JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

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Enzyme-Controlled Nitrogen-Atom Transfer Enables Regiodivergent C−H Amination

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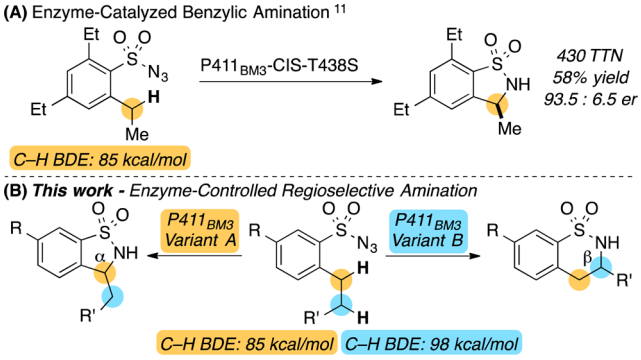
Supporting Information

ABSTRACT: We recently demonstrated that variants of cytochrome $P450_{BM3}$ (CYP102A1) catalyze the insertion of nitrogen species into benzylic C−H bonds to form new C−N bonds. An outstanding challenge in the field of C−H amination is catalyst-controlled regioselectivity. Here, we report two engineered variants of $P450_{BM3}$ that provide divergent regioselectivity for C−H amination—one favoring amination of benzylic C−H bonds and the other favoring homo-benzylic C−H bonds. The two variants provide nearly identical kinetic isotope effect values (2.8−3.0), suggesting that C−H abstraction is rate-limiting. The 2.66-Å crystal structure of the most active enzyme suggests that the engineered active site can preorganize the substrate for reactivity. We hypothesize that the enzyme controls regioselectivity through localization of a single C−H bond close to the iron nitrenoid.

The presence of nitrogen atoms in the vast majority of drugs drives the search for efficient and selective methods to form new C−N bonds.[1] Traditional approaches for forming aliphatic C−N bonds utilize the intrinsic nucleophilicity of nitrogen and the electrophilicity of a vast array of carbon species to facilitate bond formation.[2] Nature utilizes a similar reactivity profile, as exemplified by transaminase and amino acid dehydrogenase enzymes.[3] An alternative means to C−N bond formation reverses the traditional reactivity profiles by utilizing an electrophilic nitrogen species.[4] This is typically achieved via generation of a transition-metal-bound nitrenoid intermediate that can react with alkenes, nucleophilic heteroatoms, and C−H bonds.[5] This approach is attractive because new C−N bonds can be accessed directly from unactivated carbon atoms.

With the exception of the unusual cytochrome P450 TxtE-catalyzed nitration of tryptophan,[6] the machinery to generate and use electrophilic nitrogen species has not been found in nature. Cytochrome P450s, however, have evolved to generate and use electrophilic oxygen species capable of reacting with alkenes, heteroatoms, and C−H bonds.[7] This feat is possible because P450s can form a highly reactive iron−oxo intermediate known as compound I.[8] Inspired by the similarity between compound I and the carbenoid and nitrenoid intermediates invoked in transition-metal-catalyzed reactions, our group recently discovered that P450s could catalyze reactions thought to proceed through these intermediates.[9−12]

Scheme 1. Enzyme-Catalyzed Amination



C−H amination activity is particularly sensitive to the nature of the residue ligating the axial position of the iron-heme prosthetic group, with serine-ligated $P411_{BM3}$ variants providing reactivity superior to that of cysteine-ligated variants in the amination of secondary benzylic C−H bonds (Scheme 1).[11] We refer to the serine-ligated enzymes as P411s, because the diagnostic Soret peak shifts from 450 nm (cysteine-ligated) to 411 nm and the enzymes no longer catalyze their native oxygenation reactions. Complementary work by Fasan showed that variants of $P450_{BM3}$ and other hemoproteins bearing different axial ligands (cysteine, histidine, or tyrosine) will catalyze this type of transformation, although they require more activated tertiary benzylic C−H bonds to afford >15% yield.[13] The need for relatively weak C−H bonds (BDE ≤ 85) can be understood, in part, by considering the reaction mechanism. Iron-catalyzed aminations are understood to proceed via a mechanism in some ways reminiscent of P450-catalyzed C−H oxidation, where C−H bond cleavage generates a radical species that can rebound to form the new C−N bond.[14] White found that the C−H cleavage is sensitive to bond strength, where C−H bonds with lower bond dissociation energies (BDEs) are aminated preferentially to C−H bonds with greater BDEs.[15] Fasan corroborated this result using enzymes, reporting a linear relationship between reaction rate and C−H bond strength.[13b]

This observation indicates that it could be challenging to aminate strong C−H bonds of substrates having alternative, weak C−H bonds. Given that C−H amination is kinetically controlled, Du Bois found that substrate geometry can

Received: September 14, 2014
Published: October 17, 2014

   dx.doi.org/10.1021/ja509308v | J. Am. Chem. Soc. 2014, 136, 15505−15508

influence selectivity.[16] Rather than substrate control, it would be more attractive to have selectivity controlled by the catalyst, such that different products could be generated from a single starting material, depending on the catalyst. In select examples, Du Bois and Katsuki found that modulating the steric properties of the ligand on rhodium and iridium catalysts can shift selectivity in favor of stronger, but less hindered, C−H bonds.[17]

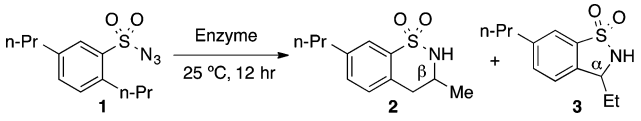
We hypothesized that enzymes could provide an elegant solution to this regioselectivity challenge. Since P450s can be engineered to alter the regioselectivity of hydroxylation,[18] we imagined that active-site engineering could similarly generate catalysts with high selectivity for amination (Scheme 1). As a model system, we selected 2,5-di-*n*-propylbenzenesulfonylazide **1** because it contains two potential sites for C−H amination—the benzylic position (*α*-position) and the homo-benzylic position (*β*-position) with disparate C−H bond strengths (85 and 98 kcal/mol).[19] Furthermore, Zhang showed that this substrate could undergo amination of both positions under cobalt−porphyrin catalysis, although amination of the *α*-position was favored.[20]

We began by testing variants from our previous reports on C−H amination and sulfimidation.[11,21] The best variant was P411$_{BM3}$-CIS-T438S (15 mutations from wild type). It gave low activity with sulfonylazide **1** (total turnover number (TTN) = 32) (Table 1, entry 1) but was modestly selective for *β*-amination (84:16), establishing that a P411-based amination biocatalyst is capable of cleaving bonds significantly stronger than previously reported.

Expanded active sites have been key for superior reactivity in some of our previous studies.[21,22] Unfortunately, low reactivity and selectivity were observed for mutants containing alanine mutations in the active site (Table 1, entries 2−4). We thus focused on engineering new variants for amination. We selected five positions in the active site—F87, L181, I263, T268, and T438—and screened libraries made by site-saturation mutagenesis at each position in the P411$_{BM3}$-CIS-T438S parent. Mutation at four of the positions failed to provide more-active variants. The I263 library, however, yielded a substantially improved enzyme: variant P411$_{BM3}$-CIS-T438S-I263F showed an 11-fold increase in activity and 97:3 selectivity favoring amination at the *β*-position (Table 1, entry 5). Reverting the previously identified activating mutations C400S and T268A decreased the desired reactivity, confirming their importance to catalysis (Table 1, entries 6 and 7).[11]

In the course of screening site saturation mutagenesis libraries, F87A was identified as a mutation that switched selectivity to favor amination at the *α*-position, albeit with low turnover (Table 1, entry 8). Since F87A is present in many P450$_{BM3}$ variants engineered as hydroxylation catalysts, we elected to screen a set of F87A variants to determine if this mutation would continue to provide selectivity for the *α*-position in those variants and whether the additional mutations could further increase activity. In all cases tested, the F87A mutation favored *α*-amination, albeit with low levels of activity (Table 1, entries 9−11). The most active variant is P411$_{BM3}$-T268A-F87A (three mutations from wild type), providing 187 TTNs and modest selectivity (30:70 (**2**:**3**)) for the *α*-amination product (Table 1, entry 9). Reverting the F87A mutation to F87V (the mutation present in the P411$_{BM3}$-CIS backbone) switched the selectivity to the *β*-position, demonstrating the importance of the residue at this position for controlling C−H amination regioselectivity (Table 1, entry 12). We were also interested in testing the impact of F87A in the presence of the I263F mutation. The double F87A+I263F variant continued to be selective for *β*-amination, but with substantially decreased selectivity and activity (Table 1, entry 13). These results support the lynchpin role of the F87 position in controlling the regioselectivity of amination.

Table 1. Comparison of Activities (TTN) and Regioselectivities of P411$_{BM3}$ Variants for the Reaction of Azide 1 to Sultams 2 and 3



entry	Catalyst	TTN[a]	**2**:**3**
1	P411$_{BM3}$-CIS-T438S	32	84:16
2	P411$_{BM3}$-H2-5-F10	56	53:47
3	P411$_{BM3}$-H2-4-D4	19	66:44
4	P411$_{BM3}$-H2-A10	31	44:54
5	**P411$_{BM3}$-CIS-T438S-I263F**	**361**	**97:3**
6	P450$_{BM3}$-CIS-T438S-I263F	<1	n.d.
7	P411$_{BM3}$-CIS-T438S-I263F-A268T	12	33:64
8	P411$_{BM3}$-CIS-T438S-F87A	70	25:75
9	**P411$_{BM3}$-T268A-F87A**	**187**	**30:70**
10	P411$_{BM3}$-B1-T268A-M263I	35	38:62
11	P411$_{BM3}$-Man1-T268A	7	34:66
12	P411$_{BM3}$-T268A-F87V	25	99:1
13	P411$_{BM3}$-CIS-T438S-I263F-F87A	10	66:34

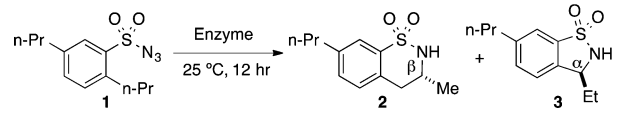
[a]TTN = Total turnover numbers. Reaction conditions and protein sequences are described in the Supporting Information. TTNs and regioselectivities were determined by HPLC analysis.

Having identified two enzyme variants with divergent regioselectivity, P411$_{BM3}$-CIS-T438S-I263F and P411$_{BM3}$-T268A-F87A, we explored the ability of these enzymes to control regioselectivity and enantioselectivity on different substrates (Table 2). In addition to providing excellent regioselectivity, P411$_{BM3}$-CIS-T438S-I263F and P411$_{BM3}$-T268A-F87A furnished sultams **2** and **3** with excellent enantioselectivity (99.5:0.5 er in both cases) (Table 2, entries 1 and 2). These enzymes are effective at controlling regioselectivity for substrates bearing longer alkyl chains, although with diminished activity (Table 2, entry 3). Surprisingly, P411$_{BM3}$-T268A-F87A affords even greater regioselectivity (3:97 (**5**:**6**)) for *α*-amination on these substrates when compared to the parent substrate (Table 2, entry 4). Changing the substituent on the aromatic ring from an alkyl group to an ester did not negatively impact the reaction. The two variants continued to strongly favor their respective regioisomers, with good yields and comparable enantioselectivies (Table 2, entries 5 and 6).

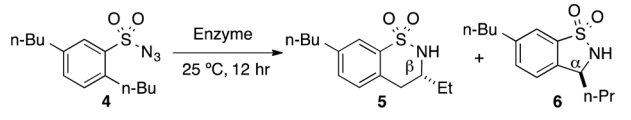
In order to gain insight into how these enzymes determine regioselectivity, we considered the possibility of mechanistic differences between amination at the *α*-position and *β*-position. To probe this, we measured the kinetic isotope effects with **1** and **D$_{14}$-1**. When P411$_{BM3}$-CIS-T438S-I263F was tested, a ^{1}H-KIE value of 2.8 was observed, whereas P411$_{BM3}$-T268A-F87A afforded a ^{1}H-KIE value of 3.0 (Figures S3 and S4). These values are consistent with C−H abstraction being rate-determining in the catalytic cycle and suggest a similar C−H

dx.doi.org/10.1021/ja509308v | J. Am. Chem. Soc. 2014, 136, 15505−15508

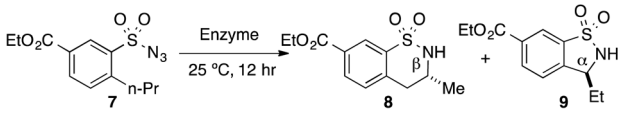
Table 2. Comparison of Activities (TTN), Regioselectivities, and Enantioselectivities for Azides 1, 4, and 7 with $P411_{BM3}$-CIS-T438S-I263F and $P411_{BM3}$-T268A-F87A



Variant	TTN	Selectivity (2:3)	er[a](2)	er[a](3)
$P411_{BM3}$-CIS-T438S-I263F	361	97:3	99.5 : 0.5	
$P411_{BM3}$-T268A-F87A	187	30:70		99 : 0.5



Variant	TTN	Selectivity (5:6)	er[a](5)	er[a](6)
$P411_{BM3}$-CIS-T438S-I263F	178	90:10	99.5 : 0.5	
$P411_{BM3}$-T268A-F87A	128	3:97		99.5 : 0.5



Variant	TTN	Selectivity (8:9)	er[a](8)	er[a](9)
$P411_{BM3}$-CIS-T438S-I263F	192	95:5	98.5 : 1.5	
$P411_{BM3}$-T268A-F87A	130	5:95		99.5 : 0.5

[a]er = enantiomeric ratio.

cleavage mechanism despite the divergent selectivities. Since C−H abstraction is kinetically controlled, reactivity depends on the proximity of the C−H bond to the metal nitrenoid. In light of the exquisite regio- and enantioselectivities provided by the two P411 variants, we hypothesize that the enzyme active sites situate the substrate such that a different C−H bond is kinetically accessible in each variant.

To aid in understanding how the active-site architecture of these P411 enzymes controls regioselectivity, we pursued their structural characterization through X-ray crystallography. Although high-quality crystals of $P411_{BM3}$-T268A-F87A were not forthcoming, crystals of $P411_{BM3}$-CIS-T438S-I263F diffracted to 2.66 Å, and molecular replacement readily yielded a structure (PDB = 4WG2). This new structure represents a substantial improvement on the previously reported $P411_{BM3}$-CIS structure, which was determined at 3.3-Å resolution.[12a] The global features remain identical, but the higher resolution data enable more-accurate placement of the side chains lining the active site, the heme vinyl and propionate moieties, and the position of the L437 side chain (Figure S1).[12a] Importantly, the F263 side chain is resolved and populates a non-favored rotamer extending into the active site. Interestingly, the location of the F263 side chain does not substantially change the location of the I-helix (on which F263 resides) by comparison to the I263 parent. It does, however, cause repacking of the flanking residues on the F-helix (Figure S1). Alignment of the structure of $P411_{BM3}$-CIS-T438S-I263F with that of wild-type $P450_{BM3}$ bound to palmitoic acid shows that I263F occludes binding of the native substrate. This is consistent with previous reports that showed that the I263F mutation shut down the native hydroxylation activity (Figure S2).[23] Docking the sultam product into the active site clearly demonstrates that I263F is positioned to pack against the benzene ring of the substrate. These complementary van der Waals interactions could decrease the conformational freedom of the nitrenoid intermediate and thereby promote reactivity at the higher-energy C−H bond (Figure S5).

In summary, we have prepared $P411_{BM3}$ enzyme variants that offer different and complementary regioselectivities for C−H amination. Mutation at the F87 position is crucial for controlling selectivity, with F87V favoring the β-amination of 2,5-disubstituted benzenesulfonyl azides whereas F87A favors α-amination. Introduction of phenylalanine at position I263 provides a 11-fold increase in β-amination activity. Given that the C−H cleavage is rate-limiting, regioselectivity is likely kinetically controlled, wherein the C−H bond cleaved is the one closest to the iron-nitrenoid, as dictated by the enzyme. Crystallographic analysis reveals that the I263F mutation has little effect on the secondary and tertiary structure of the protein and primarily decreases the volume of the active site.

While catalyst-controlled divergent regioselectivity remains a challenge for small-molecule catalysts, enzyme catalysts can be engineered readily by mutagenesis and screening for the desired selectivity. Combined with their ability to take on non-natural activities such as direct C−H amination,[24] enzymes represent a versatile platform for catalyst development to solve challenging selectivity problems in organic chemistry.

■ ASSOCIATED CONTENT

Supporting Information

Text, tables, and figures describing methods and results for reaction setup and quantitation, determination of reaction rates, and enzyme engineering. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. S. Virgil and the 3CS Catalysis Center at Caltech for assistance with HPLC and LC-MS analyses, and Pavle Nikolovski and the Molecular Observatory for assistance with X-ray crystallography and the Gordon and Betty Moore Foundation, the Beckman Institute, and the Sanofi-Aventis Bioengineering Research Program at Caltech for their generous support of the Molecular Observatory at Caltech. Operations at SSRL are supported by the US DOE and NIH. This work was supported by the Department of the Navy, Office of Naval Research (grant N00014-11-1-0205), and by the Jacobs Institute for Molecular Engineering for Medicine at Caltech. T.K.H. and J.A.M. are supported by Ruth L. Kirschstein National Research Service Awards (F32GM108143 and F32GM101792). C.C.F. is supported by an NSF Graduate Research Fellowship.

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