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Review

A unique P450 peroxygenase system facilitated by a dual-functional small molecule: concept, application, and perspective

Siyu Di,[1,2] Shengxian Fan,[1,2] Fengjie Jiang,[1,2] and Zhiqi Cong[1,2]*

1 CAS Key Laboratory of Biofuels, and Shandong Provincial Key Laboratory of Synthetic Biology, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences
2 University of Chinese Academy of Sciences, Beijing, 100049, China
* Correspondence: congzq@qibebt.ac.cn; Tel.: (0086-532-80662758)

Abstract: Cytochrome P450 monooxygenases (P450s) are promising versatile oxidative biocatalysts. However, the practical use of P450s in vitro is limited by their dependence on the co-enzyme NAD(P)H and the complex electron transport system. Using H_2O_2 simplifies the catalytic cycle of P450s; however, most P450s are inactive in the presence of H_2O_2. By mimicking the molecular structure and catalytic mechanism of natural peroxygenases and peroxidases, an artificial P450 peroxygenase system has been designed with the assistance of a dual-functional small molecule (DFSM). DFSMs, such as *N*-(ω-imidazolyl fatty acyl)-*L*-amino acids, use an acyl amino acid as an anchoring group to bind the enzyme, and the imidazolyl group at the other end functions as a general acid-base catalyst in the activation of H_2O_2. In combination with protein engineering, the DFSM-facilitated P450 peroxygenase system has been used in various oxidation reactions of non-native substrates, such as alkene epoxidation, thioanisole sulfoxidation, and alkanes and aromatic hydroxylation, which showed unique activities, and regio- and enantioselectivities when compared with native P450s. Moreover, the DFSM-facilitated P450 peroxygenase system can switch to the peroxidase mode by mechanism-guided protein engineering. In this short review, the design, mechanism, evolution, application, and perspective of these novel non-natural P450 peroxygenases for the oxidation of non-native substrates are discussed.

Keywords: cytochrome P450 monooxygenase; peroxygenase; peroxidase; protein engineering; oxidation; hydroxylation; epoxidation; sulfoxidation; dual-functional small molecule

1. Introduction

Cytochrome P450s (CYPs or P450s), a broad class of heme-containing enzymes, play important roles in drug metabolism, detoxification of xenobiotics, and steroid biosynthesis [1]. These enzymes are ubiquitous in nature, being found in animals, plants, bacteria, fungi, and other organisms [2]. P450s have potential use in the catalytic monooxygenation of various organic substrates, including aliphatic and aromatic compounds, alkenes, and heteroatoms (*N*-, *S*-oxidation) [3]. In particular, their ability to regio- and stereoselectively oxidize inert C–H bonds makes P450s an attractive enzyme class in the development of practical biocatalysts for the oxidation of compounds with commercial or high added value [4].

Thus, a variety of approaches have been developed to solve the intrinsic drawbacks of P450s, e.g., poor enzyme stability, low turnover rates, narrow substrate scope, and the need for expensive cofactors (NAD(P)H). Protein engineering, including rational design and directed evolution, represents a first choice for solving most of these issues [5]. Moreover, effective strategies have emerged to overcome some specific problems during P450-driven catalysis [6-10]. For example, researchers have constructed a substrate

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engineering approach to improve the acceptance and/or the stereo-/regioselectivity of non-native substrates of P450s by introducing protecting/anchoring/directing groups to the substrate [6]. Watanabe and co-workers used a dummy co-substrate (so-called decoy molecule) to modulate substrate promiscuity of P450s, enabling wild-type P450s to hydroxylate various small molecules that are not accepted in the absence of the decoy molecule (such as gas alkanes and benzene) [7]. Biological and chemical regeneration of NAD(P)H has been used widely to support catalysis by P450s [8]. In addition, the surrogate oxidants (e.g., hydrogen peroxide, *tert*-butyl hydroperoxide, and iodosylbenzene) are also used to drive P450 instead of molecular oxygen and reduced NAD(P)H [9-10]. Despite being useful supplements to protein engineering, these strategies often play a role in combination with protein engineering. There have been many reviews discussing the abovementioned topics [11-12]. Herein, we focus on a unique H_2O_2-dependent P450 peroxygenase system facilitated by a dual functional small molecule (DFSM). The design, construction, mechanism, and catalytic application of the DFSM-facilitated P450 peroxygenases are reviewed, and current issues and future perspectives are also discussed.

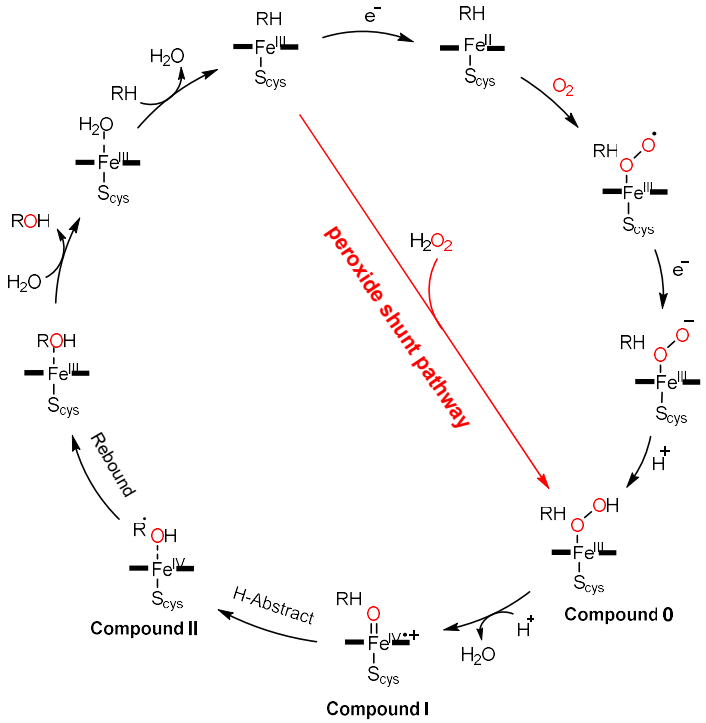

Figure 1. The catalytic cycle of cytochrome P450 monooxygenase and the peroxide-shunt pathway.

2. Proof-of-concept of the DFSM-facilitated P450 peroxygenase

The complex catalytic cycle of P450s needs reduced co-enzyme NAD(P)H and a redox partner to support the activation of molecular oxygen. Thus, it had been suggested that surrogate peroxide species can be used to drive P450-catalysis through its shunt pathway (Figure 1), with low-cost H_2O_2 being one of the best choices. However, only a few native P450 peroxygenases (e.g., CYP 152 family) can use the unique substrate-assisted mechanism to activate H_2O_2 successfully [13-15], with most P450s examined (e.g., rat liver microsomal P450, human P450s such as CYP1A2 and 3A4, thermophilic archaea CYP119, CYP175A1, and P450cam) generally showing low efficiency for the H_2O_2-dependent reaction (shunt pathway in Figure 1) [16-21]. Although the peroxygenase and peroxidase activity of P450s can be partially improved by directed evolution, the catalytic

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efficiency of the evolved P450 variants is still not comparable to natural NAD(P)H-dependent P450s [22]. This may be caused by the inherent structural characteristics of P450s. Indeed, those enzymes that make good use of H_2O_2 in nature have acid-base amino acid residue pairs that play the role of an acid-base catalyst in their active site (Figure 2) [23-24]. In contrast, the crystal structures of other P450s have revealed that such amino acid residues are not present on the distal side of their heme centers. Previous reports have suggested that the introduction of a basic residue can modify myoglobin into a peroxidase through site-directed mutagenesis [25]. Similar strategies have been applied to improve the peroxygenase/peroxidase activity of P450s [26-28]; however, the catalytic efficiency was not always satisfactory. Crystal structure studies have provided hints for the poor activity in some cases, namely, the basic group on the side chain of the introduced residue is distal from the heme center such that this residue cannot efficiently activate H_2O_2 as the general acid-base catalyst [27].

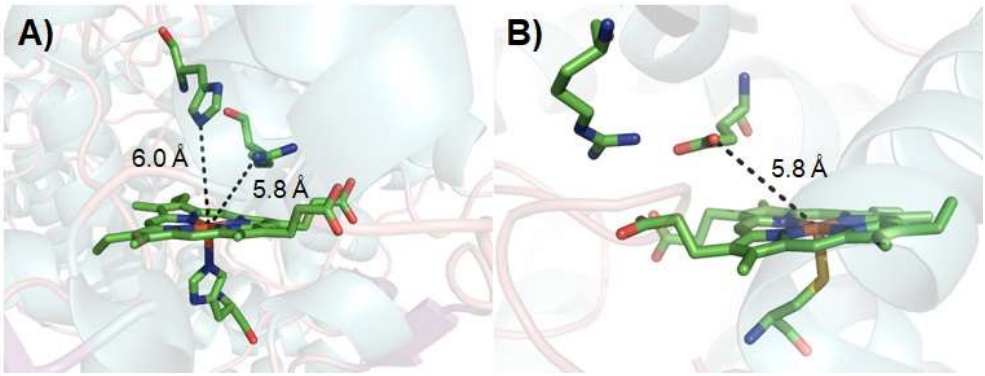

Figure 2. Active site structures of HRP (PDB ID: 1ATJ) [23] and unspecific peroxygenase (PDB ID: 2YOR) [24].

Based on previous reports, it has become clear that to use the shunt pathway of P450s successfully, two points should be met: 1) A basic group located on the distal side of the heme center is necessary; and 2) The basic group should be placed at a suitable position to ensure that this residue plays the role as an acid-base catalyst efficiently. To this end, Ma et al. designed a class of dual-functional small molecules that act as co-catalysts to aid the activation of H_2O_2 by a modified long-chain fatty acid hydroxylase P450BM3 from *Bacillus megaterium* (Figure 3) [29-31]. Typical structures of DFSMs, such as *N*-(ω-imidazolyl)-fatty acyl-*L*-amino acid (Im-Cn-AA), are shown in Figure 3B [30]. These DFSMs have and acyl amino acid moiety at one end as an anchoring group, i.e., for binding to the enzyme, and an imidazolyl group at the other end as a basic group, which acts as the general acid-base catalyst to activate H_2O_2 within the heme center. More importantly, the position of the introduced base can be finely tuned by modifying the chain length of a flexible CH_{2n} spacer. This circumvents problems caused by introducing amino acid residues (commonly installed *via* site-directed mutagenesis) that do not extend sufficiently into a suitable catalytic site [27]. Ma et al. reasoned that the DFSM-facilitated P450BM3-H_2O_2 system was capable of running smoothly with a catalytic cycle that was similar to the native UPO peroxygenase under ideal conditions (Figure 3C) [32].

This concept was then validated by using styrene epoxidation as a model reaction. The results indicated that the catalytic efficiency of the H_2O_2-dependent P450BM3_F87A mutant was improved more than 30-fold in the presence of the best DFSM, *N*-(ω-imidazolyl)-hexanoyl-*L*-phenylalanine (Im-C6-Phe), when compared with that of the F87A mutant alone. Two classes of control experiments were performed using various molecules without the terminal imidazolyl group or acyl amino acid group (mono-functional small molecules, MFSMs). These MFSMs did not improve the catalytic turnover numbers (TON) of styrene epoxidation, but inhibited the reactions in some cases,

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indicating the importance of both the inbuilt base and anchoring group for ensuring H_2O_2 activation of P450BM3. Control experiments using DFSMs with different chain lengths demonstrated the importance of a suitable length flexible spacer. Furthermore, the authors found that mutation of T268 to valine, a distal residue that was hypothesized to play key roles in the formation of the active compound I species [33-35], abolished the catalytic activity of the F87A-H_2O_2 system for styrene epoxidation. Interestingly, the addition of DFSM rescued the peroxygenase activity of the double mutant F87A/T268V. This discovery is important for the DFSM-facilitated P450 peroxygenase, which strongly supports the participation of the DFSM molecule in the activation of H_2O_2 to generate peroxygenase activity and provides a unique choice of protein engineering sites for developing catalytic promiscuity of the current peroxygenase system (will be discussed below by combination with other results.

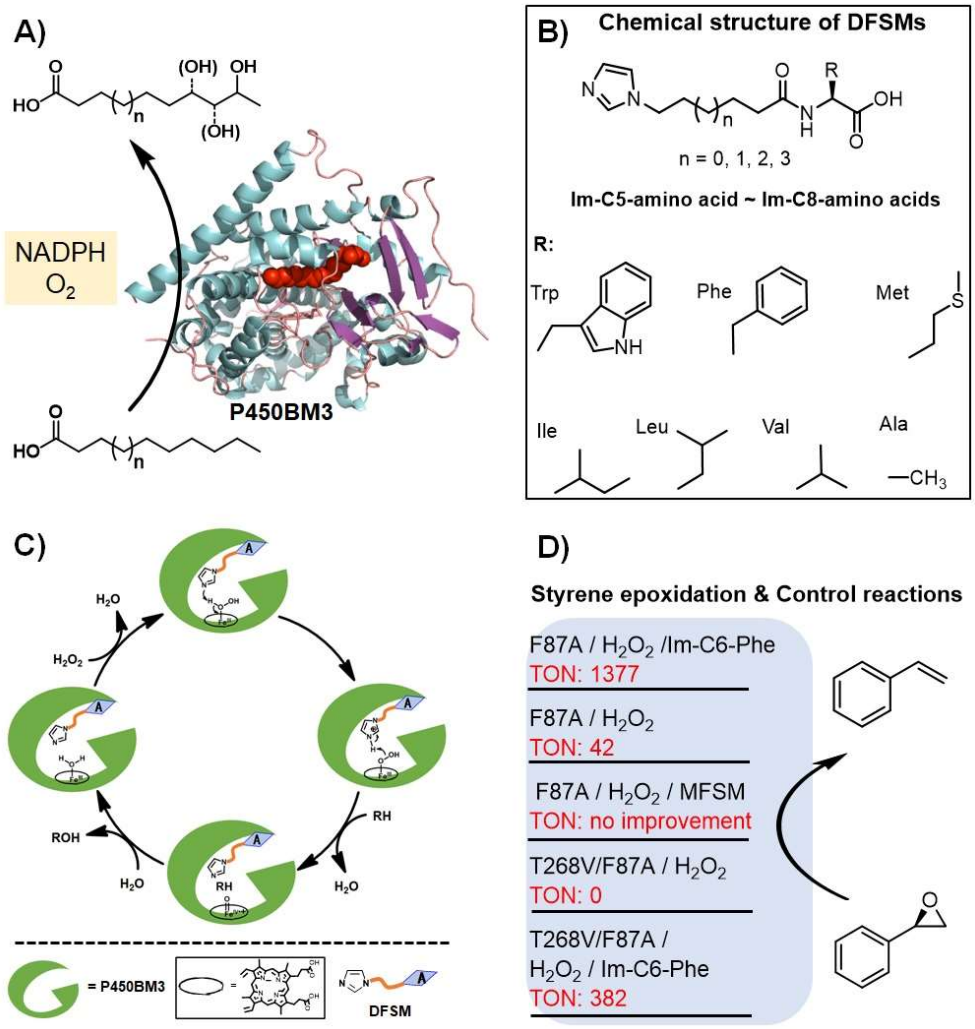

Figure 3. Proof-of-concept of DFSM-facilitated P450 peroxygenase. (A) The NADPH-dependent P450BM3 monooxygenase. (B) Proposed catalytic cycle of the DFSM-facilitated P450 peroxygenase. (C) Chemical structures of the DFSM molecules. (D) Styrene epoxidation in the presence of the DFSM and control experiments.

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The catalytic role and mechanism of DFSMs have been further disclosed by combining structural biology and computational chemistry [36]. To mimic the pre-reaction state of P450-bounded H_2O_2 and avoid the H_2O_2-initiated reaction, Jiang et al. skillfully adopted the NH_2OH molecule as the analog of H_2O_2 to prepare the co-crystal (Figure 4A-B). As a result, they successfully reported the first X-ray structure of the P450BM3 heme domain F87A mutant in complex with the DFSM, *N*-(ω-imidazolyl)-hexanoyl-*L*-phenylalanine (Im-C6-Phe) and NH_2OH at 2.70 Å resolution (PDB ID: 7EGN, Figure 4C). The crystal structure clearly shows that Im-C6-Phe bound to P450BM3 through a H-bond network formed by interactions of its terminal carboxyl group with Arg47 and Tyr51, and hydrophobic interactions between its benzyl moiety and a hydrophobic pocket composed of Pro25, Val26, Leu29, Met185, and Leu188 (Figure 4D). The unique binding mode that involves additional hydrophobic interactions is distinct from those observed in the co-crystals of P450BM3 in complex with fatty acids (native substrates) or perfluoroacyl amino acids (decoy molecules) [37-38]. This binding mode plays a crucial role in positioning the imidazolyl group of the DFSM above the heme center, where the distance between the heme iron atom and the terminal nitrogen atom of the imidazolyl group is ~5 Å, indicating the imidazolyl group of the DFSM may act as the general acid-base catalyst in H_2O_2 activation. This crystallographic information provides the structural basis for the catalytic role of DFSM in the unique artificial P450 peroxygenase system, and is consistent with the original hypothesis by Ma et al. [30].

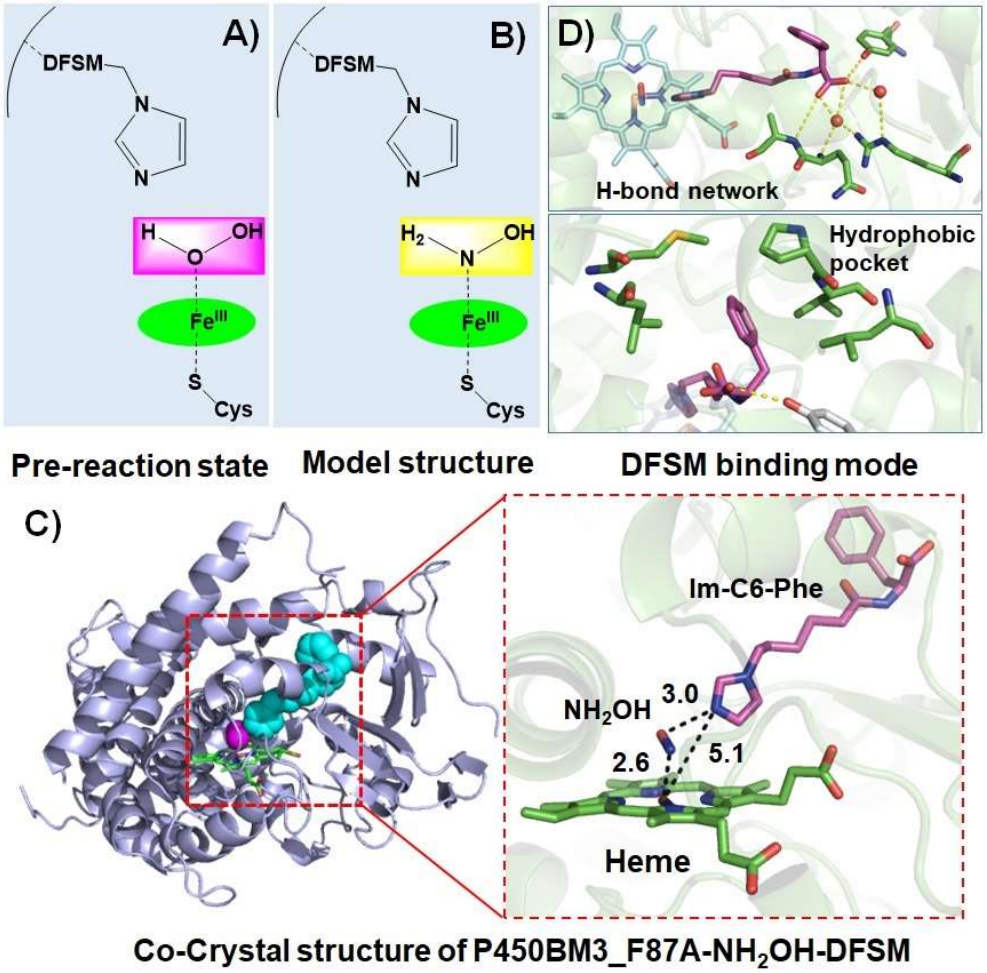

Figure 4. Structural basis of the DFSM-facilitated P450 peroxygenase. (A) Proposed pre-reaction state of P450BM3 in the presence of H_2O_2 and DFSM. (B) The model structure with NH_2OH

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instead of H_2O_2. (C) The co-crystal structure of P450BM3_F87A in complex with NH_2OH and Im-C6-Phe. (D) The binding interactions of Im-C6-Phe with P450BM3.

The mechanism for H_2O_2 activation was further elucidated by QM-MM computational investigations. These computational chemistry results revealed the crucial role of DFSM in promoting a heterolytic O–O cleavage to favor Cpd I formation [36]. The DFSM facilitates formation of a proton channel between the imidazolyl group of the DFSM and proximal H of H_2O_2, thus enabling a heterolytic O–O cleavage and Cpd I formation, which is similar to the proposed mechanism for H_2O_2 activation in natural peroxygenases (e.g., UPO) or peroxidases (e.g., HRP). In contrast, the formation of Cpd I is apparently sluggish via the O–O homolysis mechanism in the absence of the DFSM. Similar results were also observed in the theoretical simulation of H_2O_2 activation by the P450cam T252A mutant [39], indicating weak H_2O_2 activation by NADPH-dependent P450s.

3. Catalytic applications of the DFSM-facilitated P450 peroxygenase

In recent years, peroxygenase UPO has attracted considerable attention because of its versatile oxidation functions and potential in synthetic applications [40-42]. Moreover, peroxygenase that Uses green and economic H_2O_2 to circumvent the use of expensive NADPH and the complex electron transfer system (redox partner proteins) has become a promising practical bio-oxidative catalyst when compared with using NAD(P)H-dependent P450 monooxygenases [40]. Despite concerns about the potential damage of H_2O_2 to enzymes, the use of a controlled fed-batch reactor or in-situ generating H_2O_2 has been demonstrated to enhance effectively the stability of peroxygenases through control of the H_2O_2 concentration in the reaction system, resulting in high catalytic turnovers [43-44]. Therefore, developing the catalytic potential of the artificial P450 peroxygenase is not only expected to expand the chemical space of P450 enzymes but also act as a beneficial supplement to the relatively scarce natural peroxygenase resources in nature. In fact, the DFSM-facilitated P450BM3-H_2O_2 system has shown versatile unique catalytic activity towards the peroxygenation reaction of various non-native substrates, such as epoxidation, hydroxylation, and sulfoxidation [30, 45-48].

Asymmetric epoxidation of unfunctionalized olefins represents an important organic transformation to prepare optically pure epoxides, which are extremely useful building blocks for synthesizing chiral organic compounds and functional polymers [49]. Despite critical progress, there is limited understanding of the highly enantioselective epoxidation of terminal olefins, including styrene. In particular, the (*R*)-enantioselective epoxidation of styrene seems more difficult to achieve than the (*S*)-enantioselective reaction through either synthetic molecular catalysts or natural enzymatic bio-catalysts [50-51]. DFSM-facilitated P450BM3 peroxygenase enabled access to (*R*)-enantioselective epoxidation of unfunctionalized styrene and its derivatives (Figure 5). In the presence of Im-C6-Phe, P450BM3 with the F87A mutation in its heme domain catalyzed the epoxidation of styrene with a TON of 1377 over a 30 min reaction [30]. This is approximately 30-fold higher than that of the F87A mutant alone. Interestingly, the presence of Im-C6-Phe yielded an enantiomeric excess of 84% for (*R*)-(+)-styrene oxide, which is significantly higher than that of the F87A mutant alone (7%). The double mutant F87A/T268V further improves the *ee* value to 91% in the presence of Im-C6-Phe, suggesting the importance of T268V in modulating the enantioselectivity. However, the TON decreased drastically by two third (Figure 5B). Zhao et al. then systematically evaluated the effect of T268, the highly conserved residue on the distal side of the heme center and disclosed the roles of the T268 mutation in tuning activity and enantioselectivity of styrene epoxidation by the NAD(P)H- and H_2O_2-dependent P450BM3 system, respectively [45]. Based on the more selective, but lower activity profile of the double mutant F87A/T268I (97% *ee*, TON = 335), a mutant library was constructed by introducing additional mutations at ten key residues around the substrate-binding pocket (Figure 5A). Two beneficial mutants were

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determined to give high (*R*)-enantioselective epoxidation of styrene (98% *ee*) with >4000 TONs (Figure 5B). Following this approach, a set of styrene derivatives, such as *o*-, *m*-, *p*-chlorostyrenes and fluorostyrenes were epoxidized with modest to very good TONs (362–3480) and high (*R*)-enantioselectivities (95%–99% *ee*) (Figure 5B). These results are comparable with the best (*R*)-enantioselective styrene monooxygenases, such as *Se*StyA from *Streptomyces exfoliatus*, *Aa*StyA from *Amycolatopsis albispora*, and *Pb*StyA from *Pseudonocardiaceae*, which have been mined from NCBI non-redundant protein sequences recently [52]. The semi-preparative scale synthesis of (*R*)-styrene oxide performed at 0 °C with high conversion, maintained enantioselectivity, and moderate isolated yields, further suggests the potential application of the current P450 enzymatic system in styrene epoxidation.

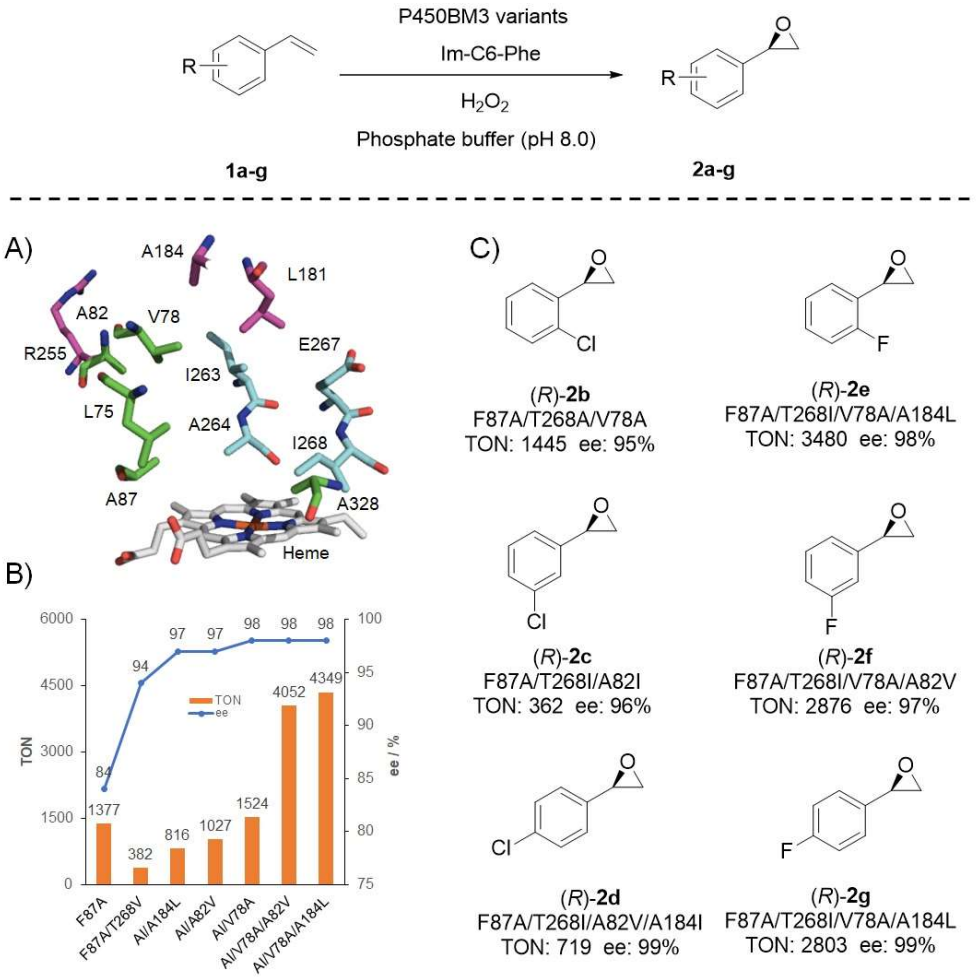

Figure 5. Protein engineering of the DFSM-facilitated P450BM3 peroxygenase for catalyzing (*R*)-enantioselective epoxidation of styrene and its derivatives. A) Key residues around the substrate-binding pocket of P450BM3; B) Protein engineering for styrene epoxidation; C) The epoxidation of styrene derivatives by the DFSM-facilitated P450 peroxygenases.

The direct hydroxylation of small alkanes to alcohols is a long-standing challenge because of the higher bond dissociation energies (BDE) of their C–H bonds when compared with that of the corresponding hydroxylated products, the latter easily leads to overoxidation [53]. Natural oxidizing enzymes, such as methane monooxygenase [54], soluble butane monooxygenase (sBMO) [55], fungal peroxygenase (*Aae*UPO) [56], and engineered P450s [38, 46, 53c, 57-59, 61], are promising biocatalysts for the selective

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hydroxylation of small alkanes. Recently, Chen et al. reported the H_2O_2-dependent hydroxylation of small alkanes (C_3–C_6) by using rationally engineered P450BM3 variants assisted by DFSMs [46]. Compared with some main results through enzymatic hydroxylation of small alkanes [56-62], DFSM-facilitated P450BM3 peroxygenase showed unique features and catalytic activities (Table 1). The hydrophobic mutation of the highly conserved T268 residue plays a key role for improving the hydroxylation activities of small alkanes, which is distinct from NADPH-dependent P450 enzymes [47]. Here, the presence of the DFSM was critical for accomplishing the catalytic functions of engineered P450BM3 variants because activity is completely lost in the absence of the DFSM. Two triple-mutants BM3_F87A/T268I/A184I and BM3_F87A/T268I/A82T showed the highest total turnover numbers (TTN) for the hydroxylation of propane and *n*-Butane (Entries 1–2 in Table 1), respectively, with better activity than *Aae*UPO, the only known H_2O_2-dependent native hydroxylase for small alkanes (Entries 20–21 in Table 1) [56], and comparable activity to the P450BM3 decoy system (Entries 3–8 in Table 1) [57-58], but far lower than P450$_{PMO}$R1 and P450$_{PMO}$R2, two evolved NADPH-dependent propane monooxygenases (Entries 13–14 in Table 1) [59]. Notably, the product formation rates (PFR) for 2-propanol and 2-butanol of the current artificial P450 peroxygenase are far better than all reported natural or engineered enzyme systems. The contradiction between high PFR and low TTN suggests that the DFSM-facilitated P450 peroxygenase may be unstable. Nonetheless, reducing instability should yield an efficient biocatalyst for the direct hydroxylation of small alkanes. In addition, this peroxygenase system is unavailable for the hydroxylation of smaller alkanes (e.g., ethane and methane), which has been achieved by natural methane monooxygenase (MMO) or other enzymes (Entries 9, 12, 16, 17 in Table 1) [38, 57b, 60-61]. Anyhow, Ciuffetti et al reported that CYP52L1 from *Graphium* sp. ATCC 58400 can oxidize propane, but without any turnover numbers or catalytic constants mentioned [62]. This may be the only known P450 enzyme that uses gaseous alkanes as natural substrates, suggesting that P450 has a weak preference for small alkanes. Therefore, further protein engineering may be necessary for the DFSM-facilitated P450BM3 peroxygenase to access the direct hydroxylation of methane or ethane.

Table 1. Main results reported in literature for enzymatic hydroxylation of small alkanes.

Entry	Enzyme	Alkanes	Final product	PFR[a]	TTN[b]	Ref.
1	BM3_F87A/T268I/A184I / Im-C6-Phe	Propane	2-Propanol	630	1775	[46]
2	BM3_F87A/T268I/A82T / Im-C6-Phe	*n*-Butane	2-Butanol	1042	2253	[46]
3	BM3 / PFC10	Propane	2-Propanol	70	700	[57a]
4	BM3 / PFC9-L-Leu	Propane	2-Propanol	256	2560	[38a]
5	BM3 / 3CCPA-Pip-Phe	Propane	2-Propanol	615	-	[57b]
6	BM3 / PFC9	*n*-Butane	2-Butanol	110	1100	[58]
7	BM3 / PFC11	Propane	2-Propanol	-	1021	[58]
8	BM3 / PFC7	*n*-Butane	2-Butanol	-	3632	[58]
9	BM3 / C7AM-Pip-Phe	Ethane	Ethanol	82.7	-	[57b]
10	P450cam_EB	*n*-Butane	2-Butanol	520	-	[61]
11	P450cam_EB_L294M/T185M/L1358P/G248A	Propane	2-Propanol	505	-	[61]
12	P450cam_EB_L294M/T185M/L1358P/G248A	Ethane	Ethanol	78.2	-	[61]
13	P450$_{PMO}$R1	Propane	2-Propanol	455	35600	[59]
14	P450$_{PMO}$R2	Propane	2-Propanol	370	45800	[59]
15[c]	CYP52L$_1$	Propane	1-Propanol	-	-	[62]
16	sMMO	Methane	Methanol	78	-	[60]
17	sMMO	Ethane	Ethanol	45.6	-	[60]
18	sMMO	Propane	2-Propanol	33-58.8	-	[60]
19	sMMO	*n*-Butane	2-Butanol	7.2-28.8	-	[60]

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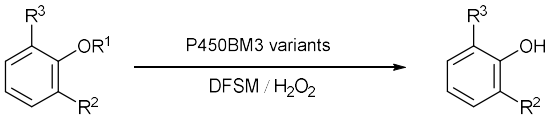
20	*Aae*UPO		Propane	2-Propanol	17	999	[56]
21	*Aae*UPO		*n*-Butane	2-Butanol	21	1258	[56]

[a]PFR: product formation rate in μmol·min^{-1}·(μmol P450)$^{-1}$. [b]TTN: total turnover number. [c]There is no catalytic turnover data reported.

The *O*-demethylation of aromatic ethers can produce value-added phenolic-type compounds and is also a key step in the formation of diols, the precursor of aromatic ring-opening reactions for coniferyl and sinapyl lignin-derivatives [63-64]. Various powerful oxidative enzymes, such as peroxidases from white-rot, soft-rot, and brown-rot fungi, as well as some bacteria, can catalyze demethylation of lignin-derived compounds and their model compounds [65-68]. A few P450 enzymes also show promise as an *O*-demethylase for lignin-derived aromatic ethers [69]. Recently, Jiang et al. successfully applied the DFSM-facilitated P450BM3 peroxygenase system to perform *O*-demethylation of various aromatic ether substrates (Table 2) [47]. These reactions show excellent regioselectivity toward the hydroxylation of the methoxy of aromatic ethers to give the demethylation product after automatically releasing formaldehyde. A suitable combination of the beneficial mutant and DFSM is important for controlling good regioselectivity. For example, some combinations examined still give aromatic hydroxylation as the main product. Although the DFSM-facilitated P450BM3 peroxygenase appears to open a new avenue for the key demethylation step in the bioconversion of lignin, it is still restricted by low TONs and narrow substrate scopes.

Table 2. Regioselective aromatic O-dealkylation by the DFSM-facilitated P450 peroxygenases.



3a-f **4a-f**

substrate				Enzyme system	Product			TON
No.	R^1	R^2	R^3		No.	R^2	R^3	
3a	Me	H	H	BM3_F87A/T268I / Im-C5-Phe	**4a**	H	H	486
3b	Me	Me	H	BM3_F87A/T268I / Im-C6-Phe	**4b**	Me	H	356
3c	Me	OH	H	BM3_F87A / Im-C6-Phe	**4c**	OH	H	539
3d	Et	OH	H	BM3_F87G/T268G / Im-C5-Phe	**4d**	OH	H	99
3e	Me	OMe	H	BM3_F87A/T268I / Im-C6-Phe	**4e**	OMe	H	287
3f	H	OMe	OMe	BM3_F87G/T268V / Im-C5-Phe	**4f**	OH	OMe	165

The DFSM-facilitated P450BM3 peroxygenase is also capable of catalyzing other types of reactions, including thioanisole sulfoxidation, aromatic hydroxylation of naphthalene and the benzylic hydroxylation of ethylbenzene (Figure 6) [30, 48]. The combination of the F87A mutant and Im-C6-Phe yielded sulfoxide as the sole product with a PFR of 888 μmol·min^{-1}·(μmol P450)$^{-1}$ and a catalytic TON of 3436, which is nearly 35-fold higher than that of the F87A mutant alone. Interestingly, a mutation at position 268 was found to influence the hydroxylation activity of the P450BM3 peroxygenase system significantly. In the presence of Im-C6-Phe and H_2O_2, the F87A mutant catalyzed the oxidation of naphthalene and ethylbenzene to give the corresponding hydroxylated products with TONs of 13 and 39, respectively. In contrast, the combination of Im-C6-Phe with the double mutants F87G/T268V and F87A/T268V gave 1-naphthol and 1-phenylethanol with TONs of 200 and 319, respectively, in addition to 2-phenylethanol and 4-ethylphenol as by-products (in a combined ratio of 15%) [30].

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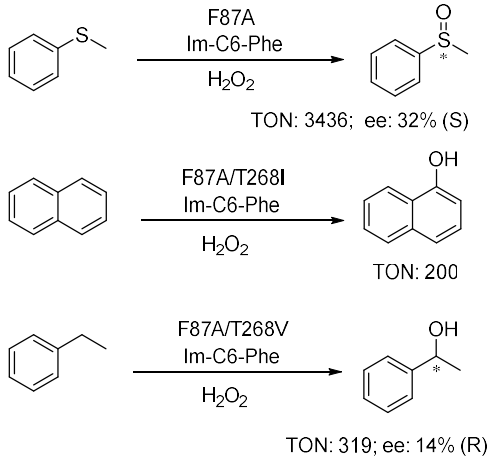

Figure 6. DFSM-facilitated P450 peroxygenases catalyzed sulfoxidation and hydroxylation.

4. Switching peroxidase activity of the DFSM-facilitated P450 peroxygenase

The catalytic promiscuity of enzymes is a fascinating topic for the biochemistry, synthetic biology, and chemical biology communities [70]. P450s have been well documented to carry out multiple catalytic functions such as monooxygenase, peroxygenase, and peroxidase activity [71]. However, research interest has focused on the monooxygenase and peroxygenase activities of P450s, and only a handful of studies have examined the catalytic peroxidase functionality of P450s. The non-natural DFSM-facilitated P450-H_2O_2 system described above mainly catalyzes various per-oxygenation reactions, including epoxidation, hydroxylation, and sulfoxidation [30, 45-48]. Interestingly, the oxidation of guaiacol, a classical substrate of peroxidases [72], catalyzed by the DFSM-facilitated P450BM3-H_2O_2 system yielded demethylated catechol as a major product, suggesting it mainly functioned as a peroxygenase but not as a peroxidase [47]. After carefully analyzing the catalytic mechanism of the potential competitive oxidation pathways in the DFSM-facilitated P450BM3-H_2O_2 system, Ma et al. hypothesized that mutation of redox-sensitive residues may enable switching of peroxygenase activity to peroxidase activity [73]. Using a semi-rational design approach, similar to FRISM (Focused Rational Iterative Site-specific Mutagenesis) named by Reetz and Wu [74], Ma et al. identified mutations of three key redox-sensitive tyrosine residues that are located on the surface of P450. Screening for activity-enhanced peroxidase mutants yielded a mutant that efficiently catalyzed one-electron oxidation of guaiacol through combination with other redox-sensitive residues located in the electron transfer pathway. The engineered system also exhibits favorable one-electron oxidation activity toward other peroxidase substrates, including 2,6-dimethoxyphenol, *o*-phenylenediamine, and *p*-phenylenediamine, and almost without peroxygenase activity for these substrates. Notably, this system attains the best peroxidase activity of any P450 reported [26, 75], and rivals most natural peroxidases [76], suggesting significant potential for catalytic promiscuity of the DFSM-facilitated P450BM3-H_2O_2 system (Figure 7). Future efforts should explore the functional applications of the DFSM-facilitated P450 peroxidase in synthetic chemistry.

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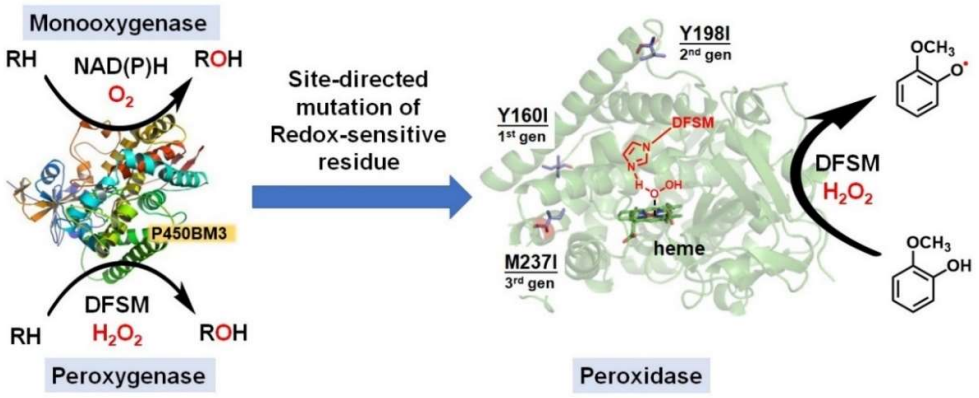

Figure 7. Native monooxygenase activity. DFSM-facilitated peroxygenase activity and switching to peroxidase activity by mechanism-guided protein engineering.

5. Summary and perspectives

In summary, although only a few natural P450s, such as CYP152 peroxygenases from *Sphingomonas paucimobilis*, *Bacillus subtilis* and *Clostridium acetobutylicum* can directly use an oxygen atom from peroxides for oxidation reactions [13-15], the engineered artificial P450 peroxygenases have significantly expanded the substrate scope and reaction types of P450-catalyzed per-oxygenation reactions. Therefore, it is no exaggeration to state that H_2O_2-driven P450 peroxygenases are emerging as powerful bio-oxidation catalysts. Among these, the DFSM-facilitated P450 peroxygenases provide a unique solution for the efficient use of H_2O_2 by P450s, which exhibit much higher H_2O_2 activities in epoxidation, sulfoxidation, and hydroxylation reactions when compared with those P450 peroxygenases that have been engineered through site-directed mutagenesis and directed evolution [22, 26-28, 77]. Moreover, the DFSM-facilitated P450 peroxygenases may offer better opportunities for enhancing the regio- and enantioselectivity in oxidation reactions of non-natural substrates. On the one hand, the introduced DFSMs can influence the orientation of substrates through interaction with each other to modulate reaction selectivity, besides its role in the activation of H_2O_2, which still requires further experimental characterization. On the other hand, the highly conserved T268 residue can be optionally mutated in the DFSM-facilitated P450 peroxygenase system. In contrast, the mutation of T268 is not favorable in NADPH-dependent P450BM3 oxidation because this residue is located on the distal side of the heme center and is thought to play multiple roles in NADPH-dependent catalysis [33-35, 78]. In fact, successful examples of the DFSM-facilitated P450 peroxygenase system have demonstrated that mutation of T268 has a significant influence on regulating the substrate pocket space when employing a protein engineering strategy [45-48]. This suggests that protein engineering of the DFSM-facilitated P450 peroxygenase system may also have its own unique advantages for controlling reaction selectivity in comparison with natural NADPH-dependent P450s. In addition, the high peroxidase activity of the DFSM-facilitated P450-system developed recently expanded the catalytic promiscuity of the system [73], whose further application in organic transformation is expected.

Despite the many advantages of the DFSM-facilitated P450 peroxygenase system worth exploring, it is important to note that there are still major drawbacks that hampers its use as a practical biocatalyst: 1) Despite high efficiency, the introduction of DFSM undoubtedly increases the cost of the catalytic reaction, especially when a large excess is required; 2) Oxidative damage of P450 caused by the presence of a large amount of H_2O_2; 3) The uncertainty associated with applying this strategy to other P450s; 4) The complex structures of DFSMs lead to an increase in the threshold of popularization and use; 5) The precise and complete catalytic reaction mechanism needs to be elucidated. In conclusion, the DFSM-facilitated P450 peroxygenase system simultaneously faces opportunities and

challenges. Maximizing the potential of the system and answering the above issues will open new avenues for developing P450-based biocatalysts.

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