Enantioselective Enzyme-Catalyzed Aziridination Enabled by Active-Site Evolution of a Cytochrome P450

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Supporting Information

ABSTRACT: One of the greatest challenges in protein design is creating new enzymes, something evolution does all the time, starting from existing ones. Borrowing from nature’s evolutionary strategy, we have engineered a bacterial cytochrome P450 to catalyze highly enantioselective intermolecular aziridination, a synthetically useful reaction that has no natural biological counterpart. The new enzyme is fully genetically encoded, functions in vitro or in whole cells, and can be optimized rapidly to exhibit high enantioselectivity (up to 99% ee) and productivity (up to 1,000 catalytic turnovers) for intermolecular aziridination, demonstrated here with tosyl azide and substituted styrenes. This new aziridination activity highlights the remarkable ability of a natural enzyme to adapt and take on new functions. Once discovered in an evolvable enzyme, this non-natural activity was improved and its selectivity tuned through an evolutionary process of accumulating beneficial mutations.

INTRODUCTION

The impressive catalytic diversity of enzymes illustrates the breadth of reaction space explored throughout biological evolution. Chemists are taking advantage of this vast repository of biocatalysts for chemical synthesis; they are also mimicking nature’s engineering strategy of mutation and selection to optimize enzyme function for a myriad of applications. A remaining challenge is to create new enzymes for the many useful reactions that are not known in the biological world. We and others have argued that learning from nature’s mechanisms for catalytic innovation—exploiting enzyme catalytic promiscuity and retaining key elements of structure and mechanism while exploring new functions—can help us discover new biocatalysts starting from nature’s vast repertoire. The discovery and optimization of enzyme catalysts for non-natural reactions has provided alternative routes to important molecules that would otherwise be unattainable through biocatalysis.

Our group and that of Fasan recently reported that heme proteins can form reactive metal–nitrenoid species and catalyze intramolecular C–H amination. We extended this novel biocatalytic activation mode to intermolecular sulfimidation and showed that the enzyme, derived from a cytochrome P450, could be tuned by mutation to catalyze nitrene transfer to various aryl sulfides with good activity and moderate enantioselectivity (Scheme 1A). Because heme proteins do not encounter nitrene precursors in their natural environments, they never had the chance to explore and evolve this capability. But the cytochrome P450’s versatile catalytic framework is clearly capable of catalytic activities well beyond what nature has explored.

The extensive literature on reactions catalyzed by metalloporphyrins provides inspiration for reactivities that should be investigated. Among the nitrene transfer reactions used in synthetic chemistry, aziridination with azide nitrene sources is particularly attractive (Scheme 1B), given its high atom economy and the utility of the aziridine functional group in chemical synthesis. Metalloporphyrin-catalyzed aziridination of olefins with iminodianes or haloamines as the nitrene source dates to the 1980s or early 2000s, respectively. Advances in the past ten years have expanded metalloporphyrin-catalyzed...
aziridination to include systems that use azide-based nitrene sources.\textsuperscript{10}

In contrast, enzymes that use electrophilic nitrogen species are extremely rare (e.g., proposed mechanism of TxtE-catalyzed nitrilation of tryptophan\textsuperscript{11}). As a result, natural aziridination follows an entirely different strategy from the metalloporphyrin-catalyzed aziridination of olefins described above. Indeed, the biosynthesis of naturally occurring aziridine rings is believed to proceed by intramolecular nucleophilic displacement.\textsuperscript{12} Enzyme catalysts for the convergent synthesis of aziridines from olefins and azide nitrene precursors would be highly attractive and would fill a significant gap in nature’s biosynthetic repertoire.

During our previous work on sulfoxide imidation catalyzed by variants of cytochrome P450BM3, two factors appeared to affect reactivity. First, the reaction was promoted by electron-rich sulfoxides, and second, enzyme-catalyzed azide reduction to sulfinamide represented a significant side reaction, particularly with less reactive, electron-deficient sulfoxides (Scheme 1).\textsuperscript{6} Increasing the sulfoxide substrate loading improved the ratio of sulfinamide to sulfoxide side product, which suggested to us that increasing the effective concentration of sulfoxide nitrene acceptor in the enzyme active site might improve sulfoxidation productivity. Given the less reactive nature of olefins relative to sulfoxides along with operational limitations to increasing olefin effective concentration (i.e., their limited solubility in aqueous media), we surmised that intermolecular aziridination would be an even more challenging activation mode for the enzyme. We hypothesized, however, that protein engineering would allow us to circumvent these limitations if the enzyme could more effectively bind and orient the substrates in the enzyme active site for productive nitrene transfer or, alternatively, catalyze the competing azide reduction less efficiently. Here we demonstrate that active-site engineering can indeed enable a heme protein to catalyze efficient, highly enantioselective intermolecular nitrene transfer to olefins to make aziridines.

### RESULTS AND DISCUSSION

We started this investigation of enzyme-catalyzed aziridination with an engineered variant of cytochrome P450BM3, P411BM3-CIS-T438S\textsuperscript{13}, previously found to be effective for intramolecular C–H amination and sulfoxide imidation (see Table S1 for mutations from wild-type P450BM3).\textsuperscript{4,6} We call this enzyme a “P411” due to the change in the characteristic CO-bound Soret peak from 450 to 411 nm effected by mutation of the cysteine residue that coordinates the heme iron to serine (C400S).\textsuperscript{5,13} This axial cysteine is completely conserved in cytochrome P450s and is required for the native monoxygenase activity.\textsuperscript{14} Thus, the P411 enzyme is no longer a “cytochrome P450”, nor does it exhibit its native hydroxylation activity.\textsuperscript{5b,15} However, the C400S mutation increases the non-native carbene and nitrene transfer activities of P450BM3\textsuperscript{4,6,13} and other P450s.\textsuperscript{16} Two crystal structures of P411 variants of P450BM3 show that S400 coordinates the iron and causes no significant structural perturbation in the substrate binding pocket.\textsuperscript{13}

Previous work on enzymatic sulfimidation suggested that electron-rich sulfoxides promote nitrene transfer.\textsuperscript{6} Reasoning that the electronic properties of the olefin substrate could influence aziridination as well, we tested the activity of P411BM3-CIS-T438S toward the electron-rich 4-methoxystyrene using tosyl azide (TsN\(_3\)) as the nitrene precursor (Table 1). Tosyl azide was completely consumed in this reaction, the major product of which was the azide reduction product p-toluensulfonamide (>300 total turnovers (TTN), not shown in Table 1). Amido-

alcohol 2 appeared as a minor product. Control experiments showed that the desired aziridine product rapidly decomposes under aqueous reaction conditions to the corresponding amido-alcohol 2 (Figure S1). Degradation of this aziridine product has also been observed in studies with small-molecule catalysts.\textsuperscript{17} We therefore inferred that production of 2 is directly related to the nitrene transfer activity of the enzyme toward olefin 1.

This low level of nitrene transfer activity to 4-methoxystyrene of the P411BM3-CIS-T438S enzyme prompted us to investigate other variants. We chose a small set of cytochrome P450BM3 variants and heme proteins prepared for other studies in order to assess how changes in the protein sequence affect nitrene transfer to olefin substrates (Tables S2 and S3). P450BM3 variants lacking the C400S and/or T268A mutations were weakly active, giving 2 at levels lower than P411BM3-CIS-T438S (3–4 TTN). Myoglobin (horse heart), cytochrome c (bovine heart), and cytochrome P450BM3 (from Rhodococcus sp. NCIMB 9784) were all inactive for this intermolecular aziridination (Table S3). An engineered variant of the thermostable cytochrome P450 from Sulfolobus acidocaldarius, CYP119, that contained an axial cysteine-to-serine mutation (C317S) did catalyze low levels of aziridination (~7 TTN). This demonstrates that mutations previously described to activate non-native nitrene-transfer activity in P450BM3 may confer measurable activity on other P450s as well. In turn, these enzymes should be suitable starting points for further engineering.\textsuperscript{16} Of all the enzymes tested, a variant of P411BM3-CIS-T438S having a single active-site substitution, I263F, was the most active toward 4-methoxystyrene by a wide margin, providing 150 total turnovers in the formation of amido-alcohol 2 from 4-methoxystyrene (Table 1). This variant, which was found to promote regioselective intramolecular C–H amination in a previous study,\textsuperscript{13a} supported aziridination at 10-fold increased

| enzyme | TTN \textsuperscript{b} |
|--------|----------------------|
| P411BM3-CIS-T438S (P) | 15 8 5 |
| P450BM3-T438S (P) | 150 160 190 |

\textsuperscript{*}Reactions were performed in 0.1 M KPi buffer pH = 8.0 using 0.2 mol % enzyme and NADPH as reductant, with 2.5 mM tosyl azide and 7.5 mM olefin. Detailed reaction conditions can be found in the Supporting Information. TTN = total turnover number. TTNs were determined by HPLC analysis.
were stable under the reaction conditions, although some samples were analyzed by LC-MS (Figure S2). P-I263F was even more productive when the reactions were carried out using whole cells with no P411 enzyme expressed, as described in the Supporting Information methods.6 Unlike the aziridine product that leads to amido-alcohol 2, aziridine products 4 and 6 were stable under the reaction conditions, although some amido-alcohol product was detected when standards and samples were analyzed by LC–MS (Figure S2).

Table 2. Improvement in Yield and % ee for Aziridine Product 4 with Active-Site Evolution of P411BM3-CIS-T438S (P)4

| entry | enzyme               | % yield | % ee of 4 |
|-------|----------------------|---------|-----------|
| 1     | no enzyme            | 0       | nd        |
| 2     | P411BM3-CIS-T438S    | 0.1     | 25        |
| 3     | P-I263F              | 40      | 55        |
| 4     | P-I263F-A328V        | 43      | 96        |
| 5     | P-I263F-L437V        | 37      | 95        |
| 6     | P-I263F-A328V-L437V  | 55      | 99        |

*Reactions were carried out using whole E. coli cells resuspended in M9-N reaction buffer under anaerobic conditions, with 2.5 mM tosyl azide and 7.5 mM 4-methylstyrene. Yield is based on tosyl azide. See Supporting Information methods for detailed reaction setup and quantification procedures. % ee determined by SFC analysis and calculated as (S – R)/(S + R). “No enzyme” reactions were carried out using whole cells with no P411 enzyme expressed, as described in the Supporting Information methods.*

C–H amination.4) The P-I263F enzyme provided enough aziridine product in whole-cell reactions to allow for screening variants in 96-well plate format. Thus, we sought further improvement by mutagenesis and screening for aziridination productivity. Site-saturation mutagenesis (SSM) libraries were created at several active site positions that were previously shown to influence productivity and enantioselectivity in other non-natural reactions (A78, L181, T438, A328).9,10,13 Screening of these single SSM libraries for aziridination of 4-methylstyrene (3) identified P-I263F-A328V, with slightly improved yield and substantially improved % ee (96% ee; entry 4, Table 2). Another round of SSM performed on this variant at additional active site positions (F87, T268, L437) resulted in P-I263F-A328V-L437V with improved aziridine yield and a further increase in enantioselectivity (99% ee (S)). The P-I263F-L437V and P-I263F-A328V mutants were both less selective than P-I263F-A328V-L437V, demonstrating that both new mutations contribute to the very high enantioselectivity. Importantly, the yield of sulfonamide side product 7 diminished over the course of active site evolution, to the extent that aziridine 4 became the major product of the reaction catalyzed by P-I263F-A328V-L437V.

Because the azide is fully consumed in these reactions, the improved aziridine yield could result from either an increase in the rate of aziridine formation or a decrease in the rate of competing azide reduction, or from a combination of both. To address this, we measured initial rates of reaction with the P-I263F, P-I263F-A328V, and P-I263F-A328V-L437V enzymes as purified holoenzymes (Table S4, Figure S4). Initial rates of aziridination for the purified enzymes reflected the yield improvements observed in whole cells: P-I263F and P-I263F-A328V have similar turnover frequencies (15–16 min⁻¹), while P-I263F-A328V-L437V, having both new mutations, was improved (TOF ~ 24 min⁻¹). The initial turnover frequency of sulfonamide formation in vitro was similar for all the enzymes, and faster than aziridine formation (TOFs ~ 26–29 min⁻¹).

Having obtained a variant capable of high productivity and enantioselectivity for the aziridination of 4-methylstyrene (3), we next investigated whole-cell reactions with different substituted styrene substrates (Table 3). In contrast to enzyme-catalyzed imidation of sulfides,6 we saw no correlation between the electronics of the aryl substituent and productivity. In general, the evolved enzyme was more productive with styrenes substituted at the 4-position, though the highest productivity was observed with styrene itself. The evolved enzyme provided 600 catalytic turnovers for the formation of aziridine 6, corresponding to a 70% yield of 6 (entry 3 in Table 3). With higher styrene and tosyl azide loading, the enzyme catalyzed 1,000 turnovers for aziridination, while retaining high (S)-selectivity (99% ee) (Figure S5). Both 3-methylstyrene and 3-chlorostyrene were significantly less reactive than their 4-substituted counterparts, giving 85 and 21 turnovers, respectively, compared to 450 and 290 turnovers (entries 2, 4, 5, 6 in Table 3). The evolved enzyme is an exceptionally enantioselective aziridination catalyst with styrene entries 2–4 (Table 3), giving 99% ee in favor of the (S)-enantiomer with these three substrates. Both 4-methoxystyrene and α-methylstyrene (entries 1 and 8 in Table 3) gave exclusively racemic amido-alcohol product. Formation of the amido-alcohol product from these substrates may result from carbocation stabilization at the benzylic position due to the resonance and hyperconjugative stabilization provided by the respective p-O Me and α-Me groups, leading to decomposition of the aziridine product and subsequent carbocation quenching with water.

The highly enantioselective P-I263F-A328V-L437V variant has three mutations in its active site relative to the P enzyme used in initial reaction characterization (P411BM3-CIS-T438S). The crystal structure of the heme domain of P-I263F was recently solved and shows how the F263 side chain is oriented toward the heme cofactor within the active site (PDB ID: 4WG2, Figure S6).13 The effect of the I263F mutation on the active site is significant: the F263 side chain fills space above the heme cofactor whereas the I263 side chain is pointed up and away from the heme (Figure S6). Given the more conservative nature of the A328V and L437V mutations, these residues likely
Table 3. Substrate Scope of Aziridination with P-I263F-A328V-L437V Showing Productivity and Selectivity for Each Product

| Entry | Olefin | Product | TTN | % yield | % ee |
|-------|--------|---------|-----|---------|------|
| 1     |        |         | 390 | 47      |      |
| 2     |        |         | 450 | 55      | 99   |
| 3     |        |         | 600 | 70      | 99   |
| 4     |        |         | 290 | 36      | 99   |
| 5     |        |         | 21  | 2       | 95   |
| 6     |        |         | 85  | 10      | 95   |
| 7     |        |         | 130 | 15      | 81   |
| 8     |        |         | 83  | 10      |      |
| 9     |        |         | 53  | 6       | 84   |

*Reactions were carried out with whole cells expressing P-I263F-A328V-L437V under anaerobic conditions, with 2.5 mM tosyl azide and 7.5 mM olefin. †% ee determined as (S – R)/(S + R). Absolute configurations were assigned based on analogy to 6. rac = racemic.

We report the first example of enzyme-catalyzed olefin aziridination. This challenging intermolecular reaction is catalyzed by a serine-ligated “P411” variant of cytochrome P450ΔMD. That the iron-heme cofactor has no measurable activity highlights the critical role of the protein in promoting this activity. Mutations in the enzyme active site resulted in a variant that exhibits significantly improved azide utilization compared to the parental enzyme and high (S)-selectivity (up to 99% ee). These results demonstrate the critical role of protein engineering in optimizing non-natural reactivity and suggest that the well-known plasticity of the P450 active site can be leveraged to target progressively more challenging non-natural reactions.

This new aziridination biocatalyst is likely just one of many new catalysts that will be discovered when researchers start systematically exploring the new functions that existing enzymes can take on. Exploiting the catalytic promiscuity of natural enzymes combined with evolutionary optimization will enable us to greatly expand the reaction space of genetically encoded biocatalysts.

**CONCLUSIONS**

**ASSOCIATED CONTENT**

Supporting Information

The following file is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.5b00056.

Figures S1–S19, Tables S1–S4, and experimental procedures (PDF)

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**Notes**

The authors declare no competing financial interest.

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