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Rational and semi-rational engineering of cytochrome P450s for biotechnological applications

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ABSTRACT

The cytochrome P450 enzymes are ubiquitous heme-thiolate proteins performing regioselective and stereoselective oxygenation reactions in cellular metabolism. Due to their broad substrate scope and catalytic versatility, P450 enzymes are also attractive candidates for many industrial and biopharmaceutical applications. For particular uses, enzyme properties of P450s can be further optimized through directed evolution, rational, and semi-rational engineering approaches, all of which introduce mutations within the P450 structures. In this review, we describe the recent applications of these P450 engineering approaches and highlight the key regions and residues that have been identified using such approaches. These "hotspots" lie within critical functional areas of the P450 structure, including the active site, the substrate access channel, and the redox partner interaction interface.

1. Introduction

The cytochrome P450 (CYP or P450) enzymes are a superfamily of heme-thiolate proteins found in most living systems from bacteria to humans [1]. Named after their peculiar spectroscopic absorption maximum at 450 nm when the ferrous heme binds carbon monoxide, P450s have been extensively studied for over 50 years since their first discovery from the mouse liver in 1940 [2,3]. By the incorporation of one oxygen atom from molecular oxygen into an organic substrate, P450s catalyze a variety of reactions including hydroxylation, epoxidation, dehydrogenation, dealkylation, desaturation, nitration, and C–C/C–N bond formation [1,3].

In a typic catalytic cycle, P450s utilize redox proteins or domain to mediate electron transfer from NAD(P)H to the heme iron, allowing the binding and activation of molecular oxygen to form a heme iron (IV) oxo species, referred to Compound I, which in turn carry out various oxygenation reactions [4]. The P450s widely participate in the metabolism of xenobiotics, such as environmental compounds, antibiotics, and various drugs, and are also involved in the biosynthetic pathways of endogenous molecules including sterols, fatty acids, prostaglandins and many specialized metabolites [1,4].

Due to their breadth of substrate range and the catalytic versatility, P450s are of high interest for pharmaceutical and chemical applications (Fig. 1) [5]. Compared with chemical catalysts, P450s offer the significant advantage of being able to regio- and stereo-specifically activate and modify inert substrate groups under mild conditions. One of the most successful industrial applications is the production process of pravastatin, a cholesterol-lowering drug used for preventing cardiovascular disease [6]. Pravastatin could be obtained by a two-step production process, in which compactin is first produced by the fermentation of *Penicillium citrinum* and followed by stereoselective hydroxylation of compactin at the C-6 position by *Streptomyces carbophilus* containing P450sca-2 (CYP105A3) [7,8]. Recently, a single-step production process was also achieved by fusing a newly-discovered compactin-hydroxylating CYP105AS1 to a redox partner and expressing the construct in the compactin-producing *Penicillium chrysogenum*, leading to a titer of 6 g/L for pravastatin at a pilot production scale [9].

Despite the huge potential, the industrial application of microbial and other P450s is limited by several technical bottlenecks including low enzyme activity, the need for electron transfer partners, and the requirement for the expensive cofactor NAD(P)H [10]. For solutions, P450s engineering, redox partner and host cell screening, redox partner engineering, regeneration of NAD(P)H are often performed. In recent years, the number of P450 crystal structures has increased dramatically, providing crucial aid to structure-function relationship studies, as well as rational and semi-rational engineering of P450s for various

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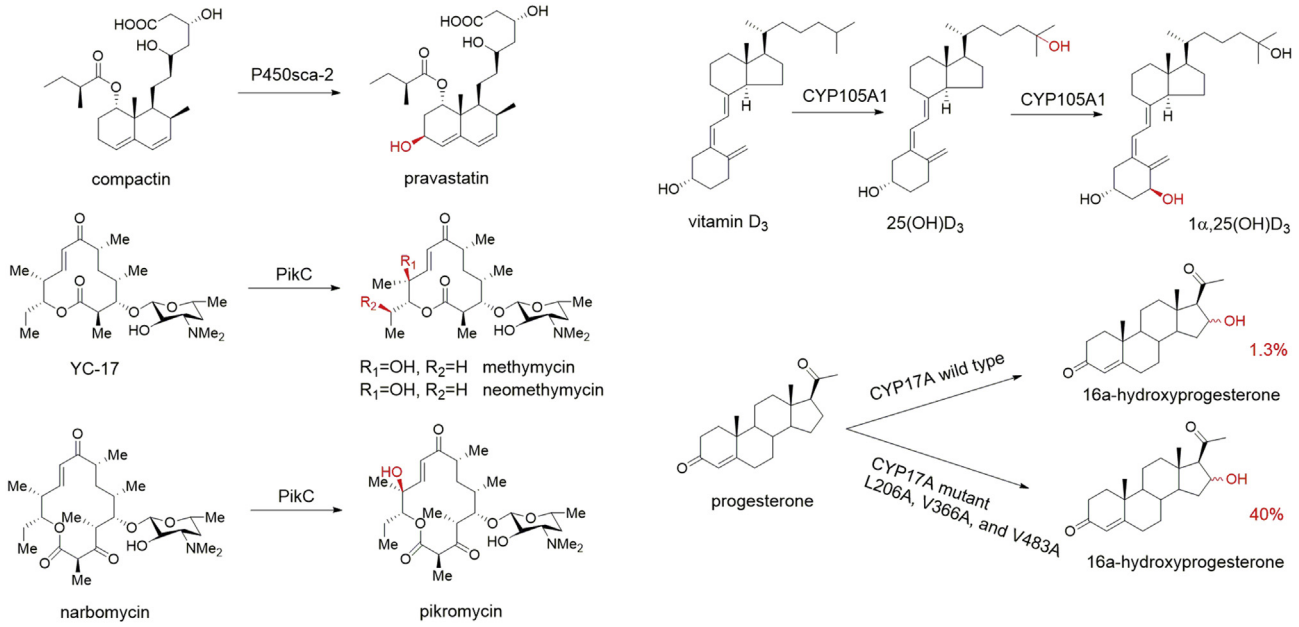

Fig. 1. Examples of cytochrome P450 enzymes involving in the production of pharmaceuticals mentioned in this review.

applications.

Recent reviews have covered the function, structure, and applications of P450s [5,10–16]. Besides, studies on P450 engineering for novel chemistry were also recently summarized [17–19]. Here, we mainly focus on the P450 engineering approaches for biotechnological applications, pay special attention to rational and semi-rational design, highlighting the critical residues in the hotspot areas including the substrate recognition sites, the substrate-access channel, and the redox partner interaction interface.

2. Structure of P450

P450cam (CYP101A1) from *Pseudomonas putida*, catalyzing the regio- and stereospecific hydroxylation of camphor to 5-*exo*-hydroxycamphor, was the first P450 to be investigated in great detail, with its crystal structure first reported in 1985 [20]. The heme domain structure of another well-characterized P450, P450BM3 (CYP102A1), a fatty acid hydroxylase from *Bacillus megaterium*, was determined in 1993 [21]. An increasing number of available P450 structures now showed that the overall P450 structure is quite conservative and generally consists of an α-helix-rich region (αA-αL) and a β-sheet-rich region (β1-β10) (Fig. 2a) [22,23]. The heme prosthetic group is located almost within the center of the structure and does not contact with the solution. A conserved cysteine residue is present on the proximal side of the heme, to which it is coordinated with a binding distance of 2.2–2.3 Å. The distal side of the heme contains a ligand-binding site that binds water, oxygen, and substrate molecules. The I helix is the most structurally conserved region in the P450s and passes through the entire P450 structure above the heme plane. The F and G helices are located above the I helix and form a V-like shape architecture. Although the overall protein structure is very similar among the different P450s, the N terminal loop, the B-C loop (the loop between the B and C helices), and the F-G loop (the loop between the F and G helices) show significant differences. Furthermore, the B′ helix and F-G loop are highly flexible and are responsible for substrate recognition and substrate binding. This structural diversity confers the high level of functional diversity observed for the P450s.

3. Engineering of the P450s

Protein engineering of enzymes is a process of modifying protein structures for desired properties, such as improved regio- and stereoselectivity, catalytic activity, thermostability, and solvent tolerance [24]. Generally, P450 engineering approaches include directed evolution, rational and semi-rational design. Directed evolution, a process that mimics natural evolution by introducing artificial random mutations and followed by screening for desired properties, is widely used

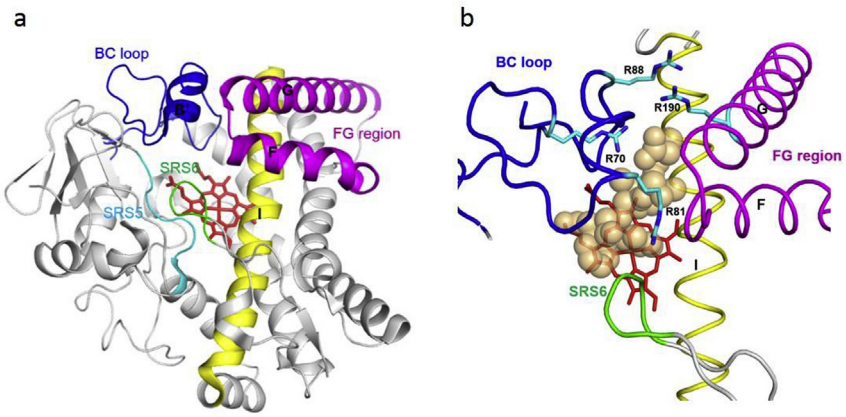

Fig. 2. (a) Structure of CYP105D7 (PDB ID: 4UBS, the ligand is removed for cleanness) from *Streptomyces avermitilis* as a representative of P450s. (b) Conserved arginine residues on the substrate entrance area of CYP105D7-diclofenac complex structure. The BC loop, FG region, I helix, SRS5 region and SRS6 region are shown in blue, magenta, yellow, cyan and green, respectively. Heme is shown as red stick. The ligand molecule in CYP105D7 (DIF-1, DIF-2, PGE) is shown as an orange sphere. Arg70, Arg81, A88 and Arg190 are shown as cyan sticks.

for investigating the structure-function relationships of both unknown P450s and well-characterized P450s [5,12,25]. Particularly, directed evolution has been frequently carried out on the full heme domain-coding regions of the wild-type P450BM3 gene and its mutants. For instance, in order to improve the catalytic activity of a drug-metabolising P450BM3 mutant (R47L/F87V/L188Q) toward substrates dextromethorphan and 3,4-methylenedioxymethylamphetamine, random mutagenesis by error-prone PCR was performed, and mutants with 200-fold increased turnover were obtained [26]. As another example, to evolve mutants with required regioselectivity for P450BM3 (F87A) that initially hydroxylates testosterone to give a 1:1 mixture of the 2β- and 15β-hydroxylated products. Reetz and co-workers carried out iterative saturation mutagenesis to the 20 residues lining the substrate-binding pocket, affording mutants that are 96–97% selective for either of the two regioisomers [27]. More recently, preparative-scale oxidation of aniline to 4-aminophenol was achieved by screening a library of P450BM3 variants containing mutations at the active site [28]. By using triple code saturation mutagenesis and iterative saturation mutagenesis, Reetz and co-workers obtained evolved P450BM3 mutants that could be combined with selected alcohol dehydrogenases to achieve one-pot, regio- and stereoselective conversion of cyclohexane to cyclohexane-1,2-diol [29]. Furthermore, Fasan et al. successfully improved the stability of the reductase domain of P450BM3 by using consensus-guided mutagenesis, a method that based on the idea that introduction of ancestral/consensus residues could contribute to the protein stability [30].

Unlike directed evolution, rational engineering usually uses site-directed mutagenesis to obtain mutants, but in this case, it is usually limited to the enzymes whose tertiary structure and structure-activity relationships are well-characterized. Rational design could effectively reduce the number of variants generated, which in turn significantly reduce the screening workload. Generally, P450 engineering by rational design starts with the identification of critical residues responsible for ligand binding based on the co-crystal structure of enzyme-ligand complex or molecular docking analysis, which is very helpful to the prediction of mutations that could be targeted to alter the catalytic activity or substrate specificity. For example, CYP260A1 from *Sorangium cellulosum* catalyzes the unique, 1α-hydroxylation of progesterone, a steroid whose hydroxylated derivatives have different medical applications [31]. To improve its selectivity, molecular docking of progesterone into the crystal structure of full-length CYP260A1 (PDB entry 5LIV) was first performed to identify potential selectivity-determining residues. Randomization of the three serine residues in active site and followed by analysis of progesterone conversion lead to the generation of two highly regio- and stereoselective mutants, S276N and S276I. The S276N mutant predominant produce 1α-hydroxy-progesterone, while the S276I mutant gave 17α-hydroxy-progesterone. Subsequent structural characterization of the S276N and S276I showed alternative binding mode of progesterone in the active site, providing a rationale for their regioselectivity.

In the case of the P450s without any crystallographic data, their structures could also be predicted using homology modeling. For example, the CYP17A1 is known to catalyze the 17α-hydroxylation of pregnenolone and progesterone as well as the subsequent C17-C20 bond cleavage, providing important precursors for different pathways [32,33]. To study the reaction of progesterone with bovine CYP17A1, a homology model was first created based on the crystal structures of human CYP17A1 (PDB codes: 4NKW, 4NKY, and 5IRQ), *Danio rerio* CYP17A1 (PDB code: 4R1Z) and CYP17A2 (PDB code: 4R20), and was then used a guide for alanine scanning of conserved active-site residues by site-directed mutagenesis, revealing that L206A, V366A, and V483A mutations alter regioselectivity, increasing the formation of the side-product, 16α-hydroxyprogesterone, up to 40% of the total product formation [34].

Semi-rational design is a combination of rational design and directed evolution, and it is a powerful method for acquiring useful variants by mutagenesis of specific amino acid(s) that was identified through the analysis of a crystal structure or homology model, and followed by mutant library screening [20]. This approach is also frequently applied for P450 engineering. For instance, P450sca-2 is known for its ability to stereoselectively hydroxylation of compactin to yield pravastatin. Based on a homology model, a more active P450sca-2 (CYP105A3) variant was subjected to site-directed saturation mutagenesis and three rounds of iterative saturation mutagenesis, focusing on the five sites that were presumably located in substrate-binding site, substrate access entrance and redox partner interaction interface. These efforts result in a mutant that showed increased whole cell biotransformation activity (7.1-fold) and overall apparent k_{cat} (10.0-fold), compared to that of the starting template [35]. Another example is the engineering of CYP153A$_{M.aq}$-CPR$_{BM3}$, a functional chimera consisting of the CYP153A heme domain from *Marinobacter aquaeolei* and the P450BM3 reductase domain, for improved terminal hydroxylation activity towards fatty acids. Semi-rational design based on a homology model of the CYP153A$_{M.aq}$ heme domain and subsequent mutagenesis of the selected residues in the substrate-binding pocket and substrate access channel was carried out. Combination of the promising variant results in a double mutant G307A/S233G showed increased activity for fatty acids with different chain length and released substrate/product inhibition effect [36].

4. “Hotspots” for rational and semi-rational engineering

When conducting rational and semi-rational design, the target residues for mutagenesis are often located within the substrate-binding active site, the substrate access channel, the redox partner interaction regions, as well as the various substrate recognition sites (SRSs). The SRSs were first identified during the experimental characterization of the CYP2 family and in the first crystal structure of P450cam [37–39]. SRS1 is located in the B-C loop (this loop can contain one to three small helices in different P450s). SRS2 is located on the C-terminal side of the F helix, SRS3 is located on the N-terminal side of the G helix, SRS4 is on the I helix, SRS5 is located in the region starting from the conserved EXXR motif to the β1-4 strand, while SRS6 spans between the β-strands 4–1 and 4–2 (Fig. 3).

4.1. Substrate-binding active site

The substrate-binding active site usually consists of the B-C loop region (including SRS1), I helix (including SRS4), and the SRS5 region. Systematic sequence-based literature mining analysis showed that residue of standard position 87, located within SRS1, is the most frequently investigated residue position for all CYPs [37]. This position (F87) is especially well-known from P450BM3, for which substitutions of F87 cause significant changes in the substrate spectrum towards unnatural substrates that include aromatic compounds, alkanes, and pharmaceuticals, with enhanced regio- and stereoselectivity, improved catalytic rates, and increased total turnover [40–43].

The I helix region of the active site has also been the focus of rational engineering. For instance, G248A substitution in a P450cam variant further improved its oxidation activity and coupling efficiency for the conversion ethane to ethanol, likely by decreasing the active site volume and facilitating the binding of small molecules [44].

CYP154E1 from *Thermobifida fusca* YX showed activity of converting (*E*)-stilbene to (*E*)-4,4′-dihydroxystilbene by double hydroxylation [45]. Substition of G239 at the corresponding position of CYP154E1 with alanine leads to a six-fold increase in activity. The CYP74 family enzymes include allene oxide synthase (AOS), divinyl ether synthase (DES) and hydroperoxide lyase (HPL), and conserved residues in the I helix central domain appeared to play a key role in their catalytic activities [46,47]. Both single mutants F295I and S297A of tomato AOS LeAOS3 dramatically alter its original enzymatic activity, and possessed new HPL activity [47]. Mutants of DES, V379F of tobacco CYP74D3 and

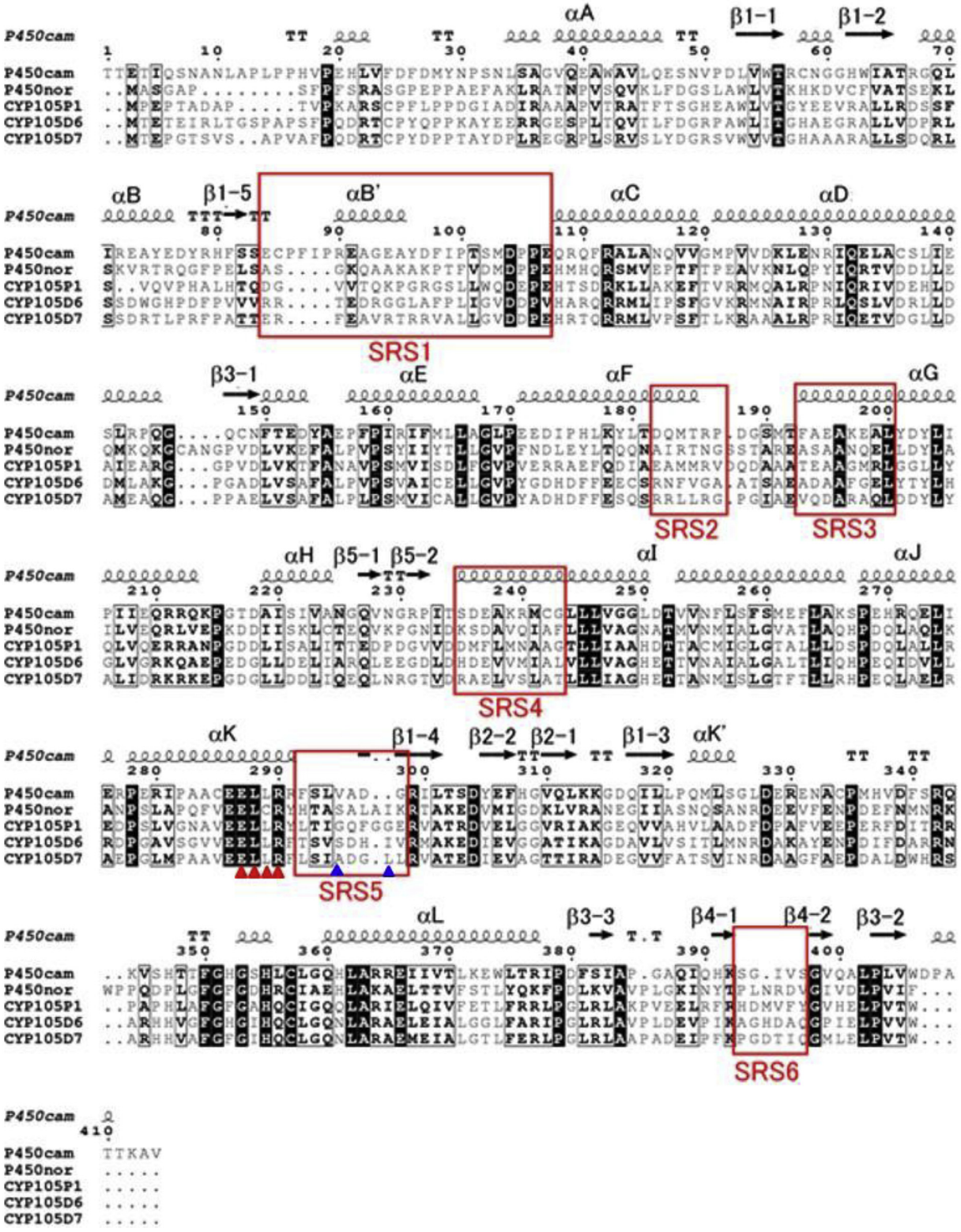

Fig. 3. Sequence alignment analysis of P450cam, P450nor, CYP105P1, CYP105D6 and CYP105D7. Red boxes indicate six substrate recognition sites (SRS). Red small triangles indicate EXXR motif. Blue small triangles indicate 5th and 9th position after EXXR motif.

E292G of flax (CYP74B16), converted their activity to AOS [46].

Another hotspot residues of P450s were identified by Seifert and Pleiss, who carried out a systematic analysis of SRS5 [48]. They showed that 98.4% of all SRS5 sequences contain a preferentially hydrophobic residue located at the fifth position after the EXXR motif and is critical for substrate specificity and regioselectivity for different P450s. Furthermore, 27% of all P450s are thought to contain a second selectivity-determining residue located at the ninth position after the EXXR motif. Despite their high sequence (43%) and structural similarity, CYP105P1 and CYP105D6 from *S. avermitilis* catalyze the hydroxylation of filipin at different positions, being C26 and C1′, respectively [49]. The residues at the fifth and ninth position after the

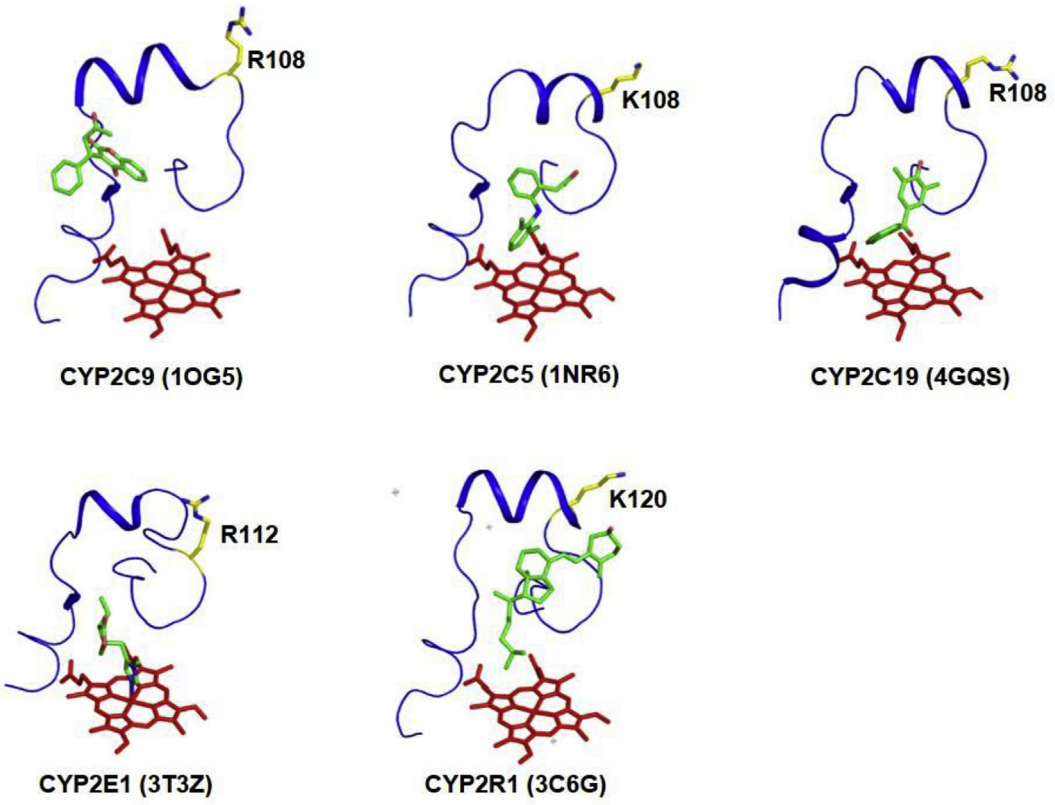

Fig. 4. Conserved arginine/lysine residues in the human drug metabolizing P450s. BC loop is shown as blue. Substrate or inhibitors are shown as green sticks. Conserved arginine/lysine residues are shown as yellow sticks.

EXXR motif in CYP105P1 are Gly284 and Gly288, are replaced by the bulky residues Ser290 and Ile293 in CYP105D6 [44] (Fig. 4). Structure analysis of these two P450s clearly showed that CYP105D6 lacks a pocket within the SRS5 region for the alkyl side chain of filipin. For canine CYP2B11, substitution of residue L363 at the fifth position after the EXXR motif, with valine (L363V), drastically shifts its regioselectivity, generating a specific progesterone 16α-hydroxylase [50]. In P450BM3, the substitution of alanine at the equivalent position with phenylalanine (A328F) influences its regioselectivity towards alkanes [42]. Futhermore, CYP153A from *Marinobacter aquaeolei* has been shown to be a fatty acid ω-hydroxylase with a broad substrate range, the G307A mutant is 2- to 20-fold more active than the wild-type [51].

4.2. Substrate access channel

In the P450 structure, the substrate access channel is generally formed by β strand 1–2, the B-C loop (includes SRS1), the F-G loop (includes SRS2 and SRS3), and the C-terminal region (includes SRS6). Altering residues along the substrate access channel is also a major component of P450 engineering. The residue Arg47 in P450BM3 lies on the B-C loop within the access channel and is one of the most frequently mentioned residues with regards to P450BM3 engineering. The crystal structures of P450BM3 bound to different substrates have showed that this residue is positioned closely to the entrance of the substrate access channel. Interestingly, the side chain of Arg47 actually faces away from the active site and has not been observed to form a hydrogen bond with the substrate in the *N*-palmitoylglycine-bound, *N*-palmitoylmethionine-bound or C7-L-Pro-L-Phe (a decoy molecule)-bound crystal structures [52–54]. However, Arg47 is undoubtedly important for substrate binding, as the R47S mutation has been shown to enhance the binding selectivity of this enzyme towards *N*-acyl homoserine lactone and its derivatives by nearly 250-fold [55,56]. As another example, although wild-type P450BM3 exhibits low activity in the oxidation of the polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene, fluoranthene, and pyrene, the double mutant R47L/Y51F increases PAH oxidation activity by up to 40-fold [57].

Besides for P450BM3, studies on many other P450s have also reported key arginine residues located along the substrate access channel. It has been shown that CYP105A1 from *Streptomyces griseolus* is capable of catalyzing a two-step hydroxylation of vitamin D3 to afford 1α, 25-dihydroxyvitamin D_3 [58]. The crystal structure of CYP105A1 indicated that three arginine residues, Arg73 (B-C loop), Arg84 (SRS1), and Arg193 (SRS3) are important for substrate binding and hydroxylation [59]. Site-directed mutagenesis experiments showed that Arg193 play a vitol role in catalysis, while the R73A and R84A display both increased 1α- and 25-hydroxylation activities. The co-crystal structure of R84A with 1α, 25-dihydroxyvitamin D_3 suggested that this mutation increases the adaptability of the B′ and F helices. Further rational engineering of this P450 by focusing on Arg73 and Arg84 generates a double mutant R73V/R84A that displayed approximately 400- and 100-fold higher k_{cat}/K_m values for the 25-hydroxylation of 1α-hydroxyvitamin D_3 and the 1α-hydroxylation of 25-hydroxyvitamin D_3, respectively [60]. In addition, this double mutant was also capable of converting vitamin D_2 to 1α,25-dihydroxyvitamin D_2 via 25-dihydroxyvitamin D_2 [61].

Interestingly, CYP105D7 from *S. avermitilis*, which catalyzes the hydroxylation of 1-deoxypentalenic acid, diclofenac, naringenin, and compactin [62–65], also has four arginine residues (Arg70, Arg81, Arg88, and Arg190) along the substrate access channel at a similar position based on crystal structure analysis (Fig. 2b). Among these, Arg70 directly recognizes the carboxylate of the distal diclofenac [65].

In early studies of the human P450s, crystal structure-function relationships demonstrated that these access channel arginine residues are also involved in substrate recognition, especially in the drug-metabolizing P450s. In the human CYP2C9, Arg108 is located within the substrate access channel and forms a salt bridge with the substrate

flurbiprofen (PDB code: 1R9O) [66]. In CYP2C5, Lys108 is located in an "outside" position similar to that in CYP2C9 in complex with S-warfarin (PDB code: 1OG5) [67]. A basic residue (Arg or Lys) is highly conserved at position 108 in various mammalian drug-metabolizing P450s, including CYP2C5, CYP2C9, CYP2C19, CYP2E1, and CYP2R1 (Fig. 4) [65]. Thus, these Arg/Lys residues located within the substrate access channel could be key targets for rational engineering using site-directed mutagenesis.

4.3. P450-redox partner interaction

In recent years, P450 engineering has not only focused on residues located within the active site and substrate access channel, but also on residues involved in P450-redox partner interactions. For instance, based on the docking and sequence alignment analysis, the residue Asp77 of CYP119 from *Salfolobus solfatarious* is predicted to be unfavourable for the interaction of CYP119 with its heterologous redox partner putidaredoxin (Pdx), due to charge repulsion [68]. The D77R mutation was expected to alleviate the charge repulsion, improving the electrostatic interaction between CYP119 and Pdx. Indeed, the D77R mutant increased both the Pdx binding (4-fold) and reaction rate (13-fold). P450moxA from *Nonomuraea recticatena* NBRC 14525 catalyzes the hydroxylation of substrates including fatty acids and steroids [69]. The T115A mutant of P450moxA, obtained after directed evolution for enhanced activity, is thought to affect the interaction of Arg107 or Arg108 with Pdx, accelerating the electron transfer. The corresponding residue involved in the interaction with ferredoxin P450cam is Arg109 or Arg112 [70]. Based on homology modeling and structural comparision, two residues (T119 and N363) of a more active P450sca-2 variant, located at the presumed Pdx interaction interface, were chosen for semi-rational engineering. Mutants T119N and N363Y exhibited improved whole cell biotransformation activity, which are 2.5- and 1.7-fold that of the starting template [35].

5. Engineering of P450-associated electron transfer systems

Redox partners are generally required to sequentially transfer two reducing equivalents from NAD(P)H to the P450. Classically, there are five major P450-associated redox partner systems (Fig. 5) [71]. Class I is a three-component system consisting of a FAD-containing reductase coupled with a small iron-sulfur redoxin, and is used by most bacterial and mitochondrial P450s. Representative of this class is putidaredoxin reductase (Pdr)/putidaredoxin (Pdx) electron transfer complex from *P. putida*. Class II is a two-component system comprising a single FAD- and FMN-containing cytochrome P450 reductase (CPR), whereas Class III, as exemplified by P450BM3, is a single-component system that is a natural fusion of P450 with CPR. Class IV systems are also single-component systems and consist of a natural fusion of P450 to an FMN- and Fe_2S_2-containing redox partner. One example from this category is the P450 RhF from the *Rhodococcus* sp. NCIMB 9784 [72,73]. Class V systems transfer electrons directly from NAD(P)H to P450 and are therefore also classed as single-component systems. For example, P450nor (cytochrome P450 nitric oxide reductase) was first isolated from *Fusarium oxysporum* MT811 and reduces nitrite to nitrous oxide [74,75].

Electron transfer is often the rate-limiting step in catalysis. In the attempt to enhance the electron transfer efficiency of P450s, a significant number of protein engineering studies have been conducted. As it is often difficult to find the native redox partner, a surrogate redox partner could be used instead. Studies have revealed that these alternative redox partners are sometimes even more efficient than their native ones. For instance, co-expression of Pdx and Pdr from *P. putida* in the host cell is a useful strategy for mediating catalytic activity and enhancing the conversion yield [76,77].

In recent years, class III and class IV systems have received increasing attention due to their self-sufficiency. The reductase domain of P450BM3 (and its homologs) has been used to construct functionally active chimeric proteins [78,79]. Kim and co-workers constructed a self-sufficient daidzein hydroxylase using the reductase domain of CYP102D1 from *S. avermitilis*. This construct catalyzes the hydroxylation of daidzein more efficiently than a three-component system [80,81]. Moreover, Li et al. generated the first self-sufficient PikC-RhFRED fusion system consisting of P450 PikC fused with the P450 reductase domain (RED) of P450RhF. This fusion protein exhibited enhanced catalytic activity that was approximately four-fold greater than that of the PikC-Fdr-Fdx three-component system towards both YC-17 and narbomycin [82]. Subsequently, this P450-RhFRED design is also applied to engineer other P450s such as P450cam and P450 MycG [83–87].

6. Conclusions

To date, structure-function relationship and protein engineering studies of P450 enzymes have been extensively conducted, especially for the mammalian P450s, P450BM3, and P450cam. New microbial P450 genes are frequently discovered and exploited in the biosynthesis of fine chemicals and in xenobiotic metabolism. These studies have contributed a significant amount of knowledge regarding the structure-

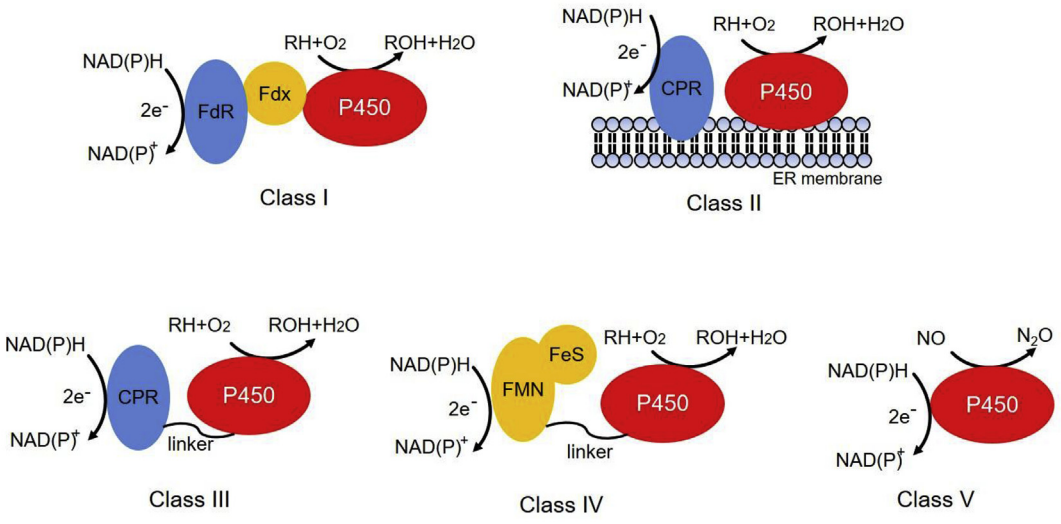

Fig. 5. Diversity of P450 redox systems.

function relationships of these P450s and P450s in general. Here, we reviewed recent examples of P450 engineering studies for various applications, and described several hotspot residues/areas for engineering in P450 structures. These residues affect enzyme properties, such as substrate selectivity, catalytic activity, and thermostability. Although rational and semi-rational engineering of the P450s has become increasingly attractive due to the rapidly expanding number of crystal structures, directed evolution is still a valuable approach, especially for identifying useful residues located outside hotspot areas, and for developing poorly-characterized proteins into useful catalysts.

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