Enzymatic C–H Amination

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Enantioselective Intramolecular C–H Amination Catalyzed by Engineered Cytochrome P450 Enzymes In Vitro and In Vivo**

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Dedicated to Peter Hayes

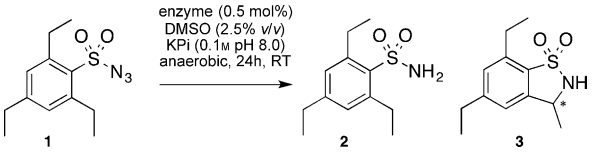
Iron-containing monooxygenases play diverse roles in nature, which range from the primary metabolic functions of alkane hydroxylases to the xenobiotic detoxification and secondary metabolic roles of cytochrome P450 enzymes.[1] Common to these enzymes is the ability to reductively activate molecular oxygen to generate highly electrophilic oxygen species, whose reactivity is comparable with that of "oxenes" (oxygen atoms that contain six valence electrons).[2] P450 enzymes in particular possess the remarkable ability to insert oxygen atoms at virtually any position within otherwise unreactive carbon skeletons, leading to the introduction of hydroxy or epoxide functionalities in diverse natural products.

Whereas enzymes are capable of inserting oxygen atoms into even unactivated C–H bonds, the sites into which nitrogen atoms can be incorporated are more constrained. Transaminases, ammonia lyases, and amino acid dehydrogenases, for example,[3] target oxidized or otherwise chemically activated carbon atoms during reaction. Enzymes that catalyze the concerted oxidative amination of C–H bonds are apparently absent from nature's repertoire of chemical catalysts.

Synthetic chemists, who are not limited to biologically accessible reagents and metals, have developed highly useful methods for the oxidative formation of C–N bonds.[4] These C–H amination reactions often proceed via a nitrenoid intermediate that has no parallel in natural enzymes. Although these reactions do not require preoxidized or otherwise activated carbon atoms, they do require specialized nitrene precursors, such as azides, haloamines, or iminoiodinanes. Of the many transition-metal catalysts based on Rh, Ru, Mn, Co, and Fe that catalyze intra- and intermolecular C–H amination, we were especially interested in the metal–porphyrin systems,[5] which react with iminoiodinanes in the +3 oxidation state to catalyze nitrene transfers that are isoelectronic with the well-established formal oxene transfer reaction of ferric P450 enzymes with iodosylbenzene.[5a,6] Trace levels (3 total turnovers; TTNs) of intramolecular C–H amination were reported more than 25 years ago for mammalian cytochrome P450 preparations reacting with iminoiodinanes.[7] We decided to revisit the possibility of finding or engineering an enzyme catalyst for this useful and challenging transformation.

Iminoiodinanes are problematic for biocatalytic application, given their polymeric nature[8] and insolubility[9] in aqueous media. As an alternative to iminoiodinanes, we decided to focus on the synthetic reaction of sulfonylazides with reduced (+2 oxidation state) metal–porphyrin systems.[10] We reasoned that such a "chemomimetic" approach to achieve direct C–H to C–N conversion could provide a biocatalytic route to amines and amides using biochemically compatible and atom-efficient azide-based nitrene precursors, with the usual advantages of enzymes, that is, selectivity and mild reaction conditions. Here we report the first highly active enzyme catalysts for C–H amination.

In initial experiments, we tested a series of 24 purified cytochrome $P450_{BM3}$ variants developed for monooxygenation reactions. Enzymes were reacted with 2,4,6-triethylbenzene-1-sulfonylazide (**1**) under anaerobic, reducing conditions at an enzyme loading of 0.5 mol% in aqueous media (phosphate buffer, 2.5% *v*/*v* DMSO; Scheme 1). Most reactions gave sulfonamide **2** as the major product, though all of the tested enzymes, including the wild type (4 TTN), gave small amounts of the C–H amination product **3**. The most active enzyme (28 TTN) for C–H amination in the initial



Scheme 1. Initial reaction used for the screening of enzymes. KPi = potassium phosphate.

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screen contained only a single mutation (T268A) relative to wild-type $P450_{BM3}$. Although mutations to the active-site residue T268 are deleterious to monooxygenation activity, given its role in promoting O–O bond scission, the T268A mutation was recently shown to promote $P450_{BM3}$-catalyzed carbene transfers to give cyclopropanes.[11] Thus, in spite of the significant differences between carbene and nitrene chemistry, we proceeded to test several $P450_{BM3}$-based cyclopropanation catalysts, including several serine–heme-ligated 'P411'[12] enzymes (so called because the Soret peak in the spectrum of the ferrous CO-bound enzyme is shifted to 411 nm rather than 450 nm for cysteine-ligated enzymes) in which the axially coordinating cysteine C400, which is absolutely required for monooxygenation activity, is mutated to serine. Given that loss of dinitrogen from azides is much more facile than O–O bond scission catalyzed by P450 enzymes, we reasoned that the "thiolate push" of the axial cysteine would be unnecessary for C–H amination.[13] The most active enzyme here was the C400S mutant $P411_{BM3}$-CIS (14 mutations from the wild type), which also contained the T268A mutation and supported over 140 total turnovers (73% yield of **3** according to HPLC analysis). Variant $P450_{BM3}$-CIS, which lacks the C400S mutation at the axial heme ligand, was significantly less active (9 TTN), indicating that serine–heme ligation strongly enhances BM3-catalyzed C–H amination. The $P450_{BM3}$-C400S single mutant ($P411_{BM3}$) also exhibited markedly improved activity (49 TTN) relative to its cysteine-ligated counterpart $P450_{BM3}$ (4 TTN).

To clarify the roles of the T268A and C400S mutations in BM3-catalyzed amination, we performed further experiments at 0.1 mol% catalyst loading with the $P450_{BM3}$-T268A and $P411_{BM3}$ (BM3-C400S) single mutants as well as the T268A/C400S double mutant in reaction with sulfonyl azide **1** (Table 1). We found that the T268A and C400S mutations combined to result in a highly active enzyme (120 TTN for the double mutant versus 310 TTN for $P411_{BM3}$-CIS; Table 1), thus indicating that the T268A and C400S mutations were major contributors to the high activity of $P411_{BM3}$-CIS. In fact, reverting the T268A mutation in $P411_{BM3}$-CIS markedly reduced activity (82 TTN).

Control experiments showed that the enzyme-catalyzed reaction was inhibited by carbon monoxide, air, and heat denaturation of the enzyme, suggesting that catalysis occurs at the enzyme-bound heme (see Table S2 in the Supporting Information). Hemin was also capable of catalyzing this reaction when reduced with dithionite (Figure 1, Table S3).

Table 1: Comparison of activities (TTN) and enantioselectivities of purified P450 and P411 variants (0.1 mol% catalyst loading) for the reaction of azide **1** to sulfonamide **2** and benzosultam **3**.

In vitro catalyst	TTN[a]	*ee* [%][b]
$P450_{BM3}$	2.1	n.d.
$P450_{BM3}$-T268A	15	36
$P411_{BM3}$	32	20
$P411_{BM3}$-T268A	120	58
$P411_{BM3}$-CIS	310	67
$P411_{BM3}$-CIS-A268T	82	47
$P411_{BM3}$-CIS-T438S	383	73

[a] TTN = total turnover number. [b] (*S*-*R*)/(*S*+*R*). n.d. = not determined. Reaction conditions described in the Supporting Information. TTNs and enantioselectivities determined by HPLC analysis.

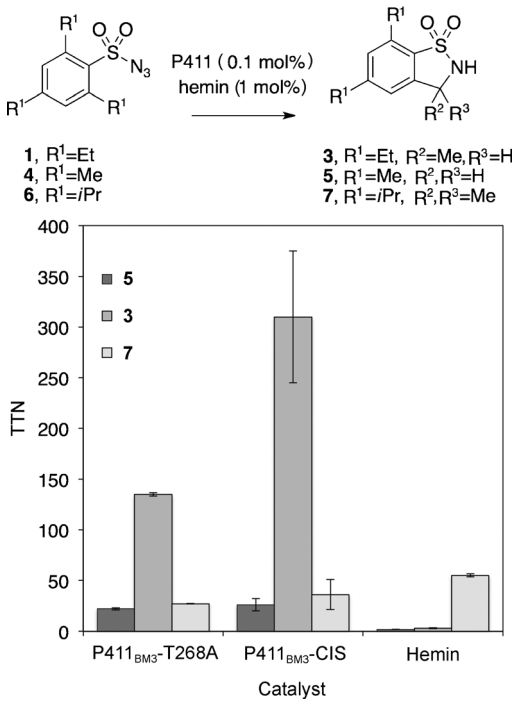

Figure 1. Substrate selectivity of P411 enzymes compared with free hemin. Compounds used to test the dependence of amination activity on C–H bond strength in reactions catalyzed by enzyme (0.1 mol%) or hemin (1 mol%) are shown. Reaction conditions described in the Supporting Information.

The observation that hemin can catalyze this reaction is consistent with earlier work in which the activity of metal–porphyrin systems in the reaction with sulfonyl azides was investigated.[14] However, whereas in vitro enzyme reactions with prochiral substrate **1** resulted in asymmetric induction (up to 73% *ee*, Table 1), reaction with hemin unsurprisingly gave only racemic **3**, strongly suggesting that BM3-catalyzed amination occurs within the chiral environment of the enzyme active site. Addition of substoichiometric amounts of NADPH was sufficient for activity (Table S4), thus supporting the hypothesis that ferrous heme is the azide-reactive state, akin to P450-catalyzed cyclopropanation.[11] Dithionite could be used in place of NADPH to drive catalysis, and its effect was comparable to that of NADPH for both cysteine- and serine-ligated enzymes $P450_{BM3}$-T268A and $P411_{BM3}$-T268A (Table S5), thus indicating that reduction to ferrous heme was not limiting.

To examine the effect of C–H bond strength on amination activity, $P411_{BM3}$-CIS and $P411_{BM3}$-T268A were reacted with the trimethyl and triisopropyl analogues of **1** (substrates **4** and **6**, respectively). The desired benzosultam products were obtained, though the productivity was lower with both substrates (Figure 1, Table S3). The activity of free hemin inversely correlated with the strength of the substrate C–H bond, consistent with earlier work on cobalt–porphyrin

catalysts,[14] showing no measurable activity on substrate **4**, minimal activity on substrate **1** (3 TTN), and the highest activity on substrate **6** (55 TTN). The differing patterns of activity observed with hemin- and enzyme-catalyzed reactions suggest that the enzyme itself plays a critical role in catalyzing C–N bond formation beyond providing a chiral active site that guides the stereochemical outcome of the reaction. In particular, enzyme reactions with triethyl and trimethyl sulfonylazide substrates **1** and **4** were markedly more productive than the corresponding hemin reactions. The reduced activity of the enzyme toward triisopropyl-bearing substrate **6** suggests that the structure of the active site currently favors smaller substrates, though it is likely that this can be modulated by further enzyme engineering.

To determine whether this new reactivity could be exploited in vivo, we next investigated whether these enzymes, expressed in intact *E. coli* cells, could catalyze amination reactions when provided with azide substrate. Remarkably, both the $P411_{BM3}$-T268A and $P411_{BM3}$-CIS enzymes were highly active on **1**, catalyzing hundreds of turnovers ($P411_{BM3}$-T268A: 245 TTN, 89% *ee*; $P411_{BM3}$-CIS: 680 TTN, 60% *ee*) under anaerobic conditions with added glucose (Table 2, and Table S6 in the Supporting Information). Lyophilized cells that contain $P411_{BM3}$-CIS could also support catalysis, with productivity similar to freshly prepared cell suspensions (750 TTN, 61% *ee*). Enantioselectivity was comparable or enhanced for whole-cell catalysts relative to purified enzymes (Table 2). The introduction of the previously characterized T438S mutation into $P411_{BM3}$-CIS strongly increased enantioselectivity (430 TTN, 86% *ee*).[11,15] Optimization of expression conditions increased the productivity of whole-cell C–H amination catalysts, enabling conversions to **3** of up to 66% in small-scale reactions (Table S7). Inspired by the simplicity of employing whole cells as amination catalysts, we performed a reaction on preparative scale (50 mg) using anaerobic resting cells that expressed the $P411_{BM3}$-CIS-T438S catalyst to afford sultam **3** (77% yield according to HPLC, 69% yield of isolated product, 87% *ee*). The level of stereoselectivity attained with whole-cell catalysts compared favorably with a previously reported chiral C–H amination catalyst, which gave 88% *ee* in reaction with substrate **1**.[16]

Table 2: Comparison of C–H amination activities (TTN) of intact *E. coli* cells expressing P450 and P411 variants.

In vivo catalyst	[P450] or [P411] [μM]	Yield of **3** [%]	TTN[a]	*ee* [%][b]
pCWori-empty	0	0	0	n.d.
$P450_{BM3}$	6.6	0.5	5.1	n.d.
$P450_{BM3}$-T268A	5.8	7.8	26	84
$P411_{BM3}$	4.3	6.7	29	16
$P411_{BM3}$-T268A	2.2	30	250	89
$P411_{BM3}$-CIS	1.4	46	680	60
$P411_{BM3}$-CIS-T438S	2.7	58	430	87

[a] TTN = total turnover number. [b] $*(S\text{-}R)/(S+R)$. n.d. = not determined. Reaction conditions described in the Supporting Information.

The beneficial effects of the T268A and C400S mutations for C–H amination is striking in that both residues play critical roles in P450-catalyzed monooxygenation.[17] While important for protonation of iron–peroxo intermediates, which occurs during dioxygen activation, T268 may sterically hinder the binding of bulkier azide substrates in C–H amination. Consistent with a steric influence, the T268A mutation enhances the stereoselectivity of C–H amination. Furthermore, it also strongly impacts the diastereo- and enantioselectivity of styrene cyclopropanation.[11] While thiolate ligation is thought to be essential for O–O bond scission and to enhance the basicity of reactive oxygen intermediates,[13,18] we found that mutation of this key residue leads to a high in vitro amination activity (Table 1). The observation that mutations to both T268 and C400 appear necessary for enzymatic C–H amination suggests that naturally occurring P450s, in which these two residues are highly conserved, will likely be poor catalysts of C–H amination.

Many enzyme-catalyzed reactions, such as ketoreduction, monooxygenation, and transamination, are increasingly useful in chemical synthesis,[19] and the development of applications with these and other naturally occurring reaction types will continue. However, it is no longer necessary to limit biocatalysis to reactions that have natural antecedents.[11,20] Rather, the scope of biocatalysis can be expanded by directing natural enzymes to imitate the artificial, thus developing new methodologies by judicial choice of reaction conditions, synthetic reagents, and protein engineering.

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