Biocatalysis Hot Paper

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Improved Cyclopropanation Activity of Histidine-Ligated Cytochrome P450 Enables the Enantioselective Formal Synthesis of Levomilnacipran**

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Abstract: *Engineering enzymes capable of modes of activation unprecedented in nature will increase the range of industrially important molecules that can be synthesized through biocatalysis. However, low activity for a new function is often a limitation in adopting enzymes for preparative-scale synthesis, reaction with demanding substrates, or when a natural substrate is also present. By mutating the proximal ligand and other key active-site residues of the cytochrome P450 enzyme from Bacillus megaterium (P450-BM3), a highly active His-ligated variant of P450-BM3 that can be employed for the enantioselective synthesis of the levomilnacipran core was engineered. This enzyme, BM3-Hstar, catalyzes the cyclopropanation of N,N-diethyl-2-phenylacrylamide with an estimated initial rate of over 1000 turnovers per minute and can be used under aerobic conditions. Cyclopropanation activity is highly dependent on the electronic properties of the P450 proximal ligand, which can be used to tune this non-natural enzyme activity.*

Enzymes in nature catalyze only a small subset of industrially relevant chemical transformations.[1] Increasing the number of activation modes accessible to enzymes is thus an important goal in biocatalysis, green chemistry, and sustainable synthesis.[2] Drawing an analogy between the mechanism of monooxygenation catalyzed by cytochrome P450 and transition metal catalyzed carbene insertions, we hypothesized that an iron–carbenoid intermediate could be generated at the heme prosthetic group of the enzyme in the presence of diazo compounds. Staring from this hypothesis, we have developed methods for the intermolecular, enantioselective cyclopropanation of styrenes and the N–H insertion of anilines catalyzed by variants of the cytochrome P450 enzyme from *Bacillus megaterium* (P450-BM3).[3] These enzymatic methods are attractive alternatives to transition metal catalysis with Rh or Cu complexes[4] for cyclopropanation because cytochrome P450 enzymes are genetically encoded, can be very stereoselective, and use inexpensive and nontoxic Fe as the catalytic center.

One challenge to using enzymes for non-natural chemistry is that the activity is often low.[3,5] Wild-type P450-BM3 has very low cyclopropanation activity, for example, and even the best reported variants are still 10–100 times less active for styrene cyclopropanation than for the epoxidation or hydroxylation of their preferred fatty acid substrates.[6,7] Although introducing mutations at amino acid residues critical for native monooxygenation readily eliminates the competing P450-catalyzed styrene epoxidation, molecular oxygen severely inhibits the desired cyclopropanation.[8] As a result, anaerobic procedures must be employed to favor carbenoid transfer reactions. Another consequence of the relatively low cyclopropanation activity is that the transformation is limited to electron-neutral and electron-rich alkenes.[3a] Increasing the activity of P450s toward these non-natural reactions would greatly expand their utility as cyclopropanation catalysts for the bench-top synthesis of biologically active molecules and even large-scale industrial use.

Recently, we reported that mutation of the proximal Cys at position 400 in P450-BM3 to Ser enabled cyclopropanation by whole cells expressing these proteins (Figure 1a).[8] The Cys to Ser mutation increased the protein redox potential by 140 mV, thereby allowing efficient reduction of the inactive Fe^{III}-heme resting state to the Fe^{II}-heme active catalyst in vivo.[9] However, in addition to modulating the redox potential, we reasoned that the axial ligand also affects the electronic properties of the proposed iron–carbenoid intermediate and thus the inherent rate of P450-catalyzed cyclopropanation. In particular, we hypothesized that by varying

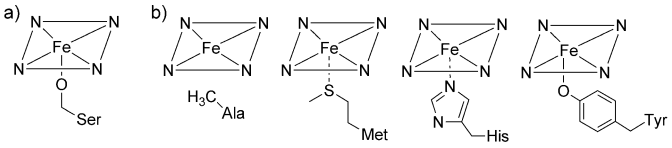

Figure 1. a) Ser-ligated heme in BM3 enables cyclopropanation in vivo.[3b] b) Proposed Ala-, Met-, His-, and Tyr-ligated heme in axX variants of BM3.

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the axial coordinating ligand, we might discover a more active enzyme, which would enable the cyclopropanation of a broader range of substrates.

To test this, we have initially focused on variants of P450-BM3 bearing His, Met, Tyr, or Ala at the proximal position (Figure 1b). This set represents a range of possible coordinating heteroatom ligands, and His, Met, and Tyr are found at the axial position of naturally occurring heme proteins such as horseradish peroxidase (HRP), cytochrome *c*, and catalase.[10] We chose Ala as well because it is an archetypal small amino acid and may allow a water molecule or hydroxide ion to coordinate to the Fe center.[11] To examine how the different axial ligands affect cyclopropanation activity, we introduced each of the four axial mutations into a P450-BM3 holoenzyme containing the additional mutation T268A. This mutation was previously found to be highly beneficial for cyclopropanation.[3a] Four variants, T268A-axX (where "X" denotes the single-letter amino acid code of each axial variant), were expressed as the His-tagged heme domains and purified and characterized by UV/Vis spectroscopy. All four variants bound CO efficiently and provided distinct Fe^{II}–CO absorbances in the 414–422 nm range (Figure S1 in the Supporting Information).

When we monitored the reactions of whole *E. coli* cells expressing the four P450-BM3 variants with styrene and ethyl diazoacetate (EDA), we found that T268A-axH was highly active and catalyzed the cyclopropanation of styrene to greater than 50% conversion within 30 min (Figure 2). Both the Cys-ligated T268A and the Ser-ligated T268A-axS (enzymes with the axS mutation were previously denoted as "P411s" owing to the characteristic Fe^{II}–CO absorbance at 411 nm of axS proteins[3b]) exhibited much slower kinetics at the same protein concentration. T268A is not expected to be highly active for the cyclopropanation of styrene in vivo because its redox potential in the absence of native substrates is more negative than that of biological reductants (−410 mV versus −310 mV for NAD(P)H). The Fe^{III} resting state thus cannot be easily reduced to the Fe^{II} active catalyst. However, the higher activity of T268A-axM and T268A-axH mutants relative to T268A-axS suggests that, among variants that can be reduced to Fe^{II} by biological reductants, those that have more electron-donating axial ligands are more active cyclopropanation catalysts in vivo.

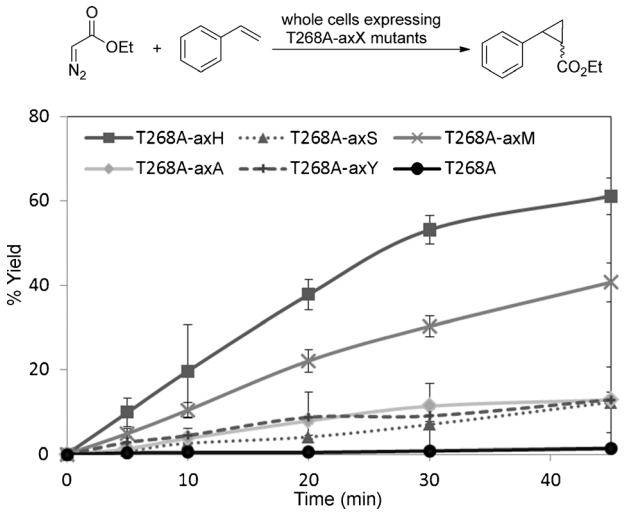
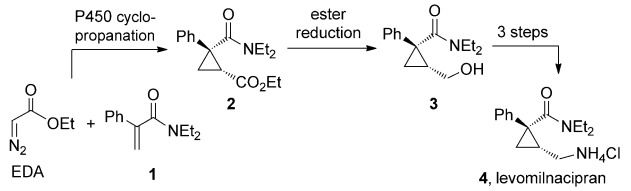


Figure 2. Reaction progress of the in vivo cyclopropanation of styrene with EDA catalyzed by axial variants of T268A.

Our finding that the cyclopropanation activity of BM3 variants increases when natural Cys ligation is replaced with His or Ser coordination contrasts with what has been previously observed for P450-catalyzed monooxygenation.[12] Mutation of the proximal Cys to Ser or His causes the monooxygenation activity of the enzyme to fall precipitously.[13] We have observed that the mutation of other highly conserved amino acids in the P450 active site, such as the distal Thr residue on the I-helix (T268 in P450-BM3) that facilitates the multiple proton transfers required for oxygen activation, also produces more active cyclopropanation catalysts. The conserved amino acids required for the activation of molecular oxygen are thus not necessarily required for the activation of diazo compounds. Indeed, mutation of the axial residue and other conserved positions in heme proteins may allow us to develop new enzymes that are better suited to carbenoid or nitrenoid modes of reaction.[14]

To showcase the improved activity of the T268A-axX mutants, we wanted to develop a method for the cyclopropanation of electron-deficient olefins, which have been challenging substrates for transition metal catalysts.[15] In particular, we hypothesized that an enantioselective BM3 catalyst could be used for the cyclopropanation of *N*,*N*-diethyl-2-phenylacrylamide (**1**) with EDA in an expedient synthesis of the levomilnacipran core (Scheme 1). Levomil-

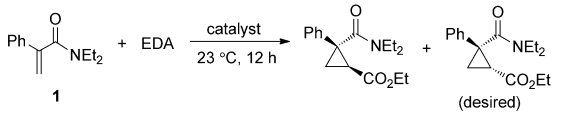


Scheme 1. Proposed formal synthesis of levomilnacipran by using P450-catalyzed enantioselective cyclopropanation in the key ring-forming step.

nacipran (Fetzima) is a selective serotonin and norepinephrine reuptake inhibitor that was recently approved by the Food and Drug Administration for the treatment of clinical depression.[16] The more active (1*R*,2*S*)-isomer of milnacipran,[17] levomilnacipran, is sold in enantiopure form. While multiple syntheses of milnacipran and levomilnacipran have been described,[18] none have used intermolecular enantioselective cyclopropanation to construct the cyclopropane core of the molecule. The most high yielding and efficient of the reported methods requires a series of alkylations and thermal rearrangements that require strong alkali bases and temperatures in excess of 80 °C.[15a,b] A concise, chemoenzymatic synthesis could constitute a mild and energy-efficient alternative to this existing procedure.

When we combined **1** (10 mM) with EDA (10 mM) in the presence of whole cells expressing the axial variants (Table 1), we found that T268A-axH catalyzed the reaction to 81%

Table 1: Reaction of **1** with EDA catalyzed by T268A-axX mutants in vivo.



Entry	Catalyst[a]	Yield [%][b]	TN[c]	*trans*/*cis*	*ee* [%][d]
1	T268A-axA	19	1600	18:82	21
2	T268A-axH	81	7100	6:94	42
3	T268A-axM	12	1000	16:84	4
4	T268A-axY	12	1000	17:83	5
5	T268A-axS	18	1500	16:84	17
6	T268A	0.8	80	16:84	n/a[e]
7	Hemin (5 μM)[f]	3.0	50	18:82	0
8	HRP (10 μM)[f]	0.4	3	n/a	n/a
9	T268A-axH + CO	0	n/a	n/a	n/a
10	E. coli w/o BM3	0	n/a	n/a	n/a

[a] For whole cells expressing T268A-axX, protein concentrations were determined by CO-assay. Cell densities were normalized such that all whole cell samples had [BM3] = 1.0 μM. [b] Reactions were performed on a 0.5 mL scale with final concentrations of 8.7 mM EDA and 10 mM **1**. Yields were determined by gas chromatography by using phenylethanol as an internal standard. [c] Turnover numbers (TN) were determined by dividing the millimolar product yield by the catalyst concentration (1 μM for BM3 variants and 5 μM for hemin). [d] Enantioselectivity was determined by chiral supercritical fluid chromatography with CO_2. [e] Could not be determined due to low product yield. [f] Used in vitro as isolated protein or complex.

yield with 6:94 diastereoselectivity and 42% enantioselectivity for the desired product (Table 1, entry 2). T268A and hemin failed to provide the desired product in synthetically useful yields (Table 1, entries 6 and 7, respectively). Although variants T268A-axA and T268A-axS also showed appreciable activity, they were less active and less enantioselective than the His mutant when normalized for catalyst expression level. T268A-axH is also more active than other axial mutants when used as the purified holoenzyme (Table S3 in the Supporting Information). Interestingly, horseradish peroxidase, which is naturally ligated by an axial His, is a poor catalyst for this reaction (Table 1, entry 8). This result suggests that heme His ligation alone is not sufficient for catalysis and that the P450 fold is privileged for this transformation. The changes in diastereo- and enantioselectivity observed for the different axial mutants suggest that the amino acid at position 400 can affect the active-site geometry as well as reactivity. When whole cells expressing T268A-axH were purged with CO prior to the addition of both substrates, no catalysis was observed (entry 9). Furthermore, control cells transformed with pET-22b(+) vector without the BM3 gene also failed to catalyze the reaction (entry 10). These controls indicate that a folded His-ligated BM3 enzyme is the active catalyst.

To create a catalyst more enantioselective than T268A-axH, we performed site-saturation mutagenesis at four active-site positions that had been shown previously to affect selectivity in cyclopropanantion or monooxygenation; F87, I263, L437 and T438. The libraries were screened in 96-well plates with whole cells and an oxygen quenching system containing glucose oxidase and catalase in sealed plates (see the Supporting Information). The enantioselectivity of each reaction was determined by chiral supercritical fluid chromatography. Mutagenesis at positions F87 and I263 failed to produce variants with higher selectivity than the parent T268A-axH. The libraries at L437 and T438, however, yielded variants T268A-axH-L437W and T268A-axH-T438W, which catalyzed the reaction to 69 and 68% enantioselectivity, respectively. Unfortunately, combining the Trp mutations at position 437 and 438 significantly reduced the yield and reduced the enantioselectivity to 23%.

Given that T268A-axH-L437W showed higher activity than T268A-axH-T438W (Table S4), we chose the former as the parent for a second round of site saturation at two sites; V78 and L181. These two positions are located in the same region of the BM3 active site as L437 but are not located on the same helix or loop as L437. We identified two variants, T268A-axH-L437W-V78M and T268A-axH-L437W-L181V, that showed improved enantioselectivity relative to T268A-axH-L437W (87% and 75% *ee*, respectively) without loss of reactivity. V78M and L181V were combined in variant T268A-axH-L437W-V78M-L181V (named "BM3-Hstar"), which provided the desired product in greater than 92% yield with 92% enantioselectivity and 2:98 diastereoselectivity (Figure 3).

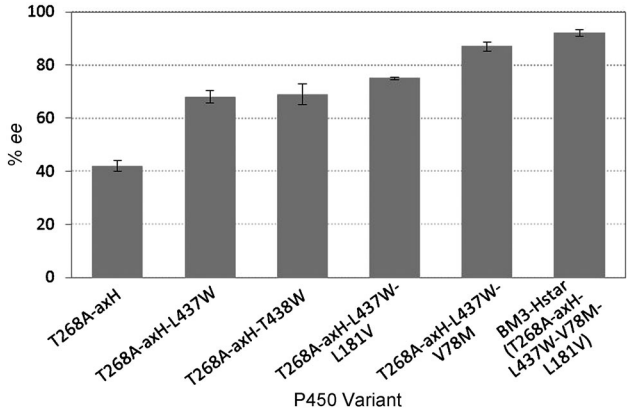
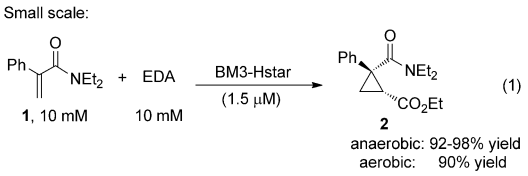


Figure 3. Sequential site-saturation mutagenesis at key active-site positions in the T268A-axH variant led to increased enantioselectivity for the cyclopropanation of **1**.

When the reaction of **1** and EDA catalyzed by BM3-Hstar in whole *E. coli* cells was monitored by gas chromatography, we found that the reaction reached greater than 80% conversion within the first 10 min. This is much faster than what we had observed for the cyclopropanation of **1** with T268A-axS (Figure S3 in the Supporting Information). The reaction catalyzed by the parent construct T268A-axH was also complete within the first 15 min, thus suggesting that the axH mutation alone dramatically increases the rate of olefin cyclopropanation. An estimate of the initial rate based on the first five minutes of reaction shows that catalysis proceeds at over 1000 turnovers per minute on average. Indeed, even under aerobic conditions, BM3-Hstar is able to catalyze the reaction of **1** and EDA to 90% yield (compared to 92–98% for an anaerobic control performed in parallel) when cells expressing BM3-Hstar to 2.0 μM are used [Eq. (1)]. This observation suggests that the cyclopropanation of **1** outcom-



petes catalyst inhibition or deactivation by molecular oxygen. No other BM3 variant has demonstrated this tolerance to atmospheric oxygen in these non-natural reactions.

The reaction of **1** with EDA could be performed on a preparative scale under aerobic conditions to provide the levomilnacipran precursor in 93% conversion and 86% yield of isolated product (204 mg) by preparative HPLC, a diastereomeric ratio of 2:98, and 92% enantioselectivity. Cyclopropane **2**, which could not be separated from unreacted **1** by column chromatography, was readily reduced to the corresponding alcohol in the presence of 1.5 equivalents of $LiBH_4$ to provide alcohol **3** in 88% yield of isolated product, without loss of enantioselectivity (Scheme 1). Elaboration of this key intermediate to levomilnacipran has been well-documented and can be achieved in two facile and high-yielding steps.[18c]

Through mutating the amino acid at the axial site of P450-BM3, we have identified a highly active and enantioselective cyclopropanation catalyst for a challenging electron-deficient olefin. Starting from a parent BM3 variant with His at position 400, we engineered BM3-Hstar through two rounds of site-saturation mutagenesis and recombination to catalyze the reaction of **1** and EDA to 92% yield, 92% enantioselectivity, and 2:98 diastereomeric ratio. Remarkably, a single mutation at the axial position dramatically increases the reaction rate such that these new variants can be used for the cyclopropanation of **1** with EDA in an aerobic environment with minimal loss of yield. Three additional mutations identified through site-saturation mutagenesis (L437W, L181V, and V78M) greatly enhanced the enantioselectivity of the transformation and further improved the activity of the catalyst, thus demonstrating that the cyclopropanation reaction of the P450-BM3 scaffold can be readily tuned for a desired substrate. The tolerance of P450-catalyzed cyclopropanation to amino acid substitution at the axial position contrasts with the strict requirement for axial Cys for monooxygenation activity. The ability of the P450 protein to accept other amino acids at this key position and still bind heme will allow us to tune the electronics of the catalytic center and thereby access a broader range of carbenoid-based transformations.

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