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, Qingdao 266101, China; disy@qibebt.ac.cn (S.D.); fansx@qibebt.ac.cn (S.F.); jiangfj@qibebt.ac.cn (F.J.)
2 University of Chinese Academy of Sciences, Beijing 100049, China
* Correspondence: congzq@qibebt.ac.cn; Tel.: +86-532-80662758

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 H2O2 simplifies the catalytic cycle of P450s; however, most P450s are inactive in the presence of H2O2. 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 H2O2. 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 selectivity. 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 compounds containing heteroatoms such as nitrogen and sulfur [3–8]. In particular, P450s can regio- and stereoselectively oxidize inert C–H bonds, thus acting as an attractive enzyme class in the development of practical biocatalysts for organic synthesis [9–11].

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 [12–14]. Moreover, effective strategies have emerged to overcome some specific problems during P450-driven catalysis [15–33]. For example, researchers have constructed a substrate 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 [15–24]. 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) [25–27]. Biological and chemical regeneration of NAD(P)H has been used widely to support catalysis by P450s [28,29]. 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 [30–33]. 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 [34–41]. Herein, we focus on a unique H$_2$O$_2$-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.

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$_2$O$_2$ 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$_2$O$_2$ successfully [42–47], 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$_2$O$_2$-dependent reaction (shunt pathway in Figure 1) [48–53]. Although the peroxygenase and peroxidase activity of P450s can be partially improved by directed evolution, the catalytic efficiency of the evolved P450 variants is still not comparable to natural NAD(P)H-dependent P450s [54–57]. This may be caused by the inherent structural characteristics of P450s. Indeed, those enzymes that make good use of H$_2$O$_2$ in nature have acid-base amino acid residue pairs that play the role of an acid–base catalyst in their active site (Figure 2) [58,59]. 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 [60–62]. Similar strategies have been applied to improve the peroxygenase/peroxidase activity of P450s [63,64]; 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$_2$O$_2$ as the general acid–base catalyst [63].

Based on previous reports, it has become clear that to use the shunt pathway of P450s efficiently, 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 DFSM approach to modify cytochrome P450BM3 monoxygenase from Bacillus megaterium into its peroxygenase mode (Figure 3) [65–67]. Typical structures of DFSMs, such as N-(ω-imidazolyl)-fatty acyl-γ-amino acid (Im-Cn-AA), are shown in Figure 3B [66]. These DFSMs have an acyl amino acid moiety at one end as an anchoring group to bind with the enzyme, and an imidazolyl group at the other end as a basic group to assist the activation of H$_2$O$_2$. Moreover, the position of imidazolyl can be optionally tuned by changing the chain length of a flexible spacer having various CH$_2$ numbers, while the introduced basic residues by site-directed mutagenesis often can’t extend sufficiently into a suitable catalytic site [63]. Ma et al. reasoned that the DFSM-facilitated P450BM3-H$_2$O$_2$ system was capable of running smoothly with a catalytic cycle that was similar to the native UPO peroxygenase under ideal conditions (Figure 3C) [68,69].
Based on previous reports, it has become clear that to use the shunt pathway of P450s efficiently, 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 DFSM approach to modify cytochrome P450BM3 monooxygenase from *Bacillus megaterium* into its peroxygenase mode (Figure 3) [65–67]. Typical structures of DFSMs, such as N-(ω-imidazolyl)-fatty acyl-L-amino acid (Im-Cn-AA), are shown in Figure 3B [66]. These DFSMs have an acyl amino acid moiety at one end as an anchoring group to bind with the enzyme, and an imidazolyl group at the other end as a basic group to assist the activation of H$_2$O$_2$. Moreover, the position of imidazolyl can be optionally tuned by changing the chain length of a flexible spacer having various CH$_2$ numbers, while the introduced basic residues by site-directed mutagenesis often can’t extend sufficiently into a suitable catalytic site [63]. Ma et al. reasoned that the DFSM-facilitated peroxygenase shunt pathway

This concept was firstly validated by the H$_2$O$_2$-dependent epoxidation of styrene catalyzed by the P450BM3_F87A mutant. The presence of the best DFSM, N-(ω-imidazolyl)-hexanoyl-L-phenylalanine (Im-C6-Phe), increased the catalytic turnover number (TON) more than 30-fold than that of the F87A alone. The roles of the DFSMs were further demonstrated by using mono-functional small molecules (MFSMs) without the terminal imidazolyl group or acyl amino acid group, the latter didn’t improve TON and even inhibit the reactions. The ability of the DFSMs to generate peroxygenase activity was further demonstrated by using the double mutant F87A/T268V. The authors found that mutating the highly conserved T268 [69–73] abolished the H$_2$O$_2$ activity of the enzyme, which can be recovered upon the addition of DFSM. This discovery 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).
P450BM3-H2O2 system was capable of running smoothly with a catalytic cycle that was similar to the native UPO peroxygenase under ideal conditions (Figure 3C) [68,69]. This concept was firstly validated by the H2O2-dependent epoxidation of styrene catalyzed by the P450BM3_F87A mutant. The presence of the best DFSM, N-(ω-imidazolyl)hexanoyl-L-phenylalanine (Im-C6-Phe), increased the catalytic turnover number (TON) more than 30-fold than that of the F87A alone. The roles of the DFSMs were further demonstrated by using mono-functional small molecules (MFSMs) without the terminal imidazolyl group or acyl amino acid group, the latter didn’t improve TON and even inhibit the reactions. The ability of the DFSMs to generate peroxygenase activity was further demonstrated by using the double mutant F87A/T268V. The authors found that mutating the highly conserved T268 [69–73] abolished the H2O2 activity of the enzyme, which can be recovered upon the addition of DFSM. This discovery 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 the dual-functional small molecule (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.

The catalytic role and mechanism of DFSMs have been further disclosed by combining structural biology and computational chemistry [74]. To mimic the pre-reaction state of P450-bounded H2O2 and avoid the H2O2-initiated reaction, Jiang et al. skillfully adopted the NH2OH molecule as the analog of H2O2 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 NH2OH at 2.70 Å resolution (PDB ID: 7EGN, Figure 4C). The crystal structure clearly shows that Im-C6-Phe bound to P450BM3 through an 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 with fatty acids (native substrates) or perfluoroacyl amino acids (decoy molecules) [75–77]. 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 a general acid–base catalyst in H$_2$O$_2$ activation, consistent with the original hypothesis by Ma et al. [66].

The mechanism for H$_2$O$_2$ 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 [74]. The DFSM facilitates the formation of a proton channel between the imidazolyl group of the DFSM and proximal H of H$_2$O$_2$, thus enabling a heterolytic O–O cleavage and Cpd I formation, which is similar to the proposed mechanism for H$_2$O$_2$ 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$_2$O$_2$ activation by the P450cam T252A mutant [78], indicating weak H$_2$O$_2$ activation by NADPH-dependent P450s.

**Figure 4.** Structural basis of the DFSM-facilitated P450 peroxygenase. (A) Proposed pre-reaction state of P450BM3 in the presence of H$_2$O$_2$ and DFSM. (B) The model structure with NH$_2$OH instead of H$_2$O$_2$. (C) The co-crystal structure of P450BM3_F87A in complex with NH$_2$OH and Im-C6-Phe. (D) The binding interactions of Im-C6-Phe with P450BM3.
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 [79–86]. Moreover, peroxygenase that uses green and economic \( \text{H}_2\text{O}_2 \) 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 [79]. Despite concerns about the potential damage of \( \text{H}_2\text{O}_2 \) to enzymes, the use of a controlled fed-batch reactor or in situ-generating \( \text{H}_2\text{O}_2 \) has been demonstrated to enhance effectively the stability of peroxygenases through control of the \( \text{H}_2\text{O}_2 \) concentration in the reaction system, resulting in high catalytic turnovers [87–91]. 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-\( \text{H}_2\text{O}_2 \) system has shown versatile unique catalytic activity towards the peroxgenation reaction of various non-native substrates, such as epoxidation, hydroxylation, and sulfoxidation [66,92–95].

Asymmetric epoxidation of unfunctionalized olefins represents an important organic transformation to prepare optically pure epoxides [96–99]; however, 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 [100–108]. DFSM-facilitated P450BM3 peroxygenase enabled access to (R)-enantioselective epoxidation of unfunctionalized styrene and its derivatives (Figure 5). In view of the potential of the double mutant F87A/T268V in the (R)-enantioselective epoxidation of styrene in the presence of Im-C6-Phe, Zhao et al. systematically evaluated the effect of T268 residue and disclosed the roles of the T268 mutation in tuning activity and enantioselectivity of the NAD(P)H- and \( \text{H}_2\text{O}_2 \)-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 determined to give high (R)-enantioselective epoxidation of styrene (98% ee) with >4000 TONs (Figure 5B). This approach also gave modest to very good TONs (362–3480) and high (R)-enantioselectivities (95–99% ee) for the epoxidation of various styrene derivatives (Figure 5C), being comparable with the best (R)-enantioselective styrene monooxygenases, such as SeStyA from *Streptomyces exfoliatus*, AaStyA from *Amycolatopsis albispora*, and PbStyA from *Pseudonocardia* reported recently [109,110]. The further semi-preparative scale experiments suggest its potential application in styrene epoxidation [92].

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 [111,112]. Natural oxidizing enzymes, such as methane monooxygenase, soluble butane monooxygenase (sBMO), fungal peroxygenase (AaeUPO), and engineered P450s, are promising biocatalysts for the selective hydroxylation of small alkanes [76,77,93,113–123]. Recently, Chen et al. reported the peroxide-driven hydroxylation of small alkanes (C\(_3\)–C\(_6\)) by using engineered P450BM3 variants assisted by DFSMs [93]. Compared with some main results through enzymatic hydroxylation of small alkanes [116–124], DFSM-facilitated P450BM3 peroxygenase showed unique features and catalytic activities (Table 1). The hydrophobic mutation of T268 residue substantially improved the hydroxylation activities of small alkanes, which is distinct from NADPH-dependent P450 enzymes [94]. Here, the presence of the DFSM was critical for accomplishing the catalytic functions of engineered P450BM3 variants because the 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 AaeUPO, the only known \( \text{H}_2\text{O}_2 \)-dependent native hydroxylase for small alkanes (entries 20–21 in Table 1) [116], and
comparable activity to the P450BM3 decoy system (entries 3–8 in Table 1) [117–120], but far lower than P450PMO R1 and P450PMO R2, two evolved NADPH-dependent propane monooxygenases (entries 13–14 in Table 1) [121]. 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) [76,77,118,122,123]. Anyhow, Ciuffetti et al. reported that CYP52L1 from Graphium sp. ATCC 58,400 can oxidize propane, but without any turnover numbers or catalytic constants mentioned [124]. 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.

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.
Table 1. Catalytic hydroxylation of small alkanes by various enzymes in literature.

| Entry | Enzyme                      | Alkanes       | Final Product | PFR  a | TTN b | Ref. |
|-------|-----------------------------|---------------|---------------|--------|-------|------|
| 1     | BM3_F87A/T268I/A184I/Im-C6-Phe | Propane       | 2-Propanol    | 630    | 1775  | [93] |
| 2     | BM3_F87A/T268I/A82T/Im-C6-Phe | n-Butane      | 2-Butanol     | 1042   | 2253  | [93] |
| 3     | BM3/PFC10                    | Propane       | 2-Propanol    | 70     | 700   | [117]|
| 4     | BM3/PFC9-L-Leu               | Propane       | 2-Propanol    | 256    | 2560  | [76] |
| 5     | BM3/3CCPA-Pip-Phe            | Propane       | 2-Propanol    | 615    | -     | [118]|
| 6     | BM3/PFC9                     | n-Butane      | 2-Butanol     | 110    | 1100  | [120]|
| 7     | BM3/PFC11                    | Propane       | 2-Propanol    | -      | 1021  | [120]|
| 8     | BM3/PFC7                     | n-Butane      | 2-Butanol     | -      | 3632  | [120]|
| 9     | BM3/C7AM-Pip-Phe             | Ethane        | Ethanol       | 82.7   | -     | [118]|
| 10    | P450cam_EB                   | n-Butane      | 2-Butanol     | 520    | -     | [123]|
| 11    | P450cam_EB_L294M/T185M/L1358P/G248A | Propane     | 2-Propanol    | 455    | 35,600 | [121]|
| 12    | P450cam_EB_L294M/T185M/L1358P/G248A | Ethane       | Ethanol       | 78.2   | -     | [123]|
| 13    | P450 PMC_R1                  | Propane       | 2-Propanol    | 370    | 45,800 | [121]|
| 14    | P450 PMC_R2                  | Propane       | 2-Propanol    | -      | -     | [124]|
| 15    | CYP52L 1                     | Propane       | 1-Propanol    | -      | -     | [124]|
| 16    | sMMO                         | Methane       | Methanol      | 78     | -     | [122]|
| 17    | sMMO                         | Ethane        | Ethanol       | 45.6   | -     | [122]|
| 18    | sMMO                         | Propane       | 2-Butanol     | 33–58.8 | -     | [122]|
| 19    | sMMO                         | n-Butane      | 2-Butanol     | 7.2–28.8 | -     | [122]|
| 20    | AaeUPO                       | Propane       | 2-Propanol    | 17     | 999   | [116]|
| 21    | AaeUPO                       | n-Butane      | 2-Butanol     | 21     | 1258  | [116]|

- PFR: product formation rate in \( \mu \text{mol} \cdot \text{min}^{-1} \cdot (\text{\mu \text{mol P450}})^{-1} \).
- TTN: total turnover number.
- There is no catalytic turnover data reported.

The O-demethylation of aromatic ethers is of important reaction to produce value-added phenolic compounds, which is also involved in aromatic ring-opening reactions of coniferyl and sinapyl lignin derivatives [125,126]. 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 [127–130]. A few P450 enzymes also show promise as an O-demethylase for lignin-derived aromatic ethers [131–136]. Recently, Jiang et al. successfully applied the DFSM-facilitated P450BM3 peroxygenase system to perform O-demethylation of various aromatic ether substrates (Table 2) [94]. 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.
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 [137–139]. P450s have been well documented to carry out multiple catalytic functions such as monooxygenase, peroxygenase, and peroxidase activity [140]. However, research interest has focused on the monooxygenase and peroxygenase activities of P450s, and only a handful of studies have examined the catalytic peroxygenase functionality of P450s. The non-natural DFSM-facilitated P450-H₂O₂ system described above mainly catalyzes various per-oxygenation reactions, including epoxidation, hydroxylation, and sulfoxidation [66,92–95]. Interestingly, the oxidation of guaiacol, a classical substrate of peroxidases [141–144], catalyzed by the DFSM-facilitated P450BM3-H₂O₂ system yielded demethylated catechol as a major product, suggesting...
it mainly functioned as a peroxynzyme but not as a peroxidase [94]. After carefully analyzing the catalytic mechanism of the potential competitive oxidation pathways in the DFSM-facilitated P450BM3-H2O2 system, Ma et al. hypothesized that mutation of redox-sensitive residues may enable switching of peroxynzyme activity to peroxidase activity [145]. Using a semi-rational design approach, similar to FRISM (focused rational iterative site-specific mutagenesis) named by Reetz and Wu [146,147], 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 peroxynzyme activity for these substrates. Notably, this system attains the best peroxidase activity of any P450 reported [56,148], and rivals most natural peroxidases [149–153], suggesting significant potential for catalytic promiscuity of the DFSM-facilitated P450BM3-H2O2 system (Figure 7). Future efforts should explore the functional applications of the DFSM-facilitated P450 peroxidase in synthetic chemistry.

Figure 7. Native monooxygenase activity. DFSM-facilitated peroxynzyme 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 peroxynzymes from Sphingomonas paucimobilis, Bacillus subtilis, and Clostridium acetobutylicum can directly use an oxygen atom from peroxides for oxidation reactions [42–47], the engineered artificial P450 peroxynzymes have significantly expanded the substrate scope and reaction types of P450-catalyzed per-oxynzyme reactions. Therefore, it is no exaggeration to state that H2O2-driven P450 peroxynzymes are emerging as powerful bio-oxidation catalysts. Among these, the DFSM-facilitated P450 peroxynzymes provide a novel and unique solution for the efficient use of H2O2 by P450s, which exhibit much higher H2O2 activities in various reactions when compared with those P450 peroxynzymes that have been engineered through site-directed mutagenesis and directed evolution [26–28,56,63,64,77,152,153]. Moreover, the DFSM-facilitated P450 peroxynzymes 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 H2O2, which still requires further experimental characterization. On the other hand, the highly conserved T268 residue can be optionally mutated in the DFSM-facilitated P450 peroxynzyme system. In contrast, the mutation of T268 is not favorable in NADPH-dependent P450BM3 oxidation because this residue is located on the proximal side of the heme center and is thought to play multiple roles in NADPH-dependent catalysis [70–73,154].
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 peroxygenase activity of the DFSM-facilitated P450-system developed recently expanded the catalytic promiscuity of the system [145], whose further application in organic transformation is expected.

The unique selectivity and activity of the DFSM-facilitated P450 peroxygenase system have shown its potential to be as a promising bio-oxidative catalyst; however, it is worth noting that there are still some drawbacks to hamper its further industrially utilization: (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 H2O2; (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 full catalytic mechanism still 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.

Author Contributions: Conceptualization and funding acquisition, Z.C.; writing—original draft preparation, review and editing, S.D., S.F., F.J. and Z.C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China, grant number 21977104 and 21778060; the Qingdao Innovative Leading Talent Project, grant number 18-1-2-9-zhc; QIBEBT, grant number I201901 and Y872361901, and Director Innovation Fund of Key Laboratory of Biofuels, Chinese Academy of Sciences, grant number Y872131901.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Ortiz de Montellano, P.R.O. *Cytochrome P450: Structure, Mechanism, and Biochemistry*, 3rd ed.; Kluwer Academic: New York, NY, USA; Plenum Publishers: New York, NY, USA, 2005.
2. Nelson, D.R. Cytochrome P450 diversity in the tree of life. *Biochim. Biophys. Acta. Proteins Proteom.* 2018, 1866, 141–154. [CrossRef] [PubMed]
3. Bernhardt, R.; Urlacher, V.B. Cytochromes P450 as promising catalysts for biotechnological application: Chances and limitations. *Appl. Microbiol. Biotechn.* 2014, 98, 6185–6203. [CrossRef] [PubMed]
4. Fasan, R. Tuning P450 Enzymes as Oxidation Catalysts. *ACS Catal.* 2012, 2, 647–666. [CrossRef]
5. de Montellano, P.R.O. Hydrocarbon Hydroxylation by Cytochrome P450 Enzymes. *Chem. Rev.* 2010, 110, 932–948. [CrossRef]
6. Auclair, K.; Polic, V. *Monooxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450*; Hrycay, E.G., Bandiera, S.M., Eds.; Springer: New York, NY, USA, 2015; pp. 209–228.
7. Nastri, F.; Chino, M.; Maglio, O.; Bhagi-Damodaran, A.; Lu, Y.; Lombardi, A. Design and engineering of artificial oxygen-activating metalloenzymes. *Chem. Soc. Rev.* 2016, 45, 5020–5054. [CrossRef]
8. Wang, T.T.; Fan, X.T.; Hou, C.X.; Liu, J.Q. Design of artificial enzymes by supramolecular strategies. *Curr. Opin. Struct. Biol.* 2018, 51, 19–27. [CrossRef]
9. Wei, Y.F.; Ang, E.L.; Zhao, H.M. Recent developments in the application of P450 based biocatalysts. *Curr. Opin. Chem. Biol.* 2018, 43, 1–7. [CrossRef]
10. McIntosh, J.A.; Farwell, C.C.; Arnold, F.H. Expanding P450 catalytic reaction space through evolution and engineering. *Curr. Opin. Chem. Biol.* 2014, 19, 126–134. [CrossRef]
11. Zhang, X.W.; Li, S.Y. Expansion of chemical space for natural products by uncommon P450 reactions. *Nat. Prod. Rep.* 2017, 34, 1061–1089. [CrossRef]
12. Kumar, S.; Zhao, Y.; Sun, L.; Negi, S.S.; Halpert, J.R.; Muralidhara, B.K. Rational engineering of human cytochrome P450 2B6 for enhanced expression and stability: Importance of a Leu264 -> Phe substitution. *Mol. Pharmacol.* 2007, 72, 1191–1199. [CrossRef]
13. Acevedo-Rocha, C.G.; Gamble, C.G.; Lonsdale, R.; Li, A.; Nett, N.; Hoebenreich, S.; Lingnau, J.B.; Wirtz, C.; Fares, C.; Hinrichs, H.; et al. P450-Catalyzed Regio- and Diastereoselective Steroid Hydroxylation: Efficient Directed Evolution Enabled by Mutability Landscaping. *ACS Catal.* 2018, 8, 3395–3410. [CrossRef]
14. Zhang, X.; Peng, Y.; Zhao, J.; Li, Q.; Yu, X.; Acevedo-Rocha, C.G.; Li, A. Bacterial cytochrome P450-catalyzed regio- and stereoselective steroid hydroxylation enabled by directed evolution and rational design. *Bioresour. Bioprocess.* 2020, 7, 2. [CrossRef]
15. Braunegg, G.; de Raadt, A.; Feichtenhofer, S.; Griengl, H.; Kopper, I.I.; Lehmann, A.; Weber, H.J. The Concept of Docking/Protecting Groups in Biohydroxylation. *Angew. Chem.* 1999, 38, 2763–2766. [CrossRef]
16. de Raadt, A.; Griengl, H.; Weber, H. The concept of docking and protecting groups in biohydroxylation. *Chem. Eur. J.* 2001, 7, 27–31. [CrossRef]
17. Munzer, D.F.; Meinhold, P.; Peters, M.W.; Feichtenhofer, S.; Griengl, H.; Arnold, F.H.; Glieder, A.; de Raadt, A. Stereoselective hydroxylation of an achiral cyclopentane-carboxylic acid derivative using engineered P450s BM-3. *Chem. Commun.* 2005, 20, 2597–2599. [CrossRef]
18. Landwehr, M.; Hochrein, L.; Otey, C.R.; Kasravan, A.; Bäckvall, J.-E.; Arnold, F.H. Enantioselective α-Hydroxylation of 2-Arylacetic Acid Derivatives and Bupiprone Catalyzed by Engineered Cytochrome P450 BM-3. *J. Am. Chem. Soc.* 2006, 128, 6058–6059. [CrossRef]
19. Narayan, A.R.H.; Jimenez-Oses, G.; Liu, P.; Negretti, S.; Zhao, W.X.; Gilbert, M.M.; Ramabhadran, R.O.; Yang, Y.F.; Furan, L.R.; Li, Z.; et al. Enzymatic hydroxylation of an unactivated methylene C-H bond guided by molecular dynamics simulations. *Nat. Chem.* 2015, 7, 653–660. [CrossRef]
20. Li, S.Y.; Chaulagain, M.R.; Krauff, A.R.; Podust, L.M.; Montgomery, J.; Sherman, D.H. Selective oxidation of carboyl C-H bonds by an engineered macrolide P450 mono-oxygenase. *Proc. Natl. Acad. Sci. USA* 2009, 106, 18463–18468. [CrossRef]
21. Negretti, S.; Narayan, A.R.; Chiu, K.C.; Kells, P.M.; Stachowski, J.L.; Hansen, D.A.; Podust, L.M.; Montgomery, J.; Sherman, D.H. Directing Group-Controlled Regioselectivity in an Enzymatic C-H Bond Oxygenation. *J. Am. Chem. Soc.* 2014, 136, 4901–4904. [CrossRef]
22. Larsen, A.T.; May, E.M.; Auclair, K. Predictable Stereoselective and Chemoselective Hydroxylations and Epoxidations with P450 3A4. *J. Am. Chem. Soc.* 2011, 133, 7853–7858. [CrossRef]
23. Polic, V.; Cheong, K.J.; Hammerer, F.; Auclair, K. Regioselective Epoxidations by Cytochrome P450 3A4 Using a Theobromine Chemical Auxiliary to Predictably Produce N-Protected β- or γ-Amino Epoxides. *Adv. Synth. Catal.* 2017, 359, 3983–3989. [CrossRef]
24. Polic, V.; Auclair, K. Controlling substrate specificity and product regio- and stereo-selectivities of P450 enzymes without mutagenesis. *Bioorg. Med. Chem.* 2014, 22, 5547–5554. [CrossRef] [PubMed]
25. Shoji, O.; Watanabe, Y. Bringing out the Potential of Wild-type Cytochrome P450s Using Decoy Molecules: Oxygenation of Nonnative Substrates by Bacterial Cytochrome P450s. *Isr. J. Chem.* 2015, 55, 32–39. [CrossRef]
26. Shoji, O.; Watanabe, Y. Monooxygenation of Nonnative Substrates Catalyzed by Bacterial Cytochrome P450s Facilitated by Decoy Molecules. *Chem. Lett.* 2017, 46, 278–288. [CrossRef]
27. Shoji, O.; Aiba, Y.; Watanabe, Y. Hoodwinking Cytochrome P450BM-3 into Hydroxylation Non-Native Substrates by Exploiting Its Substrate Misrecognition. *Acc. Chem. Res.* 2019, 52, 925–934. [CrossRef]
28. Hara, M.; Ohkawa, H.; Narato, M.; Shirai, M.; Asada, Y.; Karube, I.; Miyake, J. Regeneration of NADPH by cactus chloroplasts: A case for spin-selective reactivity. *Chem. Eur. J.* 2007, 13, 4103–4115. [CrossRef] [PubMed]
29. Lee, S.H.; Kwon, Y.C.; Kim, D.M.; Park, C.B. Cytochrome P450-catalyzed O-dealkylation coupled with photochemical NADPH regeneration. *Proc. Natl. Acad. Sci. USA* 2009, 106, 18463–18468. [CrossRef]
30. Shoji, O.; Watanabe, Y. Monooxygenation of Nonnative Substrates Catalyzed by Bacterial Cytochrome P450s Facilitated by Decoy Molecules. *Chem. Lett.* 2017, 46, 278–288. [CrossRef]
31. Wang, X.; Chen, J.; Zhou, L.; Cong, Z. Progress in cytochrome P450 monooxygenase driven by hydrogen peroxide. *Biot. Resour.* 2017, 39, 75–84.
32. Hiiroya, K.; Murakami, Y.; Shishido, T.; Hatano, M.; Demontellano, P.R.O. Differential roles of Glu318 and Thr319 in cytochrome P450 P4501A2 catalysis supported by NADPH-cytochrome P450 reductase and tert-butyl hydroperoxide. *Arch. Biochem. Biophys.* 1994, 310, 397–401. [CrossRef]
33. Cho, K.B.; Moreau, Y.; Kumar, D.; Rock, D.A.; Jones, J.P.; Shaik, S. Formation of the active species of cytochrome p450 by using tolylbenzene: A case for spin-selective reactivity. *Chem. Eur. J.* 2007, 13, 4103–4115. [CrossRef] [PubMed]
34. Dunham, N.P.; Arnold, F.H. Nature’s Machinery, Repurposed: Expanding the Repertoire of Iron-Dependent Oxygenases. *ACS Catal.* 2020, 10, 12239–12255. [CrossRef] [PubMed]
35. Xu, J.; Wang, C.; Cong, Z. Strategies for Substrate-Regulated P450 Catalysis: From Substrate Engineering to Co-catalysis. *Chem. Eur. J.* 2019, 25, 6853–6863. [CrossRef]
36. Shoji, O.; Watanabe, Y. Peroxyreactions catalyzed by cytochromes P450. *J. Biol. Inorg. Chem.* 2014, 19, 529–539. [CrossRef]
37. Zhang, L.B.; Wang, Q. Harnessing P450 Enzyme for Biotechnology and Synthetic Biology. *ChemBioChem* 2022, 23, e202100439. [CrossRef] [PubMed]
38. Uralcher, V.B.; Girhard, M. Cytochrome P450 Monooxygenases in Biotechnology and Synthetic Biology. *Trends Biotechnol.* 2019, 37, 882–897. [CrossRef]
39. Munro, A.W.; McLean, K.J.; Grant, J.L.; Makris, T.M. Structure and function of the cytochrome P450 peroxidase enzymes. *Biochem. Soc. Trans.* 2018, 46, 183–196. [CrossRef]
40. Bornscheuer, U.T.; Pohl, M. Improved biocatalysts by directed evolution and rational protein design. *Curr. Opin. Chem. Biol.* **2001**, *5*, 137–143. [CrossRef]
41. Wang, X.D.; Saba, T.; Yiu, H.H.P.; Howe, R.E.; Anderson, J.A.; Shi, J.F. Cofactor NAD(P)H Regeneration Inspired by Heterogeneous Pathways. *Chem. 2017*, *2*, 621–654. [CrossRef]
42. Lee, D.S.; Yamada, A.; Sugimoto, H.; Matsunaga, I.; Ogura, H.; Ichihara, K.; Adachi, S.; Park, S.Y.; Shiro, Y. Substrate recognition and molecular mechanism of fatty acid hydroxylation by cytochrome P450 from Bacillus subtilis. Crystallographic, spectroscopic, and mutational studies. *J. Biol. Chem.** 1993*, *278*, 9761–9767. [CrossRef]
43. Matsunaga, I.; Yokotani, N.; Gotoh, O.; Kusunose, E.; Yamada, M.; Ichihara, K. Molecular Cloning and Expression of Fatty Acid α-Hydroxylase from Sphingomonas paucimobilis. *J. Biol. Chem.** 1997*, *272*, 23592–23596. [CrossRef] [PubMed]
44. Fujishiro, T.; Shoji, O.; Nagano, S.; Sugimoto, H.; Shiro, Y.; Watanabe, Y. Crystal structure of H_{2}O_{2}-dependent cytochrome P450Swp alpha with its bound fatty acid substrate: Insight into the regioselective hydroxylation of fatty acids at the alpha position. *J. Biol. Chem.** 2011*, *286*, 29941–29950. [CrossRef] [PubMed]
45. Goyal, S.; Banerjee, S.; Mazumdar, S. Oxygenation of monoenoic fatty acids by CYP175A1, an orphan cytochrome P450 from *Lactobacillus plantarum* WCFS1. *Bull. Chem. Soc. Jpn.** 2012*, *85*, 209–216. [CrossRef]
46. Markel, U.; Lanvers, P.; Sauer, D.F.; Wittwer, M.; Dhoke, G.V.; Davari, M.D.; Schiffs, J.; Schwaneberg, U. A Photoclick-Based High-Throughput Screening for the Directed Evolution of Decarboxylase OleT. *Chem. Eur. J.** 2021*, *27*, 954–958. [CrossRef] [PubMed]
47. Girhard, M.; Schuster, S.; Dietrich, M.; Duerr, P.; Uhracher, V.B. Cytochrome P450 P450 monoxygenase from Clostridium acetobutylicum: A new alpha-fatty acid hydroxylase. *Biochem. Biophys. Res. Commun.** 2007*, *362*, 114–119. [CrossRef] [PubMed]
48. Hrycay, E.G.; Gustafsson, J.A.; Ingelmannsundberg, M.; Ernster, L. Sodium periodate, sodium chlorite, organic hydroperoxides, and H2O2 as hydroxylating agents in steroid hydroxylation reactions catalyzed by partially purified cytochrome P-450. *Biochem. Biophys. Res. Commun.** 1975*, *66*, 209–216. [CrossRef]
49. Anari, M.R.; Josephy, P.D.; Henry, T.; Obrien, P.J. Hydrogen peroxide supports human and rat cytochrome P450 1A2-catalyzed 2-amino-3-methylindazo 4,5-t quinoline bioactivation to mutagenic metabolites: Significance of cytochrome P-450 peroxides. *Chem. Res. Toxicol.** 1997*, *10*, 582–588. [CrossRef]
50. Zhang, Z.P.; Li, Y.; Stearns, R.A.; de Montellano, P.R.O.; Baillie, T.A.; Tang, W. Cytochrome P450 3A4-mediated oxidative conversion of a cyano to an amide group in the metabolism of pinacilid. *Biochemistry** 2002*, *41*, 2712–2718. [CrossRef]
51. Rabe, K.S.; Kiko, K.; Niemeyer, C.M. Characterization of the peroxidase activity of CYP119, a thermostable P450 from Sulfolobus acidocaldarius. *Chembiochem** 2008*, *9*, 420–425. [CrossRef]
52. Goyal, S.; Banerjee, S.; Mazumdar, S. Oxygenation of monoenoic fatty acids by CYP175A1, an orphan cytochrome P450 from *Thermus thermophilus* HB827. *Biochemistry** 2012*, *51*, 7880–7890. [CrossRef]
53. Gelb, M.H.; Heimbrock, D.C.; Malkonen, P.; Sligar, S.G. Stereochemistry and deuterium isotope effects in camphor hydroxylation by the cytochrome P450cam monooxygenase system. *Biochemistry** 1982*, *21*, 370–377. [CrossRef]
54. Cirino, P.C.; Arnold, F.H. A Self-Sufficient Peroxide-Driven Hydroxylation Biocatalyst. *Angew. Chem. Int. Ed.** 2003*, *42*, 3299–3301. [CrossRef] [PubMed]
55. Kumar, S.; Chen, C.S.; Waxman, D.J.; Halpert, J.R. Directed evolution of mammalian cytochrome P4502B1. *J. Biol. Chem.** 2005*, *280*, 19569–19575. [CrossRef] [PubMed]
56. Behera, R.K.; Goyal, S.; Mazumdar, S. Modification of the heme active site to increase the peroxidase activity of thermophilic cytochrome P450: A rational approach. *J. Inorg. Biochem.** 2010*, *104*, 1185–1194. [CrossRef] [PubMed]
57. Joo, H.; Lin, Z.L.; Arnold, F.H. Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation. *Nature** 1999*, *399*, 670–673. [CrossRef]
58. Gajhede, M.; Schuller, D.J.; Henrikson, A.; Smith, A.T.; Poulos, T.L. Crystal structure of horseradish peroxidase C at 2.15 angstrom resolution. *Nat. Struct. Biol.** 1997*, *4*, 1032–1038. [CrossRef]
59. Piuntke, K.; Strittmatter, E.; Ullrich, R.; Grobe, G.; Pecyna, M.J.; Kluge, M.; Scheibner, K.; Hofrichter, M.; Plattner, D.A. Structural basis of substrate conversion in a new aromatic peroxidase: Cytochrome P450 functionality with benefits. *J. Biol. Chem.** 2013*, *288*, 34767–34776. [CrossRef]
60. Ozaki, S.I.; Roach, M.P.; Matsui, T.; Watanabe, Y. Investigations of the roles of the distal heme environment and the proximal heme iron ligand in peroxide activation by heme enzymes via molecular engineering of myoglobin. *Acc. Chem. Res.** 2001*, *34*, 818–825. [CrossRef]
61. Watanabe, Y.; Ueno, T. Introduction of P450, Peroxidase, and Catalase Activities into Myoglobin by Site-Directed Mutagenesis: Diverse Reactivities of Compound I. *Bull. Chem. Soc. Jpn.** 2003*, *76*, 1309–1322. [CrossRef] [PubMed]
90. Choi, D.S.; Lee, H.; Tieves, F.; Lee, Y.W.; Son, E.J.; Zhang, W.; Shin, B.; Hollmann, F.; Park, C.B. Bias-Free in situ H$_2$O$_2$ Generation in a Photovoltaic-Photoelectrochemical Tandem Cell for Biocatalytic Oxyfunctionalization. *ACS Catal.* 2019, 9, 10562–10566. [CrossRef]

91. Yayci, A.; Baraibar, A.G.; Krewing, M.; Fueyo, E.F.; Hollmann, F.; Alcalde, M.; Kourist, R.; Bandow, J.E. Plasma-Driven in situ Production of Hydrogen Peroxide for Biocatalysis. *ChemSusChem* 2020, 13, 2072–2079. [CrossRef]

92. Zhao, P.; Chen, J.; Ma, N.; Chen, J.; Qin, X.; Liu, C.; Yao, F.; Yao, L.; Jin, L.; Cong, Z. Enabling highly (R)-enantioselective epoxidation of styrene by engineering unique non-natural P450 peroxogenases. *Chem. Sci.* 2021, 12, 6307–6314. [CrossRef]

93. Chen, Z.; Chen, J.; Ma, N.; Zhou, H.; Cong, Z. Peroxide-Driven Hydroxylation of Small Alkanes Catalyzed by an Artificial P450BM3 Peroxygenase System. *ACS Catal.* 2019, 9, 7350–7355. [CrossRef]

94. Cui, C.; Guo, C.; Lin, H.; Ding, Z.Y.; Wu, Z.L. Functional characterization of an (R)-selective styrene monooxygenase from *Thauera butanivorans*, formerly *Pseudomonas butanovora*. *J. Porphyr. Phthalocyanines*. 2018, 22, 831–836. [CrossRef]

95. Yu, D.; Wang, J.B.; Reetz, M.T. Exploiting Designed Oxidase-Peroxygenase Mutual Benefit System for Asymmetric Cascade Reactions. *J. Am. Chem. Soc.* 2019, 141, 5655–5658. [CrossRef] [PubMed]

96. Heine, T.; Scholtissek, A.; Hofmann, S.; Koch, R.; Tischler, D. Accessing Enantiopure Epoxides and Sulfoxides: Related Flavin-Dependent Monooxygenases Provide Reversed Enantioselectivity. *ChemCatChem* 2020, 12, 199–209. [CrossRef]

97. Lin, H.; Tang, Y.; Dong, S.; Lang, R.; Chen, H. A new monooxygenase from *Herbaspirillum huttiense* catalyzed highly enantioselective epoxidation of allylbenzenes and allylic alcohols. *Catal. Sci. Technol.* 2020, 10, 2145–2151. [CrossRef] [PubMed]

98. van Schie, M.; Paul, C.E.; Arends, I.; Hollmann, F. Photoenzymatic epoxidation of styrenes. *Chem. Commun.* 2019, 55, 1790–1792. [CrossRef]

99. Cui, C.; Guo, C.; Lin, H.; Ding, Z.Y.; Wu, Z.L. Functional characterization of an (R)-selective styrene monooxygenase from streptomycetes sp. NRRL S-31. *Enzym. Microb. Technol.* 2019, 132, 109391. [CrossRef]

100. Luo, Y.R. *Handbook of Bond Dissociation Energies in Organic Compounds*; CRC Press: Boca Raton, FL, USA, 2002.

101. Cooley, R.B.; Dubbel, B.L.; Sayavedra-Soto, L.A.; Bottomley, P.J.; Arp, D.J. Kinetic characterization of the soluble butane monooxygenase from *Thauera butanivorans*, formerly *Pseudomonas butanovora*. *Microbiology* 2009, 155, 2086–2096. [CrossRef] [PubMed]

102. Kawakami, N.; Shoji, O.; Watanabe, Y. Use of Perfluorocarboxylic Acids to Trick Cytochrome P450BM3 into Initiating the Hydroxylation of Gaseous Alkanes. *Angew. Chem. Int. Ed.* 2011, 50, 5315–5318. [CrossRef] [PubMed]
118. Yonemura, K.; Ariyasu, S.; Stanfield, J.K.; Suzuki, K.; Onoda, H.; Kasai, C.; Sugimoto, H.; Aiba, Y.; Watanabe, Y.; Shoji, O. Systematic Evolution of Decoy Molecules for the Highly Efficient Hydroxylation of Benzene and Small Alkanes Catalyzed by Wild-Type Cytochrome P450BM3. ACS Catal. 2020, 10, 9136–9144. [CrossRef]

119. Ariyasu, S.; Kodama, Y.; Kasai, C.; Cong, Z.; Stanfield, J.K.; Aiba, Y.; Watanabe, Y.; Shoji, O. Development of a High-Pressure Reactor Based on Liquid-Flow Pressurisation to Facilitate Enzymatic Hydroxylation of Gaseous Alkanes. ChemCatChem 2019, 11, 4709–4714. [CrossRef]

120. Zilly, F.E.; Acevedo, J.P.; Augustyniak, W.; Deege, A.; Haeusig, U.W.; Reetz, M.T. Tuning a P450 Enzyme for Methane Oxidation. Angew. Chem. Int. Ed. 2011, 50, 2720–2724. [CrossRef]

121. Patel, R.N.; Hou, C.T.; Laskin, A.I.; Felix, A. Microbial Oxidation of Hydrocarbons: Properties of a Soluble Methane Monoxygenase from a Facultative Methane-Utlizing Organism, Methylobacterium sp. Strain CRL-26. Appl. Environ. Microbiol. 1982, 44, 1130–1137. [CrossRef]

122. Trippe, K.M.; Wolpert, T.J.; Hyman, M.R.; Ciuffettì, L.M. RNAi silencing of a cytochrome P450 monoxygenase disrupts the ability of a filamentous fungus, Graphium sp., to grow on short-chain gaseous alkanes and ethers. Biodegradation 2014, 25, 137–151. [CrossRef]

123. Gazi, S. Valorization of wood biomass-lignin via selective bond scission: A minireview. Appl. Catal. B Environ. 2019, 257, 117936. [CrossRef]

124. Trippe, K.M.; Wolpert, T.J.; Hyman, M.R.; Ciuffettì, L.M. RNAi silencing of a cytochrome P450 monoxygenase disrupts the ability of a filamentous fungus, Graphium sp., to grow on short-chain gaseous alkanes and ethers. Biodegradation 2014, 25, 137–151. [CrossRef]

125. Fasan, R.; Chen, M.M.; Crook, N.C.; Arnold, F.H. Engineered alkane-hydroxylating cytochrome P450(BM3) exhibiting nativelike catalytic properties. Angew. Chem. Int. Ed. 2007, 46, 8414–8418. [CrossRef]

126. Venkatesagowda, B. Enzymatic demethylation of lignin for potential biobased polymer applications. JACS Au 2021, 13, 1057–1066. [CrossRef] [PubMed]

127. Lanfranchi, E.; Trajkovic, M.; Barta, K.; de Vries, J.G.; Janssen, D.B. Exploring the Selective Demethylation of Aryl Methyl Ethers. Biogenetics 2017, 9, 2487. [CrossRef]

128. Chan, J.C.; Paice, M.; Zhang, X. Enzymatic Oxidation of Lignin: Challenges and Barriers toward Practical Applications. ChemCatChem 2020, 12, 401–425. [CrossRef]

129. Venkatasegowda, B. Enzymatic demethylation of lignin for potential biobased polymer applications. Fungal Biol. Rev. 2019, 33, 190–224. [CrossRef]

130. Richter, N.; Zepeck, F.; Kroutil, W. Cobalamin-dependent enzymatic O-, N-, and S-demethylation. Trends Biotechnol. 2015, 33, 371–373. [CrossRef]

131. Lanfranchi, E.; Trajkovic, M.; Barta, K.; de Vries, J.G.; Janssen, D.B. Exploring the Selective Demethylation of Aryl Methyl Ethers with a Pseudomonas Rieske Monoxygenase. ChemBioChem 2019, 20, 118–125. [CrossRef]

132. Mallinson, S.J.B.; Machovina, M.M.; Silveira, R.L.; Garcia-Borras, M.; Gallup, N.; Johnson, C.W.; Allen, M.D.; Skaf, M.S.; Crowley, M.F.; Neidle, E.L.; et al. A promiscuous cytochrome P450 aromatic O-demethylase for lignin bioconversion. Nat. Commun. 2018, 9, 2487. [CrossRef]

133. Machovina, M.M.; Mallinson, S.J.B.; Knott, B.C.; Meyers, A.W.; Garcia-Borras, M.; Bu, L.; Gado, J.E.; Oliver, A.; Schmidt, G.P.; Hinch, D.J.; et al. Enabling microbial syringol conversion through structure-guided protein engineering. Proc. Natl. Acad. Sci. USA 2019, 116, 13970–13976. [CrossRef]

134. Ellis, E.S.; Hinch, D.J.; Bleem, A.; Bu, L.; Mallinson, S.J.B.; Allen, M.D.; Streit, B.R.; Machovina, M.M.; Doolin, Q.V.; Michener, W.E.; et al. Engineering a Cytochrome P450 for Demethylation of Lignin-Derived Aldehyde Hydrides. JACS Au 2021, 1, 252–261. [CrossRef]

135. Dardas, A.; Gal, D.; Barreille, M.; Sauretignazi, G.; Sterjiades, R.; Pelmont, J. The demethylation of guaiacol by a new bacterial cytochrome P450. Arch. Biochem. Biophys. 1985, 256, 585–592. [CrossRef]

136. Eltis, L.D.; Karlson, U.; Timmis, K.N. Purification and characterization of cytochrome P450RR1 from Rhodococcus rhodochrous. Eur. J. Biochem. 1993, 213, 211–216. [CrossRef] [PubMed]

137. Bell, S.G.; Zhou, R.; Yang, W.; Tan, A.B.H.; Gentleman, A.S.; Wong, L.-L.; Zhou, W. Investigation of the Substrate Range of CYP199A4: Modification of the Partition between Hydroxylation and Desaturation Activities by Substrate and Protein Engineering. Chem. Eur. J. 2012, 18, 16677–16688. [CrossRef] [PubMed]

138. Leveson-Gower, R.B.; Mayer, C.; Roelfes, G. The importance of catalytic promiscuity for enzyme design and evolution. Nat. Rev. Chem. 2019, 3, 687–705. [CrossRef]

139. Sandovol, B.A.; Hyster, T.K. Emerging strategies for expanding the toolbox of enzymes in biocatalysis. Curr. Opin. Chem. Biol. 2020, 55, 45–51. [CrossRef]

140. Bornscheuer, U.T.; Kazlauskas, R.J. Catalytic promiscuity in biocatalysis: Using old enzymes to form new bonds and follow new pathways. Angew. Chem. Int. Ed. 2004, 43, 6032–6040. [CrossRef]

141. Hrycay, E.G.; Bandiera, S.M. The monoxygenase, peroxidase, and peroxygenase properties of cytochrome P450. Arch. Biochem. Biophys. 2012, 522, 71–89. [CrossRef]

142. Zhang, P.; Yuan, H.; Xu, J.K.; Wang, X.J.; Gao, S.Q.; Tan, X.S.; Lin, Y.W. A Catalytic Binding Site Together with a Distal Tyr in Myoglobin Affords Catalytic Efficiencies Similar to Natural Peroxidases. ACS Catal. 2020, 10, 891–896. [CrossRef]
143. Yamada, M.; Hashimoto, Y.; Kumano, T.; Tsujimura, S.; Kobayashi, M. New function of aldoxime dehydratase: Redox catalysis and the formation of an expected product. *PLoS ONE* 2017, 12, e0175846. [CrossRef]

144. Chien, S.C.; Shoji, O.; Morimoto, Y.; Watanabe, Y. Use of apomyoglobin to gently remove heme from a H$_2$O$_2$-dependent cytochrome P450 and allow its reconstitution. *New J. Chem.* 2017, 41, 302–307. [CrossRef]

145. Ma, N.N.; Fang, W.H.; Liu, C.A.F.; Qin, X.Q.; Wang, X.L.; Jin, L.Y.; Wang, B.J.; Cong, Z.Q. Switching an Artificial P450 Peroxygenase into Peroxidase via Mechanism-Guided Protein Engineering. *ACS Catal.* 2021, 11, 8449–8455. [CrossRef]

146. Xu, J.; Cen, Y.X.; Singh, W.; Fan, J.J.; Wu, L.; Lin, X.F.; Zhou, J.H.; Huang, M.L.; Reetz, M.T.; Wu, Q. Stereodivergent Protein Engineering of a Lipase To Access All Possible Stereoisomers of Chiral Esters with Two Stereocenters. *J. Am. Chem. Soc.* 2019, 141, 7934–7945. [CrossRef] [PubMed]

147. Li, D.Y.; Wu, Q.; Reetz, M.T. Focused rational iterative site-specific mutagenesis (FRISM). In *Enzyme Engineering and Evolution: General Methods*; Methods in Enzymology; Tawfik, D.S., Ed.; Academic Press: New York, NY, USA, 2020; Volume 643, pp. 225–242.

148. Vidal-Limon, A.; Aguila, S.; Ayala, M.; Batista, C.V.; Vazquez-Duhalt, R. Peroxidase activity stabilization of cytochrome P450BM3 by rational analysis of intramolecular electron transfer. *J. Inorg. Biochem.* 2013, 122, 18–26. [CrossRef] [PubMed]

149. Morales, M.; Mate, M.J.; Romero, A.; Martinez, M.J.; Martinez, A.T.; Ruiz-Duenas, F.J. Two oxidation sites for low redox potential substrates: A directed mutagenesis, kinetic, and crystallographic study on Pleurotus eryngii versatile peroxidase. *J. Biol. Chem.* 2012, 287, 41053–410675. [CrossRef]

150. Mandelman, D.; Jamal, J.; Poulos, T.L. Identification of two electron-transfer sites in ascorbate peroxidase using chemical modification, enzyme kinetics, and crystallography. *Biochemistry* 1998, 37, 17610–17617. [CrossRef]

151. Murphy, E.J.; Metcalfe, C.L.; Nnamchi, C.; Moody, P.C.E.; Raven, E.L. Crystal structure of guaiacol and phenol bound to a heme peroxidase. *FEBS J.* 2012, 279, 1632–1639. [CrossRef]

152. Li, Q.S.; Ogawa, J.; Shimizu, S. Critical role of the residue size at position 87 in H$_2$O$_2$-dependent substrate hydroxylation activity and H$_2$O$_2$ inactivation of cytochrome P450BM-3. *Biochem. Biophys. Res. Commun.* 2001, 280, 1258–1261. [CrossRef]

153. Hsieh, C.H.; Makris, T.M. Expanding the substrate scope and reactivity of cytochrome P450 OleT. *Biochem. Biophys. Res. Commun.* 2016, 476, 462–466. [CrossRef]

154. Roberts, A.G.; Katayama, J.; Kaspara, R.; Ledwith, K.V.; Trong, I.L.; Stenkamp, R.E.; Thompson, J.A.; Totah, R.A. The role of cytochrome P450 BM3 phenylalanine-87 and threonine-268 in binding organic hydroperoxides. *Biochim. Biophys. Acta (BBA)—Gen. Subj.* 2016, 1860, 669–677. [CrossRef]