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Unlike the excellent (S)-enantioselective epoxidation of styrene performed by natural styrene monooxygenase (ee >99%), the (R)-enantioselective epoxidation of styrene has not yet achieved a comparable efficiency using natural or engineered oxidative enzymes. This report describes the H₂O₂-dependent (R)-enantioselective epoxidation of unfunctionalized styrene and its derivatives by site-mutated variants of a unique non-natural P450BM3 peroxygenase, working in tandem with a dual-functional small molecule (DFSM). The observed (R)-enantiomeric excess of styrene epoxidation is up to ~99%, which is unprecedented relative to natural or engineered oxidative enzymes. The catalytic turnover number is up to ~4500 (with ~98% ee), representing the best activity of a P450 peroxygenase towards styrene epoxidation, to date. This study indicates that the synergistic use of protein engineering and an exogenous DFSM constitutes an efficient strategy to control enantioselectivity of styrene epoxidation, thus substantially expanding the chemical scope of P450 enzyme functions as useful bio-oxidative catalysts.
Enabling Highly \((R)\)-Enantioselective Epoxidation of Styrene by Engineering Unique Non-Natural P450 Peroxygenases

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ABSTRACT: Unlike the excellent \((S)\)-enantioselective epoxidation of styrene performed by natural styrene monooxygenase (\(ee >99\%\)), the \((R)\)-enantioselective epoxidation of styrene has not yet achieved a comparable efficiency using natural or engineered oxidative enzymes. This report describes the \(\text{H}_2\text{O}_2\)-dependent \((R)\)-enantioselective epoxidation of unfunctionalized styrene and its derivatives by site-mutated variants of a unique non-natural P450BM3 peroxygenase, working in tandem with a dual-functional small molecule (DFSM). The observed \((R)\)-enantiomeric excess of styrene epoxidation is up to ~99\%, which is unprecedented relative to natural or engineered oxidative enzymes. The catalytic turnover number is up to ~4500 (with ~98\% \(ee\)), representing the best activity of a P450 peroxygenase towards styrene epoxidation, to date. This study indicates that the synergistic use of protein engineering and an exogenous DFSM constitutes an efficient strategy to control enantioselectivity of styrene epoxidation, thus substantially expanding the chemical scope of P450 enzyme functions as useful bio-oxidative catalysts.

Optically-pure styrene oxides are extremely useful building blocks for synthesizing chiral organic compounds. The asymmetric epoxidation of styrenes represents one of the most direct ways to prepare such compounds, thus, it has attracted significant attention in recent years.\(^1\) However, despite critical progress in the development of asymmetric epoxidations based on synthetic molecular catalysts or biocatalysts, there is limited understanding regarding the highly-enantioselective epoxidation of terminal olefins, including styrene. Most catalytic systems demonstrate excellent enantioselective excess (\(ee\)) for \((S)\)-styrene oxide generation, but not for the \((R)\)-configuration.\(^2\) For example, the proline-derived C1-symmetric salen ligand with a Ti(OiPr)\(_4\) catalyst effectively converted several styrene derivatives to \((S)\)-epoxides with 96\%–98\% \(ee\).\(^2\) The famous Jacobsen catalyst only generated 57\% \(ee\) for \((R)\)-styrene oxide, but that value improved to 86\% when the reaction was performed at ~78 °C.\(^3\) Similarly, a modified Shi epoxidation produced \((R)\)-styrene oxide with decent enantioselectivity (71\%-85\% \(ee\)).\(^4\) Some biomimetic porphyrin-ligand-based metal catalysts also suffer from insufficient enantioselectivity issues.\(^5\)
Figure 1. Asymmetric epoxidation of unfunctionalized styrenes catalyzed by DFSM-facilitated P450BM3 peroxygenases. The DFSM is proposed to play a role in activating \( \text{H}_2\text{O}_2 \) and tuning substrate orientation.

In nature, styrene monoxygenases (SMO) are the most notable biocatalysts performing styrene epoxidation reactions; however, their outstanding selectivity produces only \((S)\)-styrene oxide (>99% \textit{ee}). Recently, the first natural SMO to generate \((R)\)-styrene oxide (with 91% \textit{ee}) was identified from the genome of \textit{Streptomyces} sp. NRRL S-31. However, the highest known \((R)\)-enantioselectivity (98% \textit{ee}) for styrene oxidation was achieved by a strain of \textit{Mycobacterium} sp. containing an ethene monoxygenase that interacts with a limited substrate scope. Certain engineered enzymes, such as cytochrome P450 monoxygenases and the \textit{Agrocybe aegerita} unspecific peroxygenase (\textit{AaeUPO}), have also been designed for the asymmetric epoxidation of olefins. Although some evolved variants exhibit excellent \((R)\)-enantioselectivities for aromatic ring-substituted styrene derivatives (up to 99% \textit{ee}), to the best of our knowledge, the highest \((R)\)-enantioselectivity toward styrene oxide (96% \textit{ee}) was observed by Reetz and Wang using a SOS mutant of the NADPH-dependent P450BM3 enzyme. However, in terms of potential synthetic applications as chiral pharmaceutical intermediates, the \textit{ee} value typically must exceed 98%. Considering the very high \((S)\)-enantioselectivity achieved by native SMO enzymes, it remains a significant challenge to develop comparable \((R)\)-enantioselectivity in the epoxidation of unfunctionalized styrene using native enzymes or rationally-designed non-natural enzymes.

Recently, peroxygenase enzymes have garnered increasing interest related to the development of novel biocatalysts. These enzymes can promote P450-like oxyfunctionalization reactions without a cofactor (i.e., NAD(P)H) or reduced partner proteins, thus exhibiting potential for practical synthetic applications. An NADPH-dependent P450 monoxygenase can be repurposed for peroxygenase activity via site-directed mutagenesis or directed evolution. We recently constructed a unique artificial P450BM3 peroxygenase system by introducing an external dual-functional small molecule (DFSM), such as \(N\)-(ω-imidazolyl)-hexanoyl-L-phenylalanine (Im-C6-Phe). The DFSM binds to P450BM3 using an acyl amino acid as the anchoring group, and its imidazolyl moiety serves as a built-in acid-base catalyst to facilitate \( \text{H}_2\text{O}_2 \) activation. Given the fact that the \textit{ee} of \((R)\)-styrene oxide increased from 7% to 84% by P450BM3_F87A mutant upon addition of Im-C6-Phe, we reasoned that the DFSM may also participate to tune substrate orientation in active site pocket to influence enantioselectivity of the epoxidation (Figure 1). Encouraged by the valuable contribution of the DFSM, herein we report further improvements to the enantioselectivity by protein engineering of the P450 peroxygenase system. This approach leads to unprecedented \((R)\)-enantioselectivity of styrene epoxidation (up to ~99% \textit{ee}), relative to enzymatic and chemical catalysts reported to date.
Figure 2. A) Proposed mechanistic effects of the T268V mutation on the formation of Compound I in the catalytic cycle of NADPH-dependent P450BM3 monooxygenase and DFSM-facilitated P450BM3 peroxygenase. B) Active site of wild-type P450BM3 (PDB No. 1JPZ), highlighting the relative positions of T268 and the axially-coordinated H2O.

Table 1. Epoxidation of styrene catalyzed by NADPH-dependent P450BM3<sup>a</sup> and H2O2-dependent P450BM3.<sup>b</sup>

| Entry | P450s         | Im-C6-Phe | Oxidant       | TON<sup>c</sup> | ee<sup>d</sup> |
|-------|---------------|-----------|---------------|-----------------|--------------|
| 1     | full_F87A     | ×         | O2/NADPH      | 142±20          | 14.2         |
| 2     | full_F87G     | ×         | O2/NADPH      | 628±24          | 63.3         |
| 3     | full_F87V     | ×         | O2/NADPH      | 788±3           | -58.3        |
| 4     | full_F87A/T268V | ×       | O2/NADPH      | 18±1            | nd           |
| 5     | full_F87G/T268V | ×       | O2/NADPH      | 27±1            | nd           |
| 6     | full_F87V/T268V | ×       | O2/NADPH      | 72±1            | -21.1        |
| 7<sup>e</sup> | H_F87A/T268V | ×         | H2O2          | nd<sup>b</sup> | nd           |
| 8     | H_F87G/T268V  | ×         | H2O2          | nd              | nd           |
| 9     | H_F87V/T268V  | ×         | H2O2          | nd              | nd           |
| 10<sup>e</sup> | H_F87A/T268V | √         | H2O2          | 382±2           | 94.2         |
| 11    | H_F87G/T268V  | √         | H2O2          | 767±2           | 83           |
| 12    | H_F87V/T268V  | √         | H2O2          | 161±3           | 52           |

<sup>a</sup>Reaction conditions: full_F87X (0.5 µm), styrene (4 mM), NADPH (5 mM) in 0.1 M pH 8.0 phosphate buffer at 25 °C; <sup>b</sup> Reaction conditions: H_F87X/T268V (0.5 µm), styrene (4 mM), Im-C6-Phe (500 µm), H2O2 (20 mM) in pH 8.0 phosphate buffer; <sup>c</sup>TON: Turnover numbers estimated for 30-minute reactions; <sup>d</sup>ee: enantiomeric excess (%) of (R)-styrene oxide determined by chiral HPLC; <sup>e</sup>Previously-reported results<sup>17</sup>. For entries 1-6, “full” denoted the mutants of full-length P450BM3; For entries 7-12, “H” denoted the mutants of P450BM3 heme domain; nd: not detected;
A threonine residue close to the heme center is highly conserved in NAD(P)H-dependent P450 enzymes (Figure 2), and is believed to play versatile roles related to the formation of the active oxygen species, Compound I, in the catalytic cycle. Many studies have shown that mutating the conserved threonine decreases or even eliminates the catalytic activity of native NADPH-dependent P450 enzymes. We examined the effect of a T268V mutation on styrene epoxidation by full-length P450BM3 enzymes upon addition of NADPH. The catalytic turnover numbers (TONs) of the variants with single mutations of phenylalanine (F87A, F87G, and F87V) reached 142, 628, and 788 together with poor enantioselectivity, respectively (Table 1, Entries 1-3, Figure S3). The double mutants (F87A/T268V, F87G/T268V, and F87V/T268V) exhibited drastically weakened catalytic activities, as evidenced by their 8-, 23-, and 11-fold decreases in TON relative to the corresponding single mutants (Table 1, Entries 4-6). Based on the proposed catalytic mechanism (Figure 2A), the lower epoxidation activity may be due to the sluggish formation of Compound I when the T268 residue is substituted with valine.

We then scrutinized the effect of the T268V mutation on the H2O2-driven P450BM3 system. Double mutations (F87A/T268V, F87G/T268V, and F87V/T268V) in the P450BM3 heme domain led to complete loss of catalytic activity towards styrene epoxidation in the absence of Im-C6-Phe (Table 1, Entries 7-9). Comparing these results to the catalytic TONs achieved by the heme domain with single mutants, F87A (42), F87G (53), and F87V (120), under identical reaction conditions, suggested that the T268 residue may also play a key role in the formation of Compound I in the H2O2-driven P450BM3 system (Figure 2A). Additionally, the catalytic TONs of the double mutants (F87X/T268V) reached 382, 767, and 161 following the addition of Im-C6-Phe (Entries 10-12). This indicated that the presence of the DFSM revived the peroxygenase activity of P450BM3, even when the highly-conserved T268 residue was mutated. Thus, the DFSM may partially adopt the function of T268 to promote the formation of Compound I (Figure 2A). We also observed that the double-mutants containing T268V demonstrated enhanced (R)-enantioselectivity for styrene epoxidation (Entries 10-12) relative to previously-reported results regarding the single mutants. These results indicated the importance of mutating T268 to further tune the enantioslectivity of styrene epoxidation. This is a distinct advantage of the DFSM-facilitated P450BM3 peroxygenase, because the NADPH-dependent P450BM3 monooxygenases lose significant epoxidation activity following T268 mutation.

We constructed a library of double mutants probing only two selected positions (F87 and T268) to avoid excess screening efforts (Table S1, Figure S4). None of the examined variants exhibited epoxidation activity in the absence of Im-C6-Phe. However, certain mutants (F87A/T268I, F87A/T268I, F87G/T268I, F87G/T268I, F87V/T268I, and F87I/T268I) demonstrated the formation of styrene oxide with moderate to good TONs (318 to 767) and good to excellent (R)-enantioselectivity (83.0% to 97.3% ee), in the presence of Im-C6-Phe.

Figure 3. (A) The substrate access channel of N-palmitoyl glycine (NPG)-bound P450BM3 and the residues around the active site (PDB No. 1JPZ). (B) Protein engineering of P450BM3 for styrene epoxidation. Experiments were performed using P450BM3 variants (µm), 4 mM styrene, 20 mM (or 80 mM) H2O2, and 2 mM Im-C6-Phe in pH 8.0 phosphate buffer at 0 °C (or 25 °C) for 30 min. “AI” denoted the double mutant F87A/T268I.
Despite its relatively low TON (335), double mutant F87A/T268I (AI) was selected as the parent enzyme on which to perform the next round of mutations because of its excellent enantioselectivity (97.3% ee). With the aim of further improving the catalytic activity and enantioselectivity, we introduced additional mutations at ten other key residues around the substrate pocket highlighted in Figure 3A (L75, Y78, A82, L181, A184, I263, A264, E267, A328, R255). Residues L75, Y78, A82, L181, and A184 are situated near the top of the active site, and A184 has specifically been reported to affect the enantioselectivity of styrene epoxidation by NADPH-dependent P450BM3. Residue R255 is located on the surface of the protein along helix I, and is known to be a site that influences the catalytic activity of P450 PMO. These residues were selectively mutated to several other amino acids with structurally- and functionally-diverse side chains to tune the substrate pocket properties. Similar approaches have been applied to construct small and smart libraries, which efficiently decrease the effort involved in developing stereoselective isozymes or multi-functional enzyme variants by directed evolution. Therefore, a library containing approximately 60 combinations of these ten mutations in AI was created and screened for styrene epoxidation activity and enantioselectivity (Table S2, Figure S5).

Most of the examined triple mutants involving the 75, 263, 264, 267, and 328 positions led to generally negative effects, i.e., reducing enantioselectivity, catalytic TONS, or both. Triple mutants involving a change at R255 increased the proportion of R-styrene oxide produced (up to >99% ee), but significantly decreased the catalytic TONs to 120 (Figure S5, Table S2). Interestingly, some triple mutants modified at the 78, 82, 181, and 184 positions, including AI/V78A, AI/A82V, AI/L181Q, AI/L181M, and AI/A184L, exhibited improved enantioselectivities and catalytic TONS compared with the parent AI (Figure 3B). Among them, AI/L181Q demonstrated the best (R)-enantioselectivity (98.8% ee), which represents the highest styrene epoxidation (R)-enantioselectivity achieved by biological or synthetic molecular catalysts, to our knowledge. The catalytic TON of AI/L181Q is also close to 1000, which is considered an important benchmark for potential practical applicability. Combining several beneficial mutations resulted in two highly-active quadruple mutants, AI/V78A/A82V (TON = 4052) and AI/V78A/A184L (TON = 4349), each achieving ~98% ee (Figure 3B and S6, Table S3).

Encouraged by these excellent results, we examined the scope of olefinic substrates that could be epoxidized using the DFSM-facilitated P450BM3 peroxynasre system. By employing different P450BM3 mutants, we discovered that the catalytic system has a broad substrate scope, with the capability to catalyze epoxidations of ortho-, meta-, and para-substituted chlorostyrenes (1b-d) and fluorostyrenes (1e-g; Figure 4 and Figures S7-S11, Table S4-S9 to generate the corresponding (R)-enantiomeric epoxide products.

Figure 4. Substrate scope of epoxidation reactions catalyzed by P450BM3 variants using H2O2 in the presence of Im-C6-Phe.
(2d-g) with moderate to excellent TONs (up to 3480) and excellent ee (up to 99.3%). It is worthwhile to note that the F87A/T268A mutant achieved better ee (86.8%) and excellent TON (1724) than F87A/T268I and F87A/T268V mutants, the latter only provided moderate ee (49.5% and 50.7%) and TON (153 and 514) for the epoxidation of o-chlorostyrene (Figure S7, Table S4). (R)-enantioselectivity of 2c can be improved to 95.3% by the triple mutant F87A/T268A/V78A. In addition, the best ee of 2d reached 96.3% by F87A/T268I/A82I with moderate TON (362). This indicated that the size of the amino acid side chain at the 268 position substantially affected the enantioselectivity and catalytic activity of certain substrates having larger Cl-substituted groups. This is also confirmed by the reaction results of 2f-2g, in which those substrates with smaller F-substituted groups (2e-2g) or less steric hindrance 2f (para-chlorostyrene) gave excellent ee and catalytic TONs.

In summary, we have achieved high (R)-enantioselectivity and activity in the H2O2-dependent epoxidation of unfunctionalized styrene and its derivatives through protein engineering of the unique DFSM-facilitated P450BM3 peroxygenase system. The enantiomeric excess of (R)-styrene oxide is unprecedented (up to ~99%) when compared with reported native or engineered oxidative enzymes. This enantioselectivity is comparable but inverse to the well-known styrene monooxygenase (styA) that oxidizes styrene to (S)-styrene oxide with >99% ee.1a,7 The catalytic TON of ~4500 (with ~98% ee) achieved with our system suggests the potential application of the current P450 enzymatic system in styrene epoxidation. The crucial mutation of T268 and addition of a DFSM comprise an effective, novel approach for controlling enantioselectivity, which cannot be applied to natural NADPH-dependent P450BM3 monooxygenases because they lose activity following T268 mutation. This work substantially expands the catalytic potential of P450 enzymes as enantioselective epoxygenases, further indicating that the discussed P450 peroxygenase system may have unique advantages for asymmetric epoxidation.

ASSOCIATED CONTENT

Supporting Information
The Supporting Information is available free of charge via the Internet at http://pubs.acs.org, Experimental section; Figures S1-S13; and Tables S1-S9 (PDF).

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Notes
The authors declare no competing financial interests.

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P450BM3

$\text{H}_2\text{O}_2$

$\text{H}_2\text{O}$

$\text{ee: 99\%}$

$\text{ee: 99\%}$
Enabling Highly (R)-Enantioselective Epoxidation of Sty... (836.81 KiB)