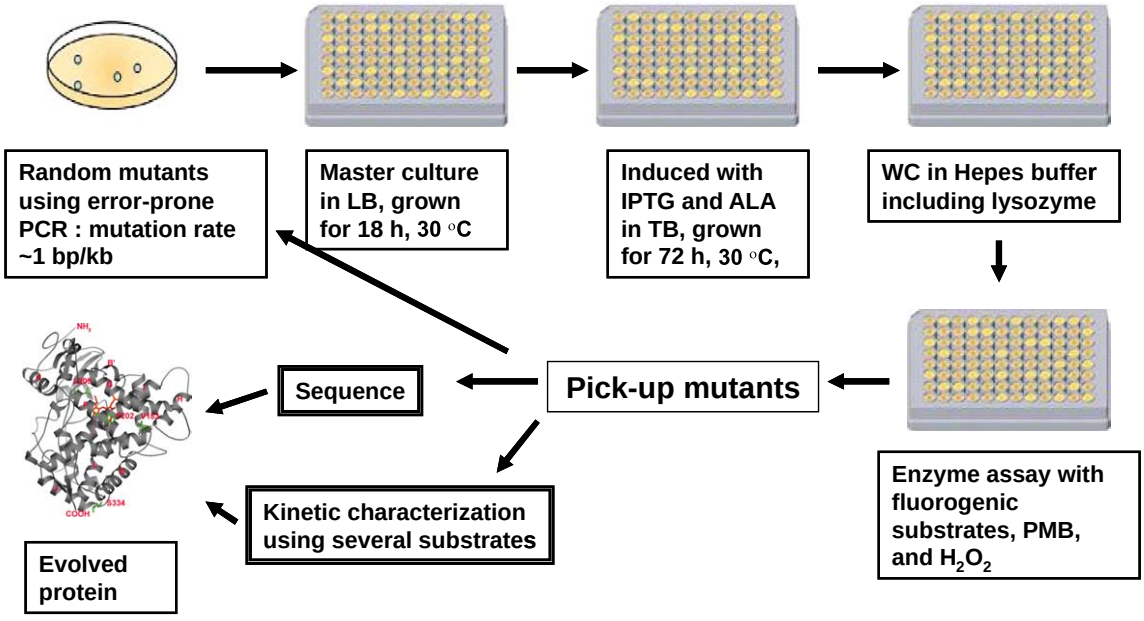
Following optimization of suitable screening methods to measure enzyme activity, error-prone PCR is performed to generate random mutants. This method is a modification of standard PCR methods designed to alter and enhance the natural error rate of the polymerase using low fidelity *Taq* DNA polymerase or Mutazyme DNA polymerase [43,46–54]. The target DNA could be either an entire reading frame of the gene of interest or a targeted sequence of the gene such as SRSs [43,46–54]. In addition, a random recombination technique, which involves splicing together different parts of homologous structures from the same structural superfamily, can be used to obtain random mutations [55,56]. A more recent development for directed evolution, however, is sequence saturation mutagenesis that randomizes a target sequence at every single position [57]. It requires four simple steps: generating a pool of DNA fragments with random length, tailing the DNA fragments with universal base using terminal transferase at the 3′-termini, elongating DNA fragments by PCR to full-length using a single-stranded template, and replacing the universal bases by standard nucleotides.

Computational approach

A semi-directed evolution approach is currently being developed to find a suitable sequence for random mutagenesis using various computational methods [58–61]. These include: (1) protein structure–activity relations (PROSAR), in which a statistical model is built based upon initial screening of sample activity-sequence relationships [59]; (2) genetic algorithm based upon the principle of evolution by natural selection [60], including initial sample screening from a combinatorial library using DNA shuffling, followed by selecting more active parents from mating and breeding; (3) sequence-independent site-directed chimeragenesis (SISDC), which includes recombination of two distantly related proteins at various sites, followed by identification of cross-over sites using SCHEMA (structural information to predict polypeptide elements that can be swapped among related proteins with minimal disruption) [60]; (4) structure-based combinatorial protein engineering (SCOPE) based upon a residue's tolerance for structure stability and/or enzyme activity, which requires theoretical analyses by partial least-squares regression, force field rotamer library, and mean-field theory [61].

Screening and selection of random mutants

Upon developing a screening/selection method and optimizing error-prone PCR, mutant libraries are created, which are further screened/selected for higher enzyme activity and characterized as depicted in Scheme 1 [62,63]. The following steps were developed in our laboratory for P450s 2B1 and 3A4 in order to find mutants with enhanced catalytic activity: (1) run an error-prone PCR, ligate the PCR product, and transfer to a supercompetent *Escherichia coli* strain; (2) transfer individual colonies to a multi-well microplate containing LB medium and grow for 18–20 h; (3) transfer 10% of the LB-grown cultures to



Scheme 1. Screening, selection, and characterization.

a multi-well microplate containing TB medium, induce P450 expression with IPTG and δ-aminolevulinic acid, and grow for 72 h; (4) prepare WC in Hepes buffer containing lysozyme; (5) measure the enzyme activity with H_2O_2 using the desired fluorogenic substrates in the presence of polymyxin B sulfate for membrane permeabilization; (6) isolate mutants with $\geqslant$2-fold higher activity than the wild-type and identify the site of mutations; (7) perform kinetic analysis of the purified mutants using H_2O_2. The selected individual mutants are combined by site-directed mutagenesis, if necessary, and are subjected to additional cycles of mutagenesis until saturation for enhanced activity in that particular pathway is achieved.

In addition, screening and selection for mutants with improved catalytic tolerance to temperature and organic solvents and enhanced utilization of H_2O_2 is carried out as described earlier for P450 BM3 [19–21]: (1) screen for mutants with ±20% of the original activity; (2) incubate the selected mutants at temperature (T_{50}), in organic solvents (C_{50}), and at H_2O_2 concentration (K_m) at which the parental P450 retains its 50% of the activity; (3) measure enzyme activity as described above to find the mutants with $\geqslant$2-fold higher than the original activity.

Characterization of random mutants

To test whether the substrate specificity is retained, kinetic analysis is also performed with several structurally diverse substrates using a standard NADPH-supported reconstituted system. Mutants with the highest catalytic efficiency are further characterized for protein stability using guanidine hydrochloride-induced heme dissociation and circular dichroism, tolerance of the catalytic activity to temperature and organic solvents, efficient utilization of H_2O_2, and structural modeling using homology modeling and substrate docking, as described for 2B1dH V183L/F202L/L209A/S334P [unpublished observations]. An X-ray crystal structure of the mutant enzyme can be solved to further understand the role of mutated active and/or non-active site residues, as described for P450 BM3 [17].

Directed evolution of mammalian cytochromes P450

Directed evolution of mammalian P450s is complicated by their requirements for redox partners and an expensive cofactor NADPH. In addition, the poor turnover (1–25 min^{-1}) limits the development of direct and cost-effective high-throughput screening/selection methods. Guengerich and co-workers [46–52] have successfully performed directed evolution of P450s 1A2 and 2A6 initially by using colony-based colorimetric and genotoxicity assays, respectively, followed by in vitro fluorescence-based enzyme assay systems. In addition, our laboratory has successfully developed a fluorescence-based in vitro activity screening method for P450s 2B1 and 3A4 ([43], unpublished observations). A summary of the approach taken and the results obtained from directed evolution of mammalian P450s is presented in Table 1.

P450 1A2

Random mutant libraries of human P450 1A2, in which mutations were made in SRS residues, were screened with *E. coli* DJ3109pNM12, a strain designed to bioactivate MeIQ and detect mutagenicity of the products [50–52]. In the first such study, 27 out of 6000 clones of P450 1A2 displaying 3- to 4-fold higher k_{cat}/K_m for the oxidation of

Table 1
Summary of directed evolution of mammalian cytochromes P450

P450	Screening/selection method	Summary of the results	References
1A2[a]	MeIQ; colony-based genotoxicity assay	3- to 4-fold increased k_{cat}/K_m with 7-ER and phenacetin	[50,51]
1A2	MeIQ; colony-based genotoxicity assay	12-fold enhanced k_{cat}/K_m with MeIQ	[52]
1A2	7-MR; fluorescence-based enzyme assay	5-fold enhanced k_{cat}/K_m	[46]
2A6[a]	Indole; colony-based colorimetric assay	Produce blue colored products	[47]
2A6	7-MR; fluorescence-based enzyme assay	Decreased K_m for the substrate	[48]
2A6	5-BR; colony-based colorimetric assay	Produce blue colored products	[49]
2C	Indole; colony-based colorimetric assay	Produce blue colored products	[6]
2B1	7-EFC; fluorescence-based enzyme assay	6- and 3-fold enhanced k_{cat}/K_m in H_2O_2- and in the NADPH- supported reactions, respectively	[43]
2B1	7-EFC; fluorescence-based enzyme assay	3-fold decreased K_m for H_2O_2 and 2-fold enhanced catalytic tolerance to DMSO and THF	Unpublished
3A4	7-BQ; fluorescence-based enzyme assay	2-fold enhanced k_{cat}/K_m in H_2O_2 system	Unpublished
3A4-CPR	7-BQ; fluorescence-based enzyme assay	Altered cooperativity	Unpublished

[a] SRSs were targeted for random mutagenesis.

7-ethoxyresorufin (7-ER) and phenacetin were selected [50,51]. Among the 27 mutations, seven were localized to SRS-5, six each to SRS-1, SRS-2, and SRS-5, two to SRS-3, and none to SRS-6. These mutants were later characterized using three substrates: 7-ER, phenacetin, and MeIQ. S126D, S119E, E225I, V322A, and T385L had significantly elevated activities towards 7-ER and phenacetin.

Recently, random mutant libraries of 1A2, in which mutations were made throughout the entire reading frame, were screened with *E. coli* DJ3109pNM12 [46]. One mutant showed 10-fold enhanced catalytic efficiency with MeIQ. Homology modeling based on an X-ray crystal structure of 2C5 suggested that two mutations, E225N and Q258H, are located in the E and F helices, and may affect the movement of helix I when the substrate binds in the active site. G437D is located in the so-called ''meander region''.

More recently, directed evolution of 1A2 using a combination of random mutagenesis of the entire open reading frame and high-throughput screening utilizing a fluorescent enzyme assay was done [52]. 1A2 yielded mutants with 5-fold enhanced catalytic efficiency with 7-methoxyresorufin (7-MR). Homology modeling based on an X-ray crystal structure of 2C5 suggested that two mutations (E136K and K170Q) were found in or near helix D and one (V193M) in helix E. So far no reports have appeared on the functional roles of helices D and E in the catalytic mechanism of P450s.

P450 2A6

Development of bicistronic systems for coexpression of P450 enzymes with CPR enabled P450 activity to be reconstituted in bacterial cells. During expression of 2E1 and some other enzymes, formation of a blue pigment was observed in bacterial cultures, which was further identified as indigo [64]. These accidental findings suggested potential applications of mammalian P450 enzymes in industrial indigo production or in the development of novel colorimetric assays based upon indole hydroxylation. As such, random mutant libraries of 2A6, which is known to metabolize indole, were generated in the SRSs regions and screened in *E. coli* on the basis of indole metabolism [47]. Interesting mutants from SRS-3 and SRS-4 libraries were identified, which showed spontaneous oxidation of 3-hydroxyindole to a colored indigo compound. F209T showed lower levels of indole 3-hydroxylation compared with wild-type, but had a 13-fold greater k_{cat} for coumarin 7-hydroxylation than wild-type. L240C/N297Q consistently produced very blue colonies.

Furthermore, mutagenesis of 2A6 was conducted to expand its capability in the oxidation of bulky-substituted indole compounds using L240C/N297Q as a template [48]. Directed evolution using the entire open reading frame yielded I140M/L240C/N297Q/I300V/I366V, which converted 5-benzyloxyindole to blue products. Further site-directed mutagenesis yielded the simplest mutant, N297Q/I300V, which oxidizes both 4- and 5-benzyloxyindole (4- and 5-BI) to colored products. Additional studies are underway to identify the products derived from 5-BI and to use active site modeling and substrate docking to further elucidate the possible mechanisms involved in the above processes.

More recently, a combination of random mutagenesis of an entire reading frame and fluorimetric high-throughput screening was used in the analysis of 2A6, utilizing a fluorescent coumarin 7-hydroxylation assay [49]. Initially, 5 of 27 mutants that showed highly decreased activity in the membrane fraction were selected for further analysis of substrate selectivity and binding affinity. All five mutants showed reduced k_{cat}/K_m for 7-methoxycoumarin (7-MC) *O*-demethylation. All mutants except one (K476E) showed highly decreased coumarin binding affinities, indicating that the decreased enzymatic activities for these mutants are mainly due to the change in the substrate binding affinities.

P450 2B1

Recently, we have developed a directed evolution approach to screen/select P450 2B1dH L209A for enhanced H_2O_2-supported 7-EFC *O*-deethylation [43]. 2B1dH L209A was used as the template for directed evolution because the engineered 2B1dH (N-terminal truncated and C-terminal His-tagged) shows >10-fold higher expression than 2B1, and L209A shows >5-fold higher activity than 2B1dH [39,65]. The engineered 2B1 V183L/F202L/L209A/S334P showed a 6-fold enhanced catalytic efficiency for H_2O_2-supported 7-EFC *O*-deethylation. F202L/L209A/S334P, however, showed the highest catalytic efficiency in the NADPH system, and it also retained the enhanced catalytic efficiency with several other substrates such as 7-benzyloxyresorufin (7-BR), benzphetamine, and testosterone [43]. F202L/L209A/S334P catalyzed testosterone 16α-hydroxylation with a k_{cat} (150 min^{-1}) that is among the highest reported for any mammalian cytochrome P450 in an NADPH-supported reaction. Interestingly, with CPA and IFA as substrates, several of the mutants showed increased metabolism and regioselectivity via the therapeutically beneficial 4-hydroxylation pathway, with L209A/S334P showing 2.8-fold enhancement of k_{cat}/K_m with CPA and V183L/L209A showing 3.5-fold enhancement with IFA. At present, 2B11 is the most efficient catalyst of CPA and IFA activation, which proceeds via 4-hydroxylation [24]. Given the ~2-fold decrease in K_m for CPA 4-hydroxylation seen with all of the L209A-containing 2B1 mutants examined, introduction of the $Leu^{209} \rightarrow Ala$ substitution into 2B11 may lead to a corresponding decrease in K_m and an increase in catalytic efficiency and/or regioselectivity for CPA 4-hydroxylation.

More recently, among a set of evolved 2B1 enzymes, V183L/F202L/L209A/S334P was further characterized with respect to activity and stability in comparison with the template L209A [unpublished observations]. V183L/F202L/L209A/S334P showed: (1) a >2.5-fold increase in k_{cat} and >3.0-fold decrease in K_m for H_2O_2 at a saturating 7-ethoxy-4-trifluoromethylcoumarin concentration, (2) an increased apparent thermal melting temperature ($T_{m,app}$) and retained catalytic tolerance to temperature, and (3) a 2-fold higher tolerance of catalytic activity over nearly the entire range of investigated DMSO and THF concentrations. V183L/F202L/L209A/S334P, therefore, can be used for further engineering by directed evolution.

The evolved enzymes have mutations outside of the active site (V183L, F202L, and S334P). Residues 183 and 334 are located in the E helix (near SRS- 2) and J–J′ loop (between SRS-5 and SRS-6), respectively, and have not previously been implicated in P450 function. Although, residue 202 is far from the active site in the P450 2B enzymes, residues 201 to 210 have been proposed to form a secondary lipid binding site in P450 2C5 and 2C3 [66]. Homology models of V183L/F202L/L209A/S334P and L209A based on the crystal structure of 2B4 showed similar backbone architecture. However, substrate docking showed that Leu-202 is in the active site <5.0 Å from 7-EFC, suggesting a possible interaction with substrates (unpublished observations). Consistent with modeling, experimental data showed that while F202L shows decreased enzyme activity, F202L/L209A shows increased activity, further suggesting that residues 202 and 209 are in close proximity [43]. $Phe^{202} \rightarrow Leu$ and $Leu^{209} \rightarrow Ala$ substitutions appear to bring both residues closer to the substrates. Therefore, further studies using site-directed mutagenesis and modeling are underway to understand the role of these residues in substrate metabolism.

P450 from the 2C subfamily

Directed evolution of 2C enzymes was recently initiated by Gillam [6] using a recombination approach to design P450 2C9, 2C11, and 2C19 in a co-expression system with CPR. Sequencing of the mutants revealed an average of 6.9 crossovers and 2.6 spontaneous mutations per 1.5 kbp sequence. In a colony-based colorimetric approach two mutants showed a blue color in liquid culture, from which only one mutant showed ~2-fold higher activity than the parent P450. No further reports on directed evolution of P450 from the 2C subfamily are available. Therefore, development of a directed evolution approach to engineer P450 2C enzymes is highly desirable.

P450 3A4

An enzyme assay using H_2O_2-supported oxidation was developed for P450 3A4 using 7-benzyloxyquinoline (7-BQ) as a substrate [67]. This led to development of a high-throughput screening method for H_2O_2-supported 3A4 activity using a WC suspension (unpublished observations). At present, two mutants with ⩾2-fold higher activity than 3A4 wild-type have been selected. However, these mutants did not show increased k_{cat} in the standard NADPH system. Further screening and selection of 3A4 is in progress.

An effort is also being made to screen/select 3A4-CPR variants with altered cooperativity using the standard NADPH system. Initially, the membranes were assayed with the fluorogenic substrates 7-BQ and 7-benzyloxy-4-(trifluoromethyl)coumarin (7-BFC). Both substrates yield sigmoidal *v* versus *S* plots, which become hyperbolic in the presence of 25 μM α-NF (unpublished observations). Therefore, a high-throughput screening method for NADPH-supported 3A4 activity was developed for 7-BQ de-benzylation in the absence and in the presence of α-NF. Our preliminary results identified a mutant with decreased cooperativity (unpublished observations). To test whether a loss or gain of cooperativity is maintained across 3A4 substrates, kinetic analysis will be performed with 7-BFC, progesterone, testosterone, and diazepam in addition to 7-BQ. Further screening and selection of 3A4-CPR is underway.

Conclusions and future prospects

Development of the described random mutagenesis and high-throughput screening methodologies, especially fluorescence-based enzyme activity, demonstrate the feasibility of directed evolution of the mammalian P450s. In addition, recent development of highly sensitive and reliable high-throughput luminescent-based P450 assays (designated as P450-Glo) by Promega may facilitate further directed evolution of mammalian P450s [68]. Luminogenic substrates for human P450s 1A1, 1A2, 1B1, 2B6, 2C8, 2C9, 2C19, 2D6, 2J2, 3A4, 3A7, 4A11, 4F3B, 4F12, and 19 have been synthesized and examined in recombinant P450 fractions and liver microsomes. Therefore, a successful directed evolution approach will serve as a basis for expansion of P450 engineering with regard to enhanced catalytic efficiency in three directions. First, the types of substrates used in screening P450s 2B1 and 3A4 can be expanded to include realistic targets of environmental, industrial, and medical importance. Second, these technologies can be easily expanded to new enzymes such as P450s from the 2C subfamily, which will be useful in expanding the range of potential environmental and medical applications. A third obvious expansion of this project is engineering P450 mutants with an enhanced capability for using H_2O_2 as an oxidant (in progress for 2B1), as described for P450 BM3 and P450cam [11,12].

Because of a growing demand for highly stable enzymes for industrial processes, the next expansion of directed evolution of mammalian P450s may be to develop a high-throughput screening method for enhanced protein stability [69]. One approach is to monitor protein unfolding transitions (induced by chemical denaturants such as GnHCl and urea or temperature) by tryptophan fluorescence at 340 nm as described previously [70,71]. Since increased heterologous expression of P450s is also important to meet the industrial demand for gram quantities, high-throughput screening to achieve a large scale preparation of P450 may be undertaken [72]. The expressed protein can be measured using a high-throughput CO-binding spectra, as described for P450 BM3 [73]. The most active and/or stable enzymes designed as described above can, in turn, be first tested and then engineered specifically for enhanced tolerance of catalytic activity to temperature and organic solvents [19–21]. Furthermore, knowledge obtained from these mutations can be utilized to re-engineer other P450s from the same or different subfamilies by introducing the corresponding mutation(s) using site-directed mutagenesis.

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