Engineering cytochrome P450 enzyme systems for biomedical and biotechnological applications

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Cytochrome P450 enzymes (P450s) are broadly distributed among living organisms and play crucial roles in natural product biosynthesis, degradation of xenobiotics, steroid biosynthesis, and drug metabolism. P450s are considered as the most versatile biocatalysts in nature because of the vast variety of substrate structures and the types of reactions they catalyze. In particular, P450s can catalyze regio- and stereoselective oxidations of nonactivated C–H bonds in complex organic molecules under mild conditions, making P450s useful biocatalysts in the production of commodity pharmaceuticals, fine or bulk chemicals, bioremediation agents, flavors, and fragrances. Major efforts have been made in engineering improved P450 systems that overcome the inherent limitations of the native enzymes. In this review, we focus on recent progress of different strategies, including protein engineering, redox-partner engineering, substrate engineering, electron source engineering, and P450-mediated metabolic engineering, in efforts to more efficiently produce pharmaceuticals and other chemicals. We also discuss future opportunities for engineering and applications of the P450 systems.

Cytochrome P450 enzymes (P450s)[3] are a superfamily of heme-thiolate–containing proteins named for the characteristic state of the reduced, carbon monoxide (CO)-bound complex displaying a maximum UV-visible absorption band at 450 nm, due to the heme iron group being linked to the apoprotein via an axial conserved cysteine (1, 2).

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[3] The abbreviations used are: P450 or CYP, cytochrome P450; FdR, ferredoxin reductase; Fdx, ferredoxin; Adx, adrenodoxin; AdR, adrenodoxin reductase; CPR, cytochrome P450 reductase; Cpd 0, I, and II, compound 0, I, and II; *ep*PCR, error-prone polymerase chain reaction; 1α,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PDB, Protein Data Bank; SRS, substrate recognition site.

Since the first discovery of P450 as a pigment (the P denoting "pigment") in rat liver microsomes in 1958 (3), more than 370,000 P450 sequences have been released (UniProt), which are found in human, animals, plants, microbes, and even viruses, demonstrating their incredible and significant diversity in nature (4). P450s play important roles in biosynthetic pathways for natural products, degradation of xenobiotics, biosynthesis of steroid hormones, and drug metabolism (5, 6). P450s are considered to be the most versatile biocatalysts in nature (7) and are involved in more than 20 different types of chemical oxidation reactions, including hydroxylation, epoxidation, decarboxylation, *N*- and *O*-dealkylation, nitration, and C–C bond coupling or cleavage, to name a few (5, 8) (plus some reductions). Furthermore, the substrate diversity of P450s covers almost all classes of organic structures found in nature (*e.g.* terpenoids, polyketides, fatty acids, alkaloids, and polypeptides) (5, 9, 10). The ubiquitous distribution and the multiplicity of reactions and substrates demonstrate the plasticity of P450 enzyme systems, providing a limitless space for mining, engineering, and designing P450 systems for practical catalysis.

Among diverse functionalities, the most important is that P450s are capable of catalyzing the regio- and stereoselective oxidation of inert C–H bonds in complex molecular scaffolds under mild conditions, making them superior to many chemical catalysts and of great interest for pharmaceutical, chemical, and biotechnological applications. However, the narrow substrate scope of some P450s, low catalytic efficiency, low stability, dependence on redox partners, high cost of cofactors, and electron uncoupling have limited the industrial applications of P450s (11, 12). More recently, innovative P450 systems have been developed to fuel industrial projects with the use of a number of new engineering strategies (*e.g.* interactions of essential elements, including P450 itself, redox partner, substrate, and cofactor). These include the powerful directed evolution approach pioneered by the Nobel Laureate Frances H. Arnold, used to build unnatural but more robust P450 systems (13).

Several excellent reviews have covered the diversity, functions, novel chemistry, and applications of P450s (5, 10, 14–17). For more insight into intriguing P450-related mechanisms and to deeply understand the strategies related to the practical application of P450 catalysis, we will focus on recent advances

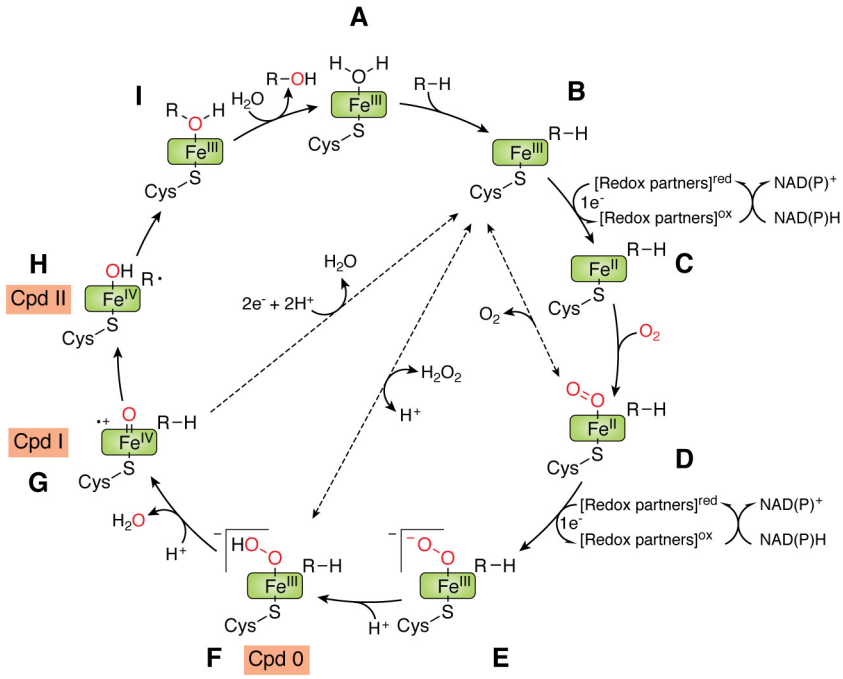


Figure 1. The catalytic cycle of P450s (*dashed arrows* indicate the peroxide shunt pathway and P450 uncoupling).

in P450 protein engineering, particularly engineering strategies for optimization of the interaction between P450s and redox partners. We will also consider substrate engineering, cofactor (NAD(P)H) regeneration, and several atypical strategies for engineering the electron transport system. Finally, a brief summary of P450-related metabolic engineering will be provided.

P450 catalytic system

In general, a P450 catalytic system includes four components: the substrate, a P450 enzyme for substrate binding and oxidative catalysis, the redox partner(s) that functions as an electron transfer shuttle, and the cofactor (NAD(P)H), which provides the reducing equivalents.

Most P450s share a common sophisticated catalytic cycle (Fig. 1) (2, 5, 18), using the typical hydroxylation reaction as a paradigm, as shown in Fig. 1. The ferric resting state (generally) of a P450 (**A**) first accepts a substrate (RH), which displaces an active-site water molecule but does not bond directly to the iron. The ferric iron (Fe^{III}) of the high-spin, substrate-bound complex (**B**) is then reduced to ferrous iron (Fe^{II}) (**C**) by one electron, transferred via a redox partner. Next, binding of dioxygen to Fe^{II} results in the [Fe^{II} O_2] complex (**D**). The complex **D** is reduced by the second electron to form complex **E**, which uses a proton from solvent to generate a ferric hydroperoxo species [Fe^{III}–OOH] (**F**), referred as to Compound 0 (Cpd 0). The O–O bond of Cpd 0 is cleaved upon the addition of the second proton and releases a molecule of water to generate the high-valent porphyrin π radical cation tetravalent iron [Fe^{IV}=O] (*i.e.* Compound I (Cpd I; **G**)). This highly reactive complex abstracts a hydrogen atom from the substrate, leading to the formation of the ferryl-hydroxo compound II (Cpd II; **H**). Subsequently, the hydroxylated product (R-OH) is formed by the reaction of the substrate radical with the hydroxyl group of Cpd II and released from the active site of complex **I**. Finally, a molecule of water returns to coordinate with Fe^{III}, restoring the resting state **A**. The same catalytic cycle is initiated repeatedly as substrate molecules bind to the heme-centered active site of P450.

It is worth noting that some P450s are capable of directly utilizing H_2O_2 as the sole electron and proton donor to form Cpd 0 and do catalysis via the so-called peroxide shunt pathway (Fig. 1, *dashed arrows*). However, this shunt pathway is greatly limited by the low efficiency and the low H_2O_2 tolerance of most P450s, except P450 peroxygenases (*e.g.* CYP152 subfamily) (19). The well-studied and established catalytic cycle provides a theoretical basis and roadmap to understand and manipulate this P450 peroxygenase subfamily by protein and substrate engineering.

Maintenance of the P450 catalytic cycle relies on continuous electron transport to the heme-iron by redox partners, which are complicated electron-transfer systems. Based on the types of redox partners and the P450-redox partner interaction relationships, P450 systems can be divided into five main classes (10, 11, 15) (Fig. 2). The Class I P450 system present in most bacterial and mitochondrial P450s has a two-component redox partner system, comprised of an FAD-containing ferredoxin reductase (FdR) and a small iron-sulfur-containing ferredoxin (Fdx) (20, 21). The Class II P450 system employed by eukaryotic organisms has a single-component redox partner, which is a membrane-bound protein containing both an FAD and an FMN domain, termed cytochrome P450 reductase (CPR). Class III P450 systems have a eukaryotic-like CPR naturally fused to the C terminus of the P450 domain through a flexible linker, represented by *Bacillus megaterium* $P450_{BM3}$ (CYP102A1) (22). Class IV P450 systems are exemplified by P450 RhF from *Rhodococcus* sp. NCIMB 9784, whose FMN/Fe_2S_2-containing reductase domain forms a natural fusion with the P450 domain

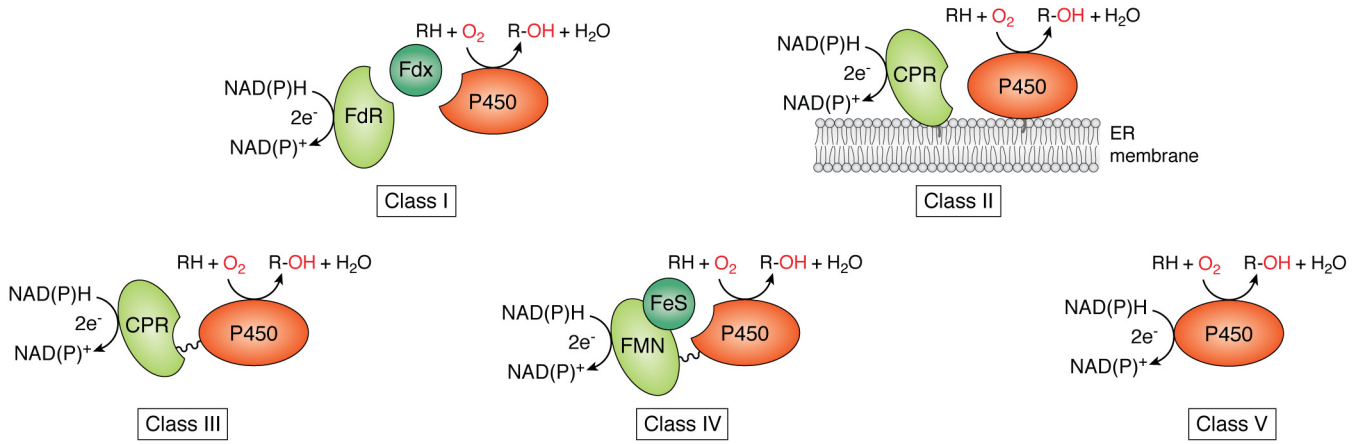


Figure 2. Classification of the P450 systems based on redox partner proteins.

(23). Interestingly, a few P450s can directly interact with their electron donors and are independent of additional redox partner proteins to accomplish the catalytic reactions; these Class V P450s include $P450_{Nor}$ (24) and P450 TxA (25). Class III–V P450s are independent of redox partner proteins and are often called self-sufficient P450s. Notably, these single-component P450 systems provide very desirable scaffolds for engineering P450 systems, due to their self-sufficiency and hence the significantly increased electron transport efficiency. It is worth noting that other classification systems also exist: Munro *et al.* (26) have categorized five other novel P450-fused redox partner systems in addition to the classical Class I and Class II types, and Bernhardt *et al.* (27) classified 10 types for P450s based on the topology of protein components involved in the electron transfer chains of P450 enzymes.

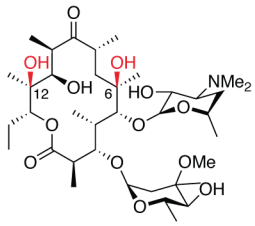
Successful applications of P450 catalytic systems

The incomparable diversity of P450s regarding substrates and reaction types provides nearly limitless application potential for production of chemicals and pharmaceuticals (28, 29), biosensor-based analysis (30), chemoenzymatic synthesis (31), and pollutant biodegradation (32). For instance, the *Saccharopolyspora erythraea* EryF and EryK P450s are involved in the production of the antibacterial agent erythromycin (Fig. 3, compound **1**) (33); *Streptomyces fradiae* TylI and TylHI P450s are involved in the biosynthesis of the antimycoplasma drug tylosin (Fig. 3, compound **2**) (34); and *Aspergillus terreus* LovA is responsible for biosynthesizing monacolin J acid (Fig. 3, compound **3**), the precursor of a series of cholesterol-lowering statin drugs (35, 36). The production of high value-added chemical intermediates from phenolic environmental pollutants has been achieved with P450 biodegradation systems (32, 37, 38), and soluble P450s have been used in bacterial cell libraries to mimic human P450 drug metabolic profiles (39, 40).

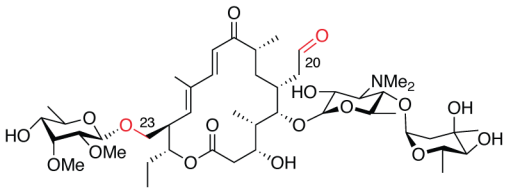
Genome mining and high-throughput screening of P450s have proven to be effective and successful strategies for seeking suitable and robust biocatalysts in industry. P450sca-2 (CYP105A3), screened from *Streptomyces carbophilus*, is able to catalyze the 6β-hydroxylation of compactin produced by *Penicillium citrinum*, generating the cholesterol-lowering drug pravastatin (Fig. 3, compound **4**) (41), considered to be one of the most successful instances of practical P450 catalysis in industry (11, 42). The bioconversion of 11-deoxycortisol into hydrocortisol by the $P450_{lun}$-containing fungus *Curvularia lunata* has been launched by Bayer on an industrial scale (11, 43) (Table 1 and Fig. 3, compound **5**). The industrially relevant P450 VD25 (CYP105A2) from *Amycolata autotrophica* (later renamed as *Pseudonocardia autotrophica*) is capable of transforming vitamin D_3 into its most bioactive form, 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$) (44) (Table 1 and Fig. 3, compound **6**). The P450 hydroxylase CYP-sb21 from the rare actinomycetes *Sebekia benihana* and CYP-pa1 from *P. autotrophica* are candidate biocatalysts for site-selective hydroxylation of the immunosuppressive drug cyclosporin A to two hair-stimulating agents with significantly decreased immunosuppressant activity, γ-hydroxy-*N*-methyl-L-Leu4-cyclosporin A and γ-hydroxy-*N*-methyl-L-Leu9-cyclosporin A, respectively (45–48) (Table 1 and Fig. 3, compounds **7**–**9**).

P450 protein engineering has been playing a vital role in developing biocatalysts for industrial applications, as exemplified by the heterologous production of artemisinic acid (Fig. 3, compound **10**), an important synthetic precursor for the potent antimalarial drug artemisinin (49). Traditional production of the anti-malarial drug artemisinin from the Chinese medicinal plant *Artemisia annua* L. is low-yield, unsustainable, and too expensive for millions of individuals suffering from malaria. A recombinant *Saccharomyces cerevisiae* strain with a heavily engineered mevalonate pathway, an amorphadiene synthase, and a key CYP71AV1 (from *A. annua*) produced 100 mg of artemisinic acid per liter (49) (Table 1 and Fig. 3, compound **10**). By applying different synthetic biology strategies, including the introduction of the cognate reductase CPR1 of CYP71AV1 and a cytochrome b_5 protein (CYB5, an electron transfer component for CYP71AV1 from *A. annua*), the titer of artemisinic acid was dramatically improved to 25 g/liter on an industrial scale, which successfully reduced the price and provided a stable artemisinin supply for the market (28).

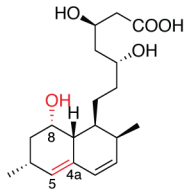
The P450 systems have also been engineered for the fragrance production. For instance, the oxidation of sesquiterpene (+)-valencene to high value-added flavor (+)-nootkatone with P450 enzymes was first accomplished in the Wong group by rationally designed mutants of $P450_{BM3}$ and $P450_{cam}$ (50) (Fig. 3, compound **11**).



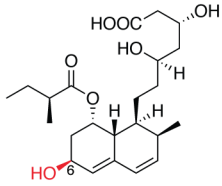
Erythromycin (1)



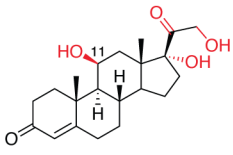
Tylosin (2)



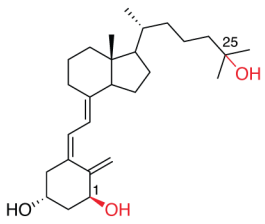
Monacolin J acid (3)



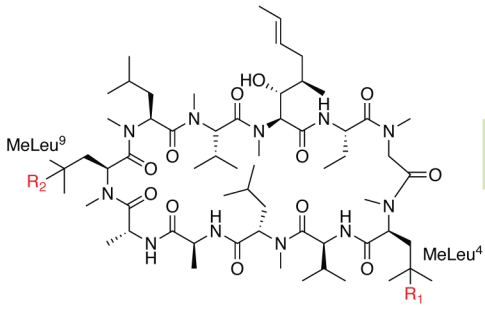
Pravastatin (4)



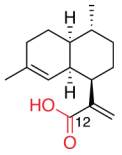
Hydrocortisol (5)



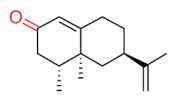
1α, 25-Dihydroxyvitamin D3 (6)



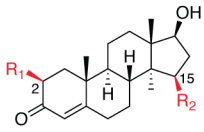
Cyclosporin A	(R_1= H, R_2= H)	(7)
CSA-4-OH	(R_1= OH, R_2= H)	(8)
CSA-9-OH	(R_1= H, R_2= OH)	(9)



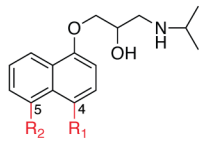
Artemisinic acid (10)



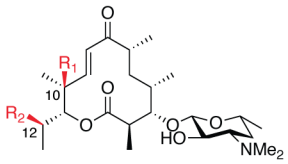
(+)-Nootkatone (11)



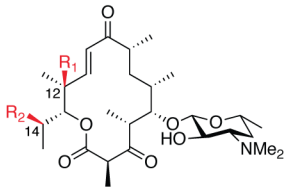
Testosterone	(R_1 =H, R_2= H)	(12)
2β-Hydroxytestosterone	(R_1 =OH, R_2= H)	(13)
15β-Hydroxytestosterone	(R_1 =H, R_2= OH)	(14)



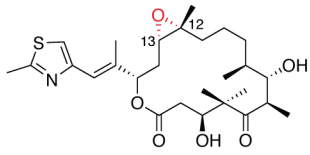
Propranolol	(R_1 =H, R_2= H)	(15)
4′-Hydroxypropranolol	(R_1 =OH, R_2= H)	(16)
5′-Hydroxypropranolol	(R_1 =H, R_2= OH)	(17)



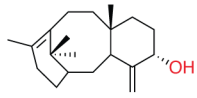
YC-17	(R_1 =H, R_2= H)	(18)
Methymycin	(R_1 =OH, R_2= H)	(19)
Neomethmycin	(R_1 =H, R_2= OH)	(20)



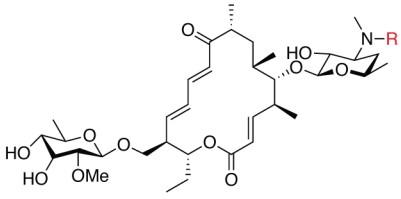
Narbomycin	(R_1 =H, R_2= H)	(21)
Pikromycin	(R_1 =OH, R_2= H)	(22)
Neopikromycin	(R_1 =H, R_2= OH)	(23)



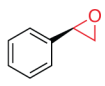
Epothilone B (24)



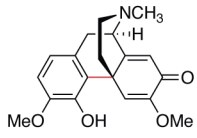
Taxadien-5α-ol (25)



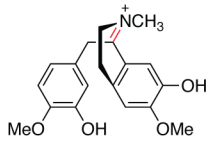
Mycinamicin IV	(R= Me)	(26)
Demethylmycinamicin IV	(R= H)	(27)



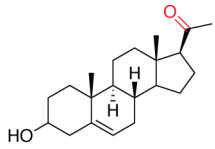
(*R*)-(+)-Styrene oxide (28)



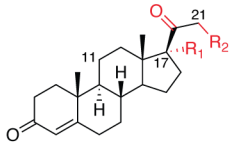
Salutaridine (29)



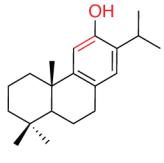
1,2-Dehydroreticuline (30)



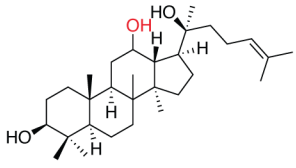
Pregnenolone (31)



Progesterone	(R_1 =H, R_2= H)	(32)
17-Hydroxyprogesterone	(R_1 =OH, R_2= H)	(33)
11-Deoxycortisol	(R_1 =OH, R_2= OH)	(34)



Ferruginol (35)



Protopanaxadiol (36)

Figure 3. Structures involved in practical catalysis of diverse P450 systems. *Red-colored groups* are introduced by P450s.

Table 1
Selected P450s involved in production of pharmaceuticals and chemical intermediates

P450	PDB code	Origin	WT/mutant	Function	Substrate	Reference
EryF (CYP107A1)	1OXA	*Saccharopolyspora erythraea*	WT	6-Hydroxylation	6-Deoxyerythronolide B	33
EryK (CYP113A1)	2JJN	*S. erythraea*	WT	12-Hydroxylation	Erythromycin D	33
TylI		*Streptomyces fradiae*	WT	C20 hydroxylation/ dehydrogenation	5-Mycaminosyl tylacone	34
TylHI	6B11	*S. fradiae*	WT	C23 Hydroxylation	23-Deoxy-5-omycaminosyl-tylonolide	34
LovA		*Aspergillus terreus*	WT	4a/5-Dehydrogenation, 8-hydroxylation	Dihydromonacolin L	35
CYP71AV1		*Artemisia annua*	WT	12-Carboxylation	Amorphadiene	49
P450sca-2 (CYP105A3)		*Streptomyces carbophilus*	WT	6β-Hydroxylation	Compactin	41
		Semi-rational design	R8–5C/T85F/ T119S/V194N/ N363Y	6β-Hydroxylation	Compactin	69
P450lun		*Curvularia lunata*	WT	11-Hydroxylation	11-Deoxycortisol	11, 43
P450VD25 (CYP105A2)		*Pseudonocardia autotrophica*	WT	25-Hydroxylation	Vitamin D_3	44
CYP-sb21 (CYP107Z14)		*Sebekia benihana*	WT	Hydroxylation at the 4th *N*-methyl leucine	Cyclosporin A	45
CYP-pa1		*P. autotrophica*	WT	Hydroxylation at the 9th *N*-methyl leucine	Cyclosporin A	47
$P450_{BM3}$ (CYP102A1)	1JPZ	*Bacillus megaterium*	WT	Hydroxylation	Fatty acids	22
	2X7Y	Site-directed mutagenesis	F87A	2β-/15β-Hydroxylation (1:1)	Testosterone	54
		Directed evolution	R47Y/T49F/ V78L/A82M/ F87A	15β-Hydroxylation (96%)	Testosterone	54
		Directed evolution	A330W/F87A	2β-Hydroxylation (97%)	Testosterone	54
Heme domain of $P450_{BM3}$		Directed evolution	9C1 A74V	4′-Hydroxypropranolol and 5′-hydroxypropranolol	Propranolol	39
P450 Vdh (CYP107BR1)	3A4G	*P. autotrophica*	WT	1α-/25-Hydroxylation	Vitamin D_3	55
		Directed evolution	T70R/V156L/ E216M/E384R	1α-/25-Hydroxylation	Vitamin D_3	56
CYP105A1	2ZBX 2ZBZ	*Streptomyces griseolus*	WT	1α-/25-Hydroxylation	Vitamin D_3	63
		Rational design	R73A/R84A	1α-/25-Hydroxylation	Vitamin D_3	64
CYP105AS1	4OQR	*Amycolatopsis orientalis*	WT	6-*epi*-Hydroxylation (97%)	Compactin	29
$P450_{Prava}$		Directed evolution	I95T/Q127R/ A180V/L263I/ A265N	6β-Hydroxylation (100%)	Compactin	29
PikC	2C6H 2C7X	*Streptomyces venezuelae* ATCC 15439		C10/C12 Hydroxylation, C12/C14 hydroxylation	YC-17/Narbomycin	65
		Rational design	D50N	C10/C12 Hydroxylation, C12/C14 hydroxylation	YC-17/Narbomycin	65
	2C6H 2C7X	Redox parent engineering	WT	C10/C12 Hydroxylation, C12/C14 hydroxylation	YC-17/Narbomycin	87
		Substrate engineering	D50N	Regio-selective hydroxylation	Unnatural substrates	105
MycG	5UHU	*Micromonospora griseorubida*	WT	C12/C13 Epoxidation, C14 hydroxylation	Mycinamicin IV	102
		Redox parent engineering		*N*-Demethylation	Mycinamicin IV	101
CYP725A4		*Taxus cuspidata*	WT	5α-Hydroxylation	Taxadiene	100
		Redox parent engineering	*N*-terminal hydrophilic modifications	5α-Hydroxylation	Taxadiene	100
CYP2C9	1OG2	Human	Self-sufficient	*N*-Demethylation	Erythromycin	89
CY2C19		Human	Self-sufficient	4-Hydroxylation	Diclofenac	89
CYP3A4	6DA2	Human	Self-sufficient	5-Hydroxylation	Omeprazole	89
1,2-Dehydroreticuline synthase (CYP82Y2-like)		*Papaver bracteatum*	PbDRS-DRR	Dehydrogenation	(*S*)-Reticuline	159
SalSyn		*Papaver somniferum*	$yPbSalSyn^{92–504}$	C–C Coupling	(*R*)-Reticuline	159
CYP76AH1		*Salvia miltiorrhiza*	WT	Hydroxylation and dehydrogenation	Miltiradiene	161
P450 protopanaxadiol synthase		*Panax ginseng*	WT	Hydroxylation	Dammarenediol-II	161
CYP11A1	3N9Y	Human	WT	C–C Cleavage	Egrosta-5-eneol/ergosta-5,22-dieneol	160
CYP17A1	3RUK	Human	WT	17α-Hydroxylation	Progesterone	160
CYP21A1		Human	WT	21-Hydroxylation	17-Hydroxyprogesterone	160
CYP11B1		Human	WT	11β-Hydroxylation	11-Deoxycortisol	160
P450 box A		*Streptomyces* sp. TM-7	WT	6β-Hydroxylation	Compactin	163
CYP106A2	4YT3	*B. megaterium* ATCC 13368	WT	15β-Hydroxylation	Progesterone, testosterone	167

Strategies and progress of engineering P450 enzyme systems

Although P450s have demonstrated amazing catalytic diversities and great prospects for application, the aforementioned limitations in industrial applications of P450s are also significant. To overcome these limitations, versatile engineering strategies have been proposed and developed to satisfy different application requirements, including protein engineering of P450s and redox partners, substrate engineering, cofactor regeneration, and P450-related metabolic engineering (Fig. 4).

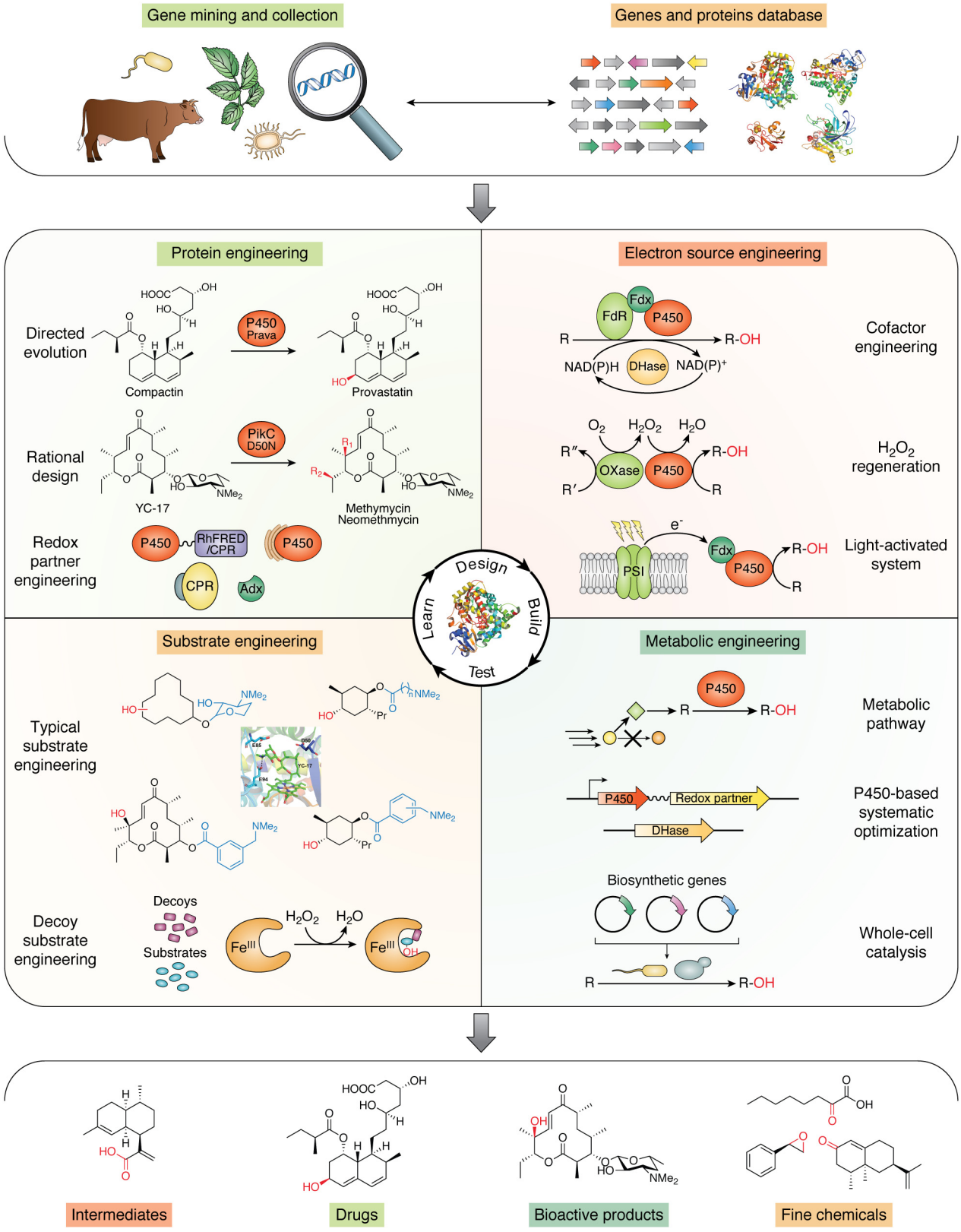


Figure 4. Engineering strategies for P450 systems discussed in the current review. *DHase* and *OXase*, dehydrogenase and oxidase, respectively.

Protein engineering

Protein engineering involves modification of the residues based on the folding principles and molecular structure of proteins, with the goal of obtaining the desired mutated proteins with enhanced properties to compensate for the poor stability, low selectivity, slow catalytic rates, and limited application space of the native proteins (51). Directed evolution and rational and semi-rational design are routinely used methods in

P450 engineering and play very important roles in the development of pharmaceutical catalysts (15, 52).

Directed evolution

Directed evolution has been widely applied to engineer P450s, the structures of which are often unknown, for desired properties under artificial selective pressure, including random mutagenesis and screening (13). To obtain the desired mutant proteins, a protein library with a large number of mutants covering sufficient molecular diversity is usually generated by error-prone PCR (*ep*PCR), combinatorial saturation mutagenesis, or DNA-shuffling methods (52). In addition, directed evolution is also an effective tool for understanding relationships between key amino acid residues surrounding catalytic pockets and catalytic abilities toward different unnatural substrates for well-characterized P450s (*e.g.* $P450_{BM3}$ and $P450_{cam}$) (53).

Reetz and co-workers (54) found that the simple $P450_{BM3}$ mutant F87A, with reduced steric hindrance for substrate, was able to catalyze the nonselective 2β- and 15β-hydroxylation of testosterone to generate a 1:1 mixture of products. To alter the regioselectivity, iterative saturation mutagenesis of 20 selected residues lining the substrate-binding pocket was done, leading to two effective mutants (A330W/F87A and R47Y/T49F/V78L/A82M/F87A) that achieved specific regio-selective production of the 15β (96%) or 2β products (97%), respectively (54) (Table 1 and Fig. 3, compounds **12–14**). P450 Vdh (CYP107BR1, Protein Data Bank (PDB) entry 3A4G) from *P. autotrophica* was also reported to produce 1α,25$(OH)_2D_3$ from vitamin D_3 (55) (Table 1 and Fig. 3, compound **6**). A Vdh-K1 mutant (T70R/V156L/E216M/E384R) was generated with 6-fold higher specific activity than WT P450 through high-throughput screening of a site-saturated mutagenesis library (56) (Table 1 and Fig. 3, compound **6**).

Directed evolution of P450s has also been applied in the generation of drug metabolites as an effective strategy for further pharmaceutical studies. Here, the strategy is to use the bacterial P450s to generate larger amounts of drug metabolites to facilitate structural analysis of the small quantities of drug metabolites, which is a regulatory requirement for further drug development. Arnold and associates (39) constructed a mutant library of $P450_{BM3}$ using *ep*PCR and combinatorial saturation mutagenesis of seven active-site residues surrounding the heme domain. The mutants selectively oxidized the antiarrhythmic drug propranolol to its active human metabolites, including 4′-hydroxypropranolol and 5′-hydroxypropranolol, via the "H_2O_2" shunt pathway (39) (Table 1 and Fig. 3, compounds **15–17**). Subsequently, a small panel of $P450_{BM3}$ variants was further subjected to site-directed mutagenesis of the active-site residues, leading to a set of metabolites of the antihypertensive drug verapamil and the antiallergic astemizole that are the same as those metabolized by mammalian P450s (57).

The Guengerich laboratory screened a series of CYP1A2 mutants generated by random mutagenesis at six substrate recognition sites (SRSs), and the obtained variants had 2–4-fold increases in k_{cat}/K_m (specificity constant) toward the analgesic and antipyretic drug phenacetin compared with the parent enzyme (58). Similarly, human CYP2A6 mutants were screened based on the production of indole oxidation products, which could find application in production of dyestuffs or as protein kinase inhibitors (59).

The protein stability of P450s, another important factor for practical applications to enhance the total turnover numbers, can also be improved via directed evolution, as exemplified by solvent tolerance optimization of the $P450_{BM3}$ variant F87A/T235A/R471A/E494K/S1024E, which was obtained from libraries constructed by saturation mutagenesis and random mutagenesis (60) (the substrates are generally hydrophobic and dissolved in organic solvents). The conversion of *p*-nitrophenoxydodecanoic acid to *p*-nitrophenol was enhanced 5.5-fold in the presence of 25% (v/v) DMSO and 10-fold in 2% THF (v/v) compared with the parental $P450_{BM3}$ F87A mutant (60).

Rational and semi-rational design

Major disadvantages of directed evolution include the dependence on a high-throughput screening method, which is not always available, the requirement of automated instruments, and high cost. Rational or semi-rational design, based on the well-characterized protein tertiary structure and the mechanistic understanding of structure-activity relationships, is regarded as an effective alternate strategy. Generally (but not always), "hot spot" residues for rational and semi-rational design are usually located within the SRS, the substrate access channel, and the P450-redox partner interaction sites (15, 61).

CYP105AS1 (from *Amycolatopsis orientalis*) catalyzes the conversion of compactin to the cholesterol-lowering drug pravastatin plus its ineffective epimer 6-*epi*-pravastatin, in a ratio of 3:97. Based on the crystal structure of CYP105AS1 (PDB entry 4OQR), a single round of *ep*PCR mutagenesis of selected residues led to a mutant with a ratio of pravastatin/6-*epi*-pravastatin of 48:52. A further two rounds of site-saturated mutagenesis produced a mutant $P450_{Prava}$ (I95T/Q127R/A180V/L263I/A265N), in which the stereoselectivity was completely inverted into a P450 pravastatin synthase with a 21-fold lower K_m value for compactin (29) (Table 1).

CYP105A1 (*S. griseolus*) hydroxylates vitamin D_3 to form 1α,25$(OH)_2$ vitamin D_3. Site-directed mutagenesis of CYP105A1 based on its three-dimensional structure (PDB entries 2ZBX and 2ZBZ) was performed. Three arginine residues (Arg-73, Arg-84, and Arg-193) located along the substrate access channel of CYP105A1 were mutated to nonpolar alanines on the basis of their important roles in substrate binding and catalysis, delineated from a crystal structure of CYP105A1 with its enzymatic product 1α,25-OH vitamin D_3 (62). As hypothesized, the double mutant R73A/R84A exhibited ~400- and 100-fold increased activity for 25-hydroxylation and 1α-hydroxylation of vitamin D_3 compared with the WT enzyme (63, 64) (Table 1).

The co-crystal structures of multifunctional P450 PikC (PDB entries 2C6H and 2C7X) bound to its native substrates (narbomycin and YC-17) suggested that Asp-50, Glu-85, and Glu-94 (located in the catalytic pocket) might be critical for substrate binding and catalytic activity (Table 1 and Fig. 3, compounds **18–23**). Accordingly, a series of mutants was constructed, and PikC D50N displayed significantly higher hydroxylation activities toward both narbomycin and YC-17 than did the WT enzyme (65) (Table 1 and Fig. 3, compounds **18–23**).

The availability of a tertiary protein structure is considered to be a major limitation in rational and semi-rational design, in that experimental structural information is not often available for the >370,000 P450 sequences. Homology modeling has often been used to bridge the "structure knowledge gap," based on the general observation that proteins with homologous sequences share similar structures (66, 67). A well-known example of P450 semi-rational design for industrial application is the development of a highly efficient mutant of P450sca-2 involved in the production of pravastatin (68). Based on homology modeling analysis of an active mutant (R8-5C) of P450sca-2 generated from directed evolution, five sites located in the substrate binding pocket (Arg-77/Thr-85), the substrate access channel (Val-194), and the redox partner interface (Asn-363/Thr-119) were selected for systematic site-directed saturation mutagenesis and three rounds of mutagenesis (69). As a result, a more active mutant (R8-5C/T85F/T119S/V194N/N363Y) was obtained, with 7-fold higher whole-cell biotransformation activity and a 10-fold higher k_{cat} value than that of R8-5C (69) (Table 1). Based on the X-ray crystal structure of the high-activity progesterone hydroxylase rabbit CYP2C5 (PDB entry 1DT6), the low progesterone hydroxylase activity of CYP2B1 was re-engineered by changing active-site residues in the three-dimensional structural model of CYP2B1 to the corresponding residues of CYP2C5 (70, 71). Finally, a CYP2B1 mutant (I114A/F206V/F297G/V363L/V130I/S294D/I477F) exhibited a 3-fold higher k_{cat} value than that of CYP2C5 for progesterone 21-hydroxylation, with 80% regioselectivity (71).

Redox partner engineering

Most P450s require redox partner proteins to sequentially transfer two electrons from NAD(P)H to the heme-iron reactive center to activate O_2 for substrate oxygenation (6), which can often be the rate-limiting step of the P450 catalytic cycle (Fig. 1). However, the reconstitution of a P450 catalytic system *in vitro* or in a recombinant host is often hampered by the lack of information about its cognate redox partners or inaccessibility of optimal surrogate redox partners. Therefore, protein-protein interactions between P450 and surrogate redox partners have been optimized to enhance the electron transfer efficiency of P450 systems, which we will term "redox partner engineering."

A comprehensive screening of redox partners to identify the best electron transport pathway for supporting the CYP105D5 activity was done in the Guengerich laboratory (72). Briefly, all four FdRs and six Fdxs encoded by the genome of *Streptomyces coelicolor* A3(2) were heterogeneously expressed and purified. A total of 24 native redox partner combinations were assembled and screened with a specific *S. coelicolor* P450, CYP105D5, which had been shown to hydroxylate free fatty acids (72). The results showed that the pair Fdx4/FdR1 functioned as the preferred redox partner system for this bacterial P450 enzyme *in vitro* (72).

Adrenodoxin and adrenodoxin reductase (Adx/AdR) were characterized as optimal redox partners in supporting the *in vitro* hydroxylation of lauric acid by CYP109D5 from *Sorangium cellulosum So ce56,* the catalytic efficiency of which was 3–4-fold higher than that of CYP109D5 supported by endogenous redox partners (Fdx2/FdR_B and Fdx8/FdR_B) (73). Interestingly, the combination of Fdx8/FdR_B was reported to be a much better pair of redox partners of P450 EpoK in the bioconversion of epothilone D to epothilone B compared with the spinach Fdx/FdR redox pair (74) (Table 1 and Fig. 3, compound **24**). Thus, a certain P450 enzyme may have a differentially preferred combination of Fdx and FdR among multiple combinations, although alternative redox partners could be functionally complementary (75). It is also worth noting that surrogate redox partners may be superior to the cognate ones; thus, it can be helpful to apply a redox partner interchange approach to determine optimal electron transfer pathways, particularly in bacterial systems, to fully exploit P450 applications.

To determine whether there are any principles for guiding the screening of optimal redox partners for a given Class I bacterial P450, Zhang *et al.* (76) constituted a reaction matrix network based on 16 Fdxs, eight FdRs, and six P450s toward seven substrates. By analyzing the reactivity profiles of 896 reactions, plastidic-type FdR and Fe_2S_2 Fdx were found to be the favored types of redox partners by Class I P450 systems. Based on the empirically derived rules, the optimal cognate Fdx of PikC from *Streptomyces venezuelae* ATCC 15439 was predicted and confirmed *in vitro* to be *Sve*Fdx1948 (76). This work has provided information about the P450-preferred redox partners, and we envision that the findings will benefit future practical applications of P450 enzymes.

Notably, the protein pair *Sel*Fdx1499 (Fe_2S_2)/*Sel*FdR0978 (plastidic-type FdR) from the cyanobacterial strain *Synechococcus elongatus* PCC 7942 has been shown to be an optimal combination for supporting *in vitro* reactions of prokaryotic P450s, including MycG, PikC, P450sca-2 and others (76). Besides the above-mentioned P450 reaction matrix network, the protein pair *Sel*Fdx1499/*Sel*FdR0978 has also been shown to be optimal for the site-selective hydroxylation of CsA by CYP-sb21 and CYP-pa1 (45, 47), the uncommon ester-to-ether transformation catalyzed by Rif16 in rifamycin biosynthesis (77), the tandem ether installation and hydroxylation by AmbV involved in neoabyssomicin/abyssomicin biosynthesis (78), and the biosynthesis of phenylserine (β-OH-Phe) unit in atratumycin by Atr27 (79).

In addition to the screening and prediction of optimal redox partners, optimization of interaction modes between P450s and redox partners through redox partner engineering provides another effective strategy for P450 activity improvement. The residues located at the P450-Fdx (Fdx directly interacts with P450) interaction interface play important roles in affecting the catalytic activity of a P450. Screening of Adx derivatives modified at N-terminal or C-terminal polypeptide sequences led to the finding that Adx(4–108) truncated at N-terminal amino acids 1–3 and C-terminal amino acids 109–128 supported the 11β-hydroxylation of 11-deoxycortisol to cortisol by CYP11B1 with a higher electron transfer rate, and the specificity constant (k_{cat}/K_m) was increased 21-fold relative to that of WT Adx (80–82). The availability of co-crystal structures of P450s and their redox partners will facilitate engineering of the P450/Fdx interface, as exemplified by the artificial fusion CYP11A1-Adx (83) and the cross-linked CYP101A1-Pdx complex (84). Based on the interaction analysis, the amino acids of ferredoxin PuxB

interacting with P450 were swapped (site-directed mutagenesis) to mimic the biogenic ferredoxin Pux of CYP199A2 from *Rhodopseudomonas palustris* CGA009. A PuxB variant with seven mutations was generated, and the rate of demethylation of 4-methoxybenzoic acid by CYP199A2 was increased 12-fold compared with WT PuxB (85). However, the semi-rational engineering approach remains challenging due to the lack of comprehensive understanding of the dynamic mechanisms for protein-protein recognition and intermolecular electron transfer. Thus, further work is needed for understanding P450-Fdx complex structures at the molecular level to address this challenge.

Inspired by the paradigm of self-sufficient P450 enzymes (Class III and IV) that contain both P450 and redox partner domains in one polypeptide chain, the construction of "unnatural" self-sufficient enzymes by making variant versions of P450-redox partner fusion proteins has been pursued (86). A self-sufficient PikC-RhFRED fusion was generated, and its catalytic activity toward YC-17 was increased ~4-fold compared with that of a three-component system (PikC + spinach Fdx/FdR) *in vitro*, likely due to enhanced intramolecular electron transfer efficiency compared with the intermolecular reaction (87). Another striking example is the construction of self-sufficient $P450_{Prava}$-RhFRED. The introduction of $P450_{Prava}$-RhFRED into the compactin-producing *P. chrysogenum* delivered more than 6 g of pravastatin per liter in a one-step fermentation (29). High-throughput generation of self-sufficient P450 libraries by fusing P450 heme domains to RhFRED via a ligation-independent cloning vector, "LICRED," was developed (88). Self-sufficient mammalian P450-reductase fusion enzymes have been prepared, mimicking the precedent of the efficient $P450_{BM3}$, including CYP2C9, CY2C19, and CYP3A4 for drug metabolism studies (89). However, establishing the optimal design and length of the linker has not been trivial (90). Among seven fused $P450_{cam}$-RhFRED (L1–L7) enzymes with varying linker regions, L4 was the most optimal, with 100% conversion of 3 mM (+)-camphor under the conditions tested (90).

Other chimeras have been made with diverse P450s from mammals, plants, and bacteria, including $P450_{cam}$ (91), P450 TxtE (92), CYP257A1 (93), $OleT_{JE}$ (94, 95), P450 isoflavone synthase (96), and CYP2E1 (97). In principle these fusion proteins can improve catalytic activity, coupling efficiency, and other electron transfer properties by simplifying the overall P450 redox system and process suitability (75, 98). More challenging are engineering and expression of eukaryotic P450s in prokaryotic systems. First, compartmentalization is one consideration for Class II P450 systems, in that interaction between P450 and CPR typically occurs in the endoplasmic reticulum. Second, the molar ratio of P450 and its redox partner in a chimeric system is fixed at 1:1, instead of 15:1 with membrane-bounded P450s and CPR in the liver (99). The construction of a chimeric protein will hamper the flexibility of modulating P450/CPR ratios. These shortcomings were circumvented during the heterologous production of oxygenated taxanes with engineered *Taxus cuspidata* P450 CYP725A4 and its native CPR in *Escherichia coli*. By optimizing the relative expression level of the CPR, physically unlinked to CYP725A4, the optimal ratio of P450 to CPR was shown to be ~12 (100) (Table 1 and Fig. 3, compound **25**). This information may be useful in further studies on the efficient redox partner engineering system of eukaryotic P450s in *E. coli in vivo*.

A change of redox partners may not only influence catalytic efficiency and product distribution (12) but also affect the type and selectivity of a P450 reaction (101). For example, the multifunctional P450 MycG interacted with a free form of the reductase domain RhFRED or the engineered *Rhodococcus*-spinach hybrid reductase RhFRED-Fdx, supporting unnatural reactions leading to the production of seven novel demethylated mycinamicin products (in addition to the physiological hydroxylation/epoxidation reactions), which were not observed with either the chimeric fusion MycG-RhFRED or the spinach Fdx/FdR- supported reaction (101, 102) (Table 1 and Fig. 3, compounds **26** and **27**). Of particular importance, these findings highlight the potential role of redox partners in modulating the function of P450 enzymes and also suggest that P450 enzymes could be made even more versatile through interaction with a variety of redox partners to gain alternative functionalities.

Substrate engineering

Limited substrate scope is a general problem with biocatalysts. The eukaryotic Class II P450s, with high substrate promiscuity, are generally not particularly suitable for synthetic and biotechnological applications due to their membrane-bound nature. To expand the substrate repertoire of prokaryotic soluble P450s, the strategy of "substrate engineering" has been practiced more often in recent years (14, 103, 104).

Typical substrate engineering is aimed toward modification of a nonnative substrate by covalently linking an anchoring/directing group to enable the productive binding of the engineered substrate. Some pioneering work on P450 substrate engineering involved PikC, based on extensive structural studies (65). The hydrogen bond network and strong ionic interactions between the desosamine moiety (a common 2-deoxy sugar in the two native PikC substrates YC-17 and narbomycin) and several residues in the P450 BC loop and FG helices were identified as key determinants in substrate recognition (80). Thus, a series of substrates was chemically engineered to contain the desosamine anchoring group, and selective C–H bond hydroxylation of a series of unnatural carbolide substrates was achieved and mechanistically interpreted (105). The regio-selectivity of PikC hydroxylation was further probed by testing the chemically modified YC-17 analogs with varied synthetic anchoring groups. As a result, the regioselectivity of PikC could be changed significantly (106). Furthermore, $PikC_{D50N}$RhFRED (a superior self-sufficient PikC mutant) was utilized to catalyze oxidation of nonactivated methylene C–H bonds of small nonnative substrates with further simplified synthetic anchors containing a dimethyl amino group (*e.g.* menthol and several bicyclic and bridged bicyclic compounds) (107). A substrate engineering approach was also successfully applied to the major drug-metabolizing human P450 CYP3A4 toward theobromine analogues (108), CYP2E1 toward nicotinate esters (109), and $P450_{BM3}$ on mono- and polysaccharides, with predictable control of the regio- and stereoselectivity (110).

Recently, based on the understanding of the structural basis for substrate recognition in 4-cresol biodegradation by *Corynebacterium glutamicum* P450 CreJ, the biocatalytically installed phosphate group (attached by a ATP-dependent two-subunit phosphatase CreHI) was harnessed as an anchoring/directing group to deliver a group of *p*- and *m*-alkyphenols into the active site of P450 CreJ, achieving the highly challenging selective oxidation of the aliphatic C–H bonds of the tested alkylphenols in a controlled manner (32). This biosynthetic approach, without any chemical modification steps, may find useful applications in the pharmaceutical, biomanufacturing, and environmental remediation industries.

Distinct from typical substrate engineering using chemically or biologically modified substrates, Watanabe and his associates have systematically developed an atypical substrate engineering strategy, "decoy" substrate engineering, in which an inactive "dummy" substrate (decoy molecule) is used to trigger the P450-catalyzed reaction on the real nonnative substrate (103, 104, 111). Notably, there is no covalent linkage between the decoy and real substrates. A decoy molecule has a similar chemical structure to native substrate, so that it can be recognized and accommodated by the P450 enzyme, and its binding can reshape the substrate-binding pocket for the binding of a nonnative substrate, which can then be oxidized more efficiently. The first generation of decoy substrates for $P450_{BS\beta}$ (CYP152A1) were short-chain fatty acids (112), followed by different types of perfluorinated fatty acids bearing shorter alkyl chains (113), *N*-perfluoroacyl amino acids (114), and nonfluorinated *N*-acyl amino acids (115) for $P450_{BM3}$. Four generations of decoy molecules have been developed, not only for expanding the substrate capabilities of P450s but also for exploring the stereoselectivity and enantioselectivity toward various substrates (*e.g.* styrene and ethylbenzene), leading to diverse chemical scaffolds that can be applied in the pharmaceutical industry (116).

Recently, a class of dual-functional small molecules containing an anchoring group for binding to the P450 and a basic group for H_2O_2 activation was elegantly designed and successfully transformed the $P450_{BM3}$ monooxygenase into a peroxygenase. *N*-(ω-Imidazolyl)-hexanoyl-L-phenylalanine (Im-C6-Phe) was the optimal co-catalyst supporting the $P450_{BM3}$-H_2O_2 system (117). The rate of epoxidation of (*R*)-(+)-styrene and the enantiomeric specificity (*ee* value) of the product were dramatically increased (to *ee* 91%) by this innovative substrate engineering approach. This engineered peroxide-driven $P450_{BM3}$ system was further utilized to hydroxylate small alkanes with the assistance of Im-C6-Phe (118) (Fig. 3, compound **28**).

As an alternative strategy to protein engineering, the observed exquisite specificity and selectivity introduced by substrate engineering of P450 enzymes has highlighted the profound influence of the substrate-anchoring groups on the functional plasticity of P450s (103). Thus, this strategy has the potential to improve the synthetic utility of P450s. For example, it could be used for building a library of chemical structures that bear hydroxyl groups at various positions as functional group handles for further synthetic transformations (*e.g.* attachment of sugars).

Electron source engineering

Almost all natural P450s are cofactor-dependent enzymes, which are often expensive and must be recycled or circumvented from a process engineering perspective. To resolve this problem, several methods have been established on a laboratory scale over several decades, including cofactor regeneration systems, peroxide replacement, electrochemical approaches, and light-activated systems (14, 119, 120).

Cofactor engineering

NAD(P)H regeneration is a popular method in cell-free biocatalysis and biotransformation, in which constant supply of reducing equivalents is achieved by introducing a second reaction system to reduce $NAD(P)^+$. Many cost-effective approaches have been widely developed in industry not only for P450s but also for many NAD(P)H-dependent oxidoreductases, including glucose dehydrogenase/glucose (121), glucose-6-phosphate dehydrogenase/glucose 6-phosphate (122), isocitrate dehydrogenase/isocitrate (123), formate dehydrogenase/formate (124), ethanol dehydrogenase/ethanol (125), and engineered phosphite dehydrogenase/phosphite (126).

The "peroxide shunt pathway" (Fig. 1) has also been successfully engineered through directed evolution because it could be industrially relevant in making P450s use the cheaper peroxides (*e.g.* H_2O_2) rather than NAD(P)H as the electron donor (38). For instance, an efficient H_2O_2 regeneration system was recently applied to the catalytic reaction of a P450 peroxygenase by coupling with an oxidase, as demonstrated by an enzyme cascade comprised of the P450 peroxygenase $P450_{CLA}$ or $P450_{Spα}$ and the enantioselective α-hydroxyacid oxidase (*S*)-α-HAO from *Aerococcus viridans* or D-lactate oxidase GO-LOX from *Gluconobacter oxydans*. This enzyme cascade efficiently converted fatty acids of various chain length ($C_{6:0}$ to $C_{10:0}$) into the chemical intermediate α-ketoacids in the presence of an internal H_2O_2-recycling system (127). Moreover, a novel P450 monooxygenase-peroxygenase cascade consisting of $P450_{BM3}$ and $OleT_{JE}$ was recently developed for asymmetric catalysis in the conversion of 3-phenylpropionic acid to (*R*)-phenyl glycol without an external supply of H_2O_2 (128).

Electrochemical approaches

Electrochemical reductions have been used to circumvent the requirement for redox partners in shuttling electrons from NADPH to P450, with the electrode being the source of reducing equivalents. Progress with electrode-adsorbed/immobilization of P450 enzymes on various electrodes has been accomplished by engineering of both electrodes and enzymes, including layer-by-layer films with polyions (129, 130), a cobalt(III) sepulchrate (Zn/CoIIIsep) mediator (131, 132), covalent immobilization to a gold (Au) self-assembled monolayer (133), and nanomaterial-modified electrodes (134, 135). Due to the limitations of applying purified soluble P450s on various electrodes, protein film electrochemistry has been considered in electrocatalytic studies. Some of the studies include engineered membrane-bound human P450s with the reductase protein CPR added to a modified gold (Au) electrode (136, 137), (membrane-bound) liver microsomes with rat and human P450s immobilized on carbon electrodes and carbon nanostructures

(138–140), and purified P450s assembled with membrane-bound CPR on pyrolytic graphite electrodes (141).

Light-activated systems

Systems have been developed by utilizing energy from light to drive the P450 catalytic cycle. Three main pathways have been designed based on the catalytic nature of P450 enzymes. The first takes advantage of the peroxide shunt pathway, with controlled generation of the reactive oxygen species *in situ*, mainly limited to the CYP152 P450 family with peroxygenase activity (*e.g.* $P450_{BS\beta}$, CYPC1a, and $OleT_{JE}$) (142, 143). The second approach mimics the native electron transfer pathway by employment of redox partners to transfer electrons from a photosensitizer instead of cofactor, exemplified by a deazaflavin-dependent photoregeneration system (144) and photosystem I with ferredoxin as an electron mediator (145–148). The third simply involves direct shuttling of electrons to the heme active site and circumvention of redox partners by the employment of a fluorescent dye, eosin Y (149, 150), and covalently attached Ru(II)-diimine complexes (151, 152). However, there are still many challenges for photobased strategies in practical P450 catalysis, including the low efficiency of light conversion, weak coupling efficiency, low protein stability and activity, and technical difficulties.

Readers are referred to details in the individual research and reviewed publications regarding enzymatic regeneration systems (153), reactive oxygen species (154), electrochemical reduction (155), and light-activated approaches (156, 157). It is fairly important to find alternative economical electron sources for development of sustainable P450 catalytic systems to reduce the production costs. Except for the NAD(P)H- and H_2O_2-regenerating systems that have found practical applications in industry, other strategies are still in the developmental phase due to several critical problems (*e.g.* low coupling efficiency and redox potential management). Advances in biotechnology, discovery, and design of novel catalytic units and more interdisciplinary approaches may help overcome these challenges.

P450-related metabolic engineering

Rapid development of synthetic biology has led to more and more P450-related metabolic engineering work that has integrated protein, substrate, and cofactor engineering of P450 systems. These efforts have enabled cost-effective bioproduction of many commercial compounds as "natural" products for variant purposes.

As some of the most important enzymes in natural product biosynthesis, numerous natural and engineered P450s have already been included in the metabolic engineering toolbox. For example, the aforementioned CYP71AV1 has been used for bioproduction of the antimalarial drug precursor artemisinic acid (49), and CYP75 enzymes have been used for the hydroxylation on the B-ring of anthocyanidins to produce commercial blue roses and carnations (158). However, in both cases P450-related metabolic engineering has not been a straightforward process. In addition to a robust P450 with the desired activity, other requirements include high-level heterologous expression, optimization of metabolic fluxes, choice of a suitable heterologous host, and the deletion or silencing of competing pathways.

P450 SalSyn from *Papaver somniferum* is a key element in the complete biosynthesis of two opioid drugs in *S. cerevisiae*. Expression of an engineered P450 SalSyn with increased activity in generating the pro-morphinan scaffold (salutaridine) (Table 1 and Fig. 3, compound **29**), together with co-expression of 19 of 21 heterologous enzymes and two native enzymes and deletion of one native yeast gene, resulted in the microbial production of thebaine/hydrocodone (159). The critical bioconversion of (*S*)-reticuline to (*R*)-reticuline was mediated by a CYP82Y2-like P450 (1,2-dehydroreticuline synthase) from *Papaver bracteatum* fused with 1,2-dehydroreticuline reductase, which was achieved by complementary approaches including gene mining, protein mutagenesis, codon optimization, and heterologous expression in yeast (159) (Table 1 and Fig. 3, compound **30**).

Szczebara *et al.* (160) fully designed a *de novo* biosynthetic pathway involving 13 engineered enzymes in recombinant *S. cerevisiae* strains, in which the total biosynthesis of hydrocortisol and several steroids was achieved. First, recombinant *S. cerevisiae* was engineered to overproduce egrosta-5-eneol and ergosta-5,22-dieneol, which was further converted into pregnenolone by CYP11A1 (Table 1, Fig. 3, compound **31**). Finally, the oxidation steps that are sequentially catalyzed by 3β-hydroxysteroid dehydrogenase/isomerase, CYP17A1, CYP21A1, and CYP11B1 were reconstituted, giving rise to the production of progesterone, 17-hydroxyprogesterone, 11-deoxycortisol, and the final product hydrocortisol (160) (Table 1, compounds **32**–**34**).

Optimization of redox partners *in vivo* is also important in P450-related metabolic engineering. Huang and co-workers (161) reconstituted the catalytic activity of CYP76AH1 in the bioconversion of miltiradiene to ferruginol, a key bioactive component of the Chinese medicinal plant *Salvia miltiorrhiza*, in a miltiradiene-overproducing yeast strain. The production of 10.5 mg of ferruginol per liter was enabled with a surrogate redox partner protein, smCPR1, from *Salvia miltiorrhiza* Bungefor (Table 1 and Fig. 3, compound **35**). Zhao *et al.* (162) designed an artificial biosynthetic pathway of protopanaxadiol (Table 1 and Fig. 3, compound **36**), the precursor of bioactive ginsenosides of *Panax ginseng*, in an engineered *S. cerevisiae* strain. The self-sufficient P450 protopanaxadiol synthase was constructed by fusing it with an AtCPR from *Arabidopsis thaliana*, which resulted in a 71% increase in protopanaxadiol production (>1400 mg/liter) compared with co-expression of the two stand-alone components, protopanaxadiol synthase and AtCPR (162) (Table 1 and Fig. 3, compound **36**).

Distinct from the *de novo* biosynthesis of high value-added compounds in recombinant cells from sugar sources, engineering a P450 system into a robust whole-cell biocatalyst is also a useful strategy. For instance, when P450 boxA from *Streptomyces* sp. TM-7 was introduced into an efflux pump inactivation mutant of *E. coli*, the production of 1.7 g of pravastatin per liter (from compactin) was achieved, which was 7-fold higher than that using WT *E. coli* (163) (Table 1). When this system was expressed in the pravastatin-tolerant actinomycetes strain *P. autotrophica*, accumulation of pravastatin reached a level of

14 g/liter, 8-fold higher than in its *E. coli* counterpart and 3-fold higher than in the original *Streptomyces* sp. TM-7 (164). These results indicate the importance of a suitable heterologous host for construction of robust whole-cell biocatalysts.

Cofactor regeneration and cofactor-free P450 systems have also found applications in whole-cell biocatalysts. Watanabe and associates developed *E. coli* as a whole-cell biocatalyst vehicle to mediate the hydroxylation of benzene into phenol by WT $P450_{BM3}$ in the presence of decoy molecules (165). A novel whole-cell P450 photobiocatalysis system driven by the electrons from eosin Y instead of redox partners and cofactors was used for the bioconversion of pharmaceuticals with engineered bacterial P450s and human P450s (150). Different cofactor regeneration systems were also applied in many cases of whole-cell biotransformation, such as CYPsb-21 (45), P450 SMO from *Rhodococcus* sp. (166), and CYP106A2 (PDB entry 4YT3) from *B. megaterium* ATCC 13368 (167) (Table 1).

Conclusions and future prospects

Compared with some robust and widely applied commercial enzymes (*e.g.* hydrolases and ligases), P450 biocatalysts are still very limited by practical disadvantages, including low activity, poor stability, narrow substrate scope, and cofactor and redox partner dependence for most P450s. However, the irresistible regio- and stereoselectivity inherent in P450s continues to attract extensive efforts to deliver more P450 systems for industrial applications in production of pharmaceuticals, fine chemicals, flavors, and fragrances.

Exciting new biotechnology approaches have contributed to breakthroughs in P450 system engineering for practical catalysis in the past decade (14, 16, 42, 103). The multiple engineering strategies mentioned in this review have significantly improved the substrate scope, stability (60), catalytic efficiency (29), and reaction specificity (54) of P450s (Fig. 4). Moreover, P450-related metabolic engineering has opened a door for industrial application of the low-stability P450 systems (14, 61). A very recent development is the application of mammalian P450s selected by mining sequences of their relatives and prediction of primordial precursors, which unexpectedly has yielded more thermostable catalysts. These have broad specificity and can be used to generate useful products at much higher temperatures, increasing their efficiency (168). Lately, the integration of P450 catalytic systems into multienzyme cascades has been shown to be useful (128).

Versatile P450s are vital elements in the enzyme toolbox gifted from nature, and they will become much more powerful in the era of synthetic biology. In the future, we envision that functional mining of new P450s, construction of systematic libraries of P450s and redox partners, design of new electron-sourcing systems, the development of stable and highly efficient redox partner–independent P450 systems, and perhaps even the *de novo* design of P450s on demand will be the frontiers of P450 system engineering. Close collaboration between biologists, chemists, physicists, engineers, computer scientists, and mathematicians will be needed for engineering future new-concept P450 systems, which can create new exciting opportunities in practical catalysis for this most versatile superfamily of enzymes.

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