Navigating the Unnatural Reaction Space: Directed Evolution of Heme Proteins for Selective Carbene and Nitrene Transfer

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CONSENSUS: Despite the astonishing diversity of naturally occurring biocatalytic processes, enzymes do not catalyze many of the transformations favored by synthetic chemists. Either nature does not care about the specific products, or if she does, she has adopted a different synthetic strategy. In many cases, the appropriate reagents used by synthetic chemists are not readily accessible to biological systems. Here, we discuss our efforts to expand the catalytic repertoire of enzymes to encompass powerful reactions previously known only in small-molecule catalysis: formation and transfer of reactive carbene and nitrene intermediates leading to a broad range of products, including products with bonds not known in biology. In light of the structural similarity of iron carbene (Fe≡C(R¹)(R²)) and iron nitrene (Fe≡NR) to the iron oxo (Fe≡O) intermediate involved in cytochrome P450-catalyzed oxidation, we have used synthetic carbene and nitrene precursors that biological systems have not encountered and repurposed P450s to catalyze reactions that are not known in the natural world. The resulting protein catalysts are fully genetically encoded and function in intact microbial cells or cell-free lysates, where their performance can be improved and optimized by directed evolution. By leveraging the catalytic promiscuity of P450 enzymes, we evolved a range of carbene and nitrene transferases exhibiting excellent activity toward these new-to-nature reactions. Since our initial report in 2012, a number of other heme proteins including myoglobin, protoglobin, and cytochromes c have also been found and engineered to promote unnatural carbene and nitrene transfer. Due to the altered active-site environments, these heme proteins often displayed complementary activities and selectivities to P450s. Using wild-type and engineered heme proteins, we and others have described a range of selective carbene transfer reactions, including cyclopropanation, cyclopropenation, Si–H insertion, B–H insertion, and C–H insertion. Similarly, a variety of asymmetric nitrene transfer processes including aziridination, sulfide imidation, C–H amidation, and, most recently, C–H amination have been demonstrated. The scopes of these biocatalytic carbene and nitrene transfer reactions are often complementary to the state-of-the-art processes based on small-molecule transition-metal catalysts, making engineered biocatalysts a valuable addition to the synthetic chemist’s toolbox. Moreover, enabled by the exquisite regio- and stereocontrol imposed by the enzyme catalyst, this biocatalytic platform provides an exciting opportunity to address challenging problems in modern synthetic chemistry and selective catalysis, including ones that have eluded synthetic chemists for decades.

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group became interested in expanding the already immense catalytic repertoire of P450s to encompass reactions not known in the biological world. Inspiration came from two sources: (1) Evolution does it—new activities arose during P450 evolution, and there was no reason to think that further innovation was not possible using evolutionary engineering methods. (2) Creative chemists had already shown that the heme cofactor and various derivatives can catalyze reactions not known in nature.26

Scheme 1. Native and Engineered Activity: P450BM3-Catalyzed C−H Hydroxylation

(A) C−H hydroxylation using wild-type P450BM3

(B) C−H hydroxylation using engineered P450BM3 35-E11

Of special interest to us were the myriad carbene30–33 and nitrene14–36 transfer reactions that synthetic chemists had developed. Metal porphyrin complexes26,27 including Fe porphyrins, are privileged catalysts for such transformations. As the vital intermediates in these metalloporphyrin-catalyzed processes, metal carbenes and nitrenes supported by the porphyrin ligand have been extensively investigated. In light of the structural similarity between these synthetic Fe carbenes and Fe nitrenes and the native Fe oxene (compound I), we thought we should be able to generate Fe carbene and nitrene intermediates in P450s by exploiting synthetic precursors not present in nature (Scheme 2). Furthermore, we posited that the selective transfer of these reactive species to organic substrates within the enzyme active site would lead to a similarly broad spectrum of synthetically useful transformations.

In 2012 and 2013, we described repurposing P450s for new-to-nature carbene and nitrene transfer reactions.1,37 Since then, a plethora of biocatalytic stereoselective C−C, C−N, C−Si, and C−B bond forming processes, including ones that are difficult or impossible to achieve with current small-molecule catalysts, have been developed by us and by other research groups who have joined this exciting journey of discovery. In this Account, we survey the evolution of heme proteins for non-natural carbene and nitrene transfer reactions in our lab. We also present the conceptual advances and strategies that were pivotal to this development.

Scheme 2. Structural Similarity of Fe Oxene, Carbene, and Nitrene

A signiificant fraction of enzymes assemble and exploit metal ions for catalysis. These metalloenzymes catalyze some of the most challenging biological reactions, including photosynthesis,9 nitrogen fixation,9 methane oxidation10 and methylmalonyl-CoA isomerization,11 with unique reactivity not observed in small-molecule organometallic complexes or enzymes based on purely organic cofactors.

Our laboratory has been fascinated by enzymes of the iron-containing cytochromes P450 (CYPs) family for decades. P450s constitute a subset of hemoproteins using the heme prosthetic group for their native functions.12,13 Found in all kingdoms of life, P450s catalyze extraordinary oxygenation reactions under exceptionally mild conditions.14–16 In these P450-catalyzed oxidation reactions, a Fe(IV)O porphyrin radical cation species (compound I) is the key oxidizing intermediate for oxene transfer reactions.17 Nature has tamed this intermediate and diversified its reactivity for a vast array of oxidative transformations.

Using P450BM3 from Bacillus megaterium (CYP102A1),18 a self-sufficient P450 naturally fused to its diheme redox partner, our group19–23 and others12,18,24,25 have engineered highly active P450 variants for the oxidation of simple alkanes and other organic substrates (Scheme 1). Starting a decade ago, our

INTRODUCTION

Nature’s malleable protein catalysts, enzymes, are capable of facilitating highly demanding transformations with exquisite control.2,5 A significant fraction of enzymes assemble and exploit metal ions for catalysis. These metalloenzymes catalyze some of the most challenging biological reactions, including photosynthesis,9 nitrogen fixation,9 methane oxidation10 and methylmalonyl-CoA isomerization,11 with unique reactivity not observed in small-molecule organometallic complexes or enzymes based on purely organic cofactors.

In the history of evolution, this promiscuity has played a central role in the emergence of new biological functions.41 In recent time, could create catalytic novelty. However, evolution has done it countless times. Less appreciated perhaps by the admirers of enzymes’ specificity and selectivity is the fact that many enzymes are also catalytically “promiscuous”38–40 they are capable of catalyzing, at some level, chemical reactions unrelated to their biological functions. In the history of evolution, this promiscuity has played a central role in the emergence of new biological functions.41 In recent years, the significance of catalytic promiscuity has been increasingly recognized, and the structural, mechanistic, and evolutionary implications of catalytic promiscuity are being
In contrast, variant P450BM3-CIS (9-10A-TS F87 V T268A) exhibited the desired activity, but a large in-house collection of P450BM3 variants accumulated during our research on P450 oxygenation proved instrumental to showing that selectivity and activity were readily tunable by protein engineering. Upon evaluating a panel of P450BM3 variants, we found that several displayed dramatically enhanced cyclopropanation activity relative to hemin in water, as well as promising enantioselectivity and complementary diastereoselectivity (Scheme 3).

Scheme 3. P450-Catalyzed Diastere- and Enantioselective Cyclopropanation of Styrenes (2012)

``TTN = total turnover number. Conditions: 30 mM styrene, 10 mM EDA, 0.2 mol % catalyst, and 10 mM Na₂S₂O₄.
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Preclinically, P450BM3 with a single T268A mutation exhibited excellent trans selectivity for the cyclopropanation of styrene. In contrast, variant P450BM3-CIS (9-10A-TS F87 V T268A) showed promising cis selectivity, which is rarely observed in small-molecule-catalyzed cyclopropanation processes. It also represented a departure from the innate diastereoselectivity of Fe porphyrin-catalyzed cyclopropanation, highlighting the role of the protein secondary coordination sphere in controlling the reaction selectivity.

Mutation T268A was found to be generally activating for cyclopropanation in a series of P450BM3 variants, likely by enlarging the active site for carbene transfer. The T268 residue in P450BM3 operates as the essential hydrogen bond donor in the cleavage of the O−O bond in the native O₂ activation and oxygenation reaction. Previous investigation showed that replacing this threonine with an alanine led to a substantial decrease in the native oxygenase activity. The activating effect of T268A in cyclopropanation thus implies fundamental mechanistic dichotomy between the native oxene transfer and the non-native carbene transfer chemistry.

AXIAL SERINE-LIGATED “P411” FOR CARBENE TRANSFER

For cyclopropanation, the P450 has to be reduced to its ferrous state to initiate the catalytic cycle. In our early work, P450s were used either as purified protein or in cell-free extract in the presence of exogenous reducing agents such as sodium dithionite (Na₂S₂O₄). We reasoned that we would be able to develop an in vivo cyclopropanation if the heme enzyme could be readily reduced by endogenous reducing agents such as NAD(P)H. Such whole-cell biocatalysts would greatly simplify the protein engineering process and enable new routes to the sustainable production of valuable compounds.

We replaced the cysteine thiolate ligand in P450BM3 with a weakly donating serine (Scheme 4). The CO-binding assay of this engineered P450 variant revealed a ferrous CO Soret peak at 411 nm instead of 450 nm in the UV–vis spectrum, which is why we refer to the axial-serine-ligated P450 variants as “P411” enzymes. Replacement of this critical Cys residue was known to abolish its monooxygenation activity; in fact, the cysteine thiolate ligand is the only amino acid residue that is conserved in all known functional P450s. Electrochemical measurements indicated that the redox potential of (Fe(III)/Fe(II)) of P411 shifted to −293 mV, which is well within the range of reduction by NADPH (E° = −320 mV). P411BM3-CIS demonstrated dramatically enhanced in vivo activity for cyclopropanation. By further increasing the substrate concentration, total turnover numbers of up to 67 800 were achieved in whole Escherichia coli cells (Scheme 4). This represented the first highly active biocatalyst for carbene transfer. The advent of P411s turned out to be a key advance for biocatalytic carbene and nitrene transfer reactions. In subsequent studies, we found that serine-ligated P411s were uniquely active for a range of new-to-nature reactions where P450s displayed no or negligible activity.

FURTHER DEVELOPMENT OF BIOCATALYTIC CYCLOPROPANATION

Expansion of biocatalytic cyclopropanation followed two directions: (1) We further engineered P450BM3 variants to broaden the substrate scope of cyclopropanation, and (2) we explored other heme proteins for biocatalytic cyclopropanation. In subsequent P450BM3 engineering, we continued to explore the role of the Fe-binding axial ligand. Heme proteins naturally use a range of axial ligands, including cysteine, histidine, and tyrosine. The identity of the axial ligand profoundly influences the catalytic activity of heme enzymes.

Starting from P450BM3 T268A C400H, iterations of site-saturation mutagenesis (SSM) and screening furnished P450BM3 Hstar (Schemes 6), a highly active catalyst for the cyclopropanation of a wide range of γ-substituted acrylamides.
and acrylates (Scheme 7).\textsuperscript{53} Subsequent studies on the inactivation mechanism of P450BM3 Hstar revealed that undesired modification of nucleophilic amino acid residues took place following reaction with the incipient electrophilic Fe carbene.\textsuperscript{54} To reduce inactivation, we performed site-directed mutagenesis of residues prone to modification, which afforded a further improved biocatalyst, P450BM3 Hstar H92N H100N (>20 000 total turnover number, TTN).\textsuperscript{54} Deep mutational screening of the serine-ligated P411BM3-CIS was also fruitful. Directed evolution of P411BM3-CIS led to a set of complementary catalysts, P411-VAC\textsubscript{cis} and P411-VAC\textsubscript{trans}, providing access to both the \textit{cis}- and \textit{trans}-diastereomers of heteroatom-substituted cyclopropanes (X = N, O, and S, Scheme 8).\textsuperscript{55} Later on, using P411-VAC\textsubscript{cis} as the template, single mutants P411-VAC\textsubscript{cis} V87C and P411-VAC\textsubscript{cis} V87F were found to furnish the \textit{cis}- and \textit{trans}-product, respectively, in the cyclopropanation of terminal aliphatic alkene.\textsuperscript{56} Cognizant of the vast structural diversity of hemoproteins, we surmised that stereoselectivities not found in P450BM3-catalyzed processes could be achieved by exploring other classes of heme proteins with altered active-site environments. Prior to this study, elegant work from the Fasan laboratory demonstrated the utility of engineered myoglobins in carbene transfer.\textsuperscript{57} We focused on thermophilic heme proteins due to their superior stability and evolvability.\textsuperscript{56} We found that wild-type Aeropyrum pernix (ApePgb) and \textit{Rhodothermus marinus} nitric oxide dioxygenase (RmaNOD) furnished measurable activity for the cyclopropanation of 1-octene (Scheme 9). Notably, RmaNOD and ApePgb displayed opposite diastereoselectivity, and evolved variants RmaNOD Q52V and ApePgb W59A Y60G F145W furnished the (1\textit{S}, 2\textit{S})- or (1\textit{R}, 2\textit{S})-product with excellent stereocontrol. Combined with P411-VAC\textsubscript{cis} V87C and P411-VAC\textsubscript{cis} V87F, we developed a stereodivergent platform to access all four stereoisomers of cyclopropanation products with excellent diastereo- and enantiocontrol.
With a series of evolved biocatalysts, we applied these heme proteins in the stereoselective preparation of key cyclopropane intermediates of pharmaceuticals, including levomilnacipran and ticagrelor (Scheme 10). In particular, a truncated globin from *Bacillus subtilis* was evolved to provide the desired stereoisomer of the ticagrelor cyclopropyl ester precursor with excellent diastereo- and enantioselectivity. Fasan’s group also demonstrated the gram-scale syntheses of tasimelteon and a TRPV1 inhibitor using their evolved myoglobin variants in whole-cell transformations. Collectively, these results showcased the potential utility of non-natural biocatalysis in the manufacturing of value-added pharmaceuticals.

### BIOCATALYTIC CYCLOPROPENATION AND BICYCLOBUTANATION

Cyclopropenes are highly strained carbocycles. With strain release, cyclopropenes can be easily converted to a broad spectrum of synthetically useful molecular architectures. Asymmetric catalytic cyclopropenation of alkynes is one of the most widely used methods for the preparation of these motifs in an enantiopure form. However, in contrast to Rh-, Cu-, Ir-, and Co-based catalysts, no Fe porphyrin complexes were known to catalyze the cyclopropenation process.

Curious whether engineered P411s could catalyze cyclopropenation, we tested a terminal aliphatic alkyne as the substrate and found that a P411 variant (P-4) previously engineered for nitrene transfer could furnish the desired cyclopropene with modest activity (260 TTN, 91% ee). Using P-4 as the parent, SSM at position 87 was carried out, as residue 87 resides above the heme cofactor, and it is known to play an essential role in controlling the stereochemistry in native oxygenation reactions. The P-4 A87F mutant showed improved activity and enantioselectivity relative to its predecessor (290 TTN, 94% ee). Surprisingly, a single A87W mutation completely reversed the enantiomeric outcome of this cyclopropenation process, furnishing the desired cyclopropene with excellent enantiocontrol (240 TTN, 94% ee). The complete reversal of stereochemistry (+94% ee → −94% ee) upon the introduction of a single mutation, rarely observed in protein engineering, highlighted the outstanding evolvability of P411 biocatalysts. With the P-4 A87F and A87W mutants, additional rounds of SSM and screening ultimately produced variants P411−K10 and P411−C6. These final variants allowed for the enantiodivergent synthesis of cyclopropenes with excellent enantiocontrol (Scheme 11). Recently, using P411 variants further evolved from the C−H alkylation lineage, internal alkynes could also be effectively transformed, affording 1,2-disubstituted cyclopropenes in uniformly outstanding enantioselectivity (>99% ee, Scheme 12).

When aromatic alkynes were subjected to these enzymatic conditions, the carbene transfer did not stop at the cyclopropene stage. Instead, sequential delivery of the carbenoid fragment to the alkyne substrate afforded the highly strained bicyclobutane as the final product. This unusual double transfer required the same enzyme to accommodate both the alkyne and the cyclopropene substrate, indicating a distinct promiscuity of evolved P411. Furthermore, the bicyclobutane product formed with complete exo, endo selectivity. This diastereoselectivity differed from the endo, endo selectivity observed in the only known intermolecular bicyclobutanation, catalyzed by an osmium porphyrin. Thus, P411-catalyzed intermolecular bicyclobutanation represents a general method to prepare interesting bicyclobutanes using easily available starting materials.

### BIOCATALYTIC SI−H AND B−H INSERTION

Silicon is the second most abundant element in Earth’s crust. Despite the abundance of silicon in nature, enzymes capable of
catalyzing the construction of carbon—silicon bonds are not known.\textsuperscript{73} Prior art in metal-carbene chemistry demonstrated that the insertion of metal carbenoids into Si—H bonds could lead to the construction of C—Si bonds. We recognized that, by repurposing heme proteins to catalyze the carbenoid insertion into Si—H bonds, we could develop a biocatalytic system to forge Si—C bonds.

We were particularly interested in the Si—H insertion of \(\alpha,\alpha\)-disubstituted diazo compounds because the use of such carbenoid precursors would engender a stereogenic center at the \(\alpha\)-carbon (Scheme 14). Although P450s were ineffective in inducing appreciable enantiodifferentiation, we found that \textit{Rhodothermus marinus} cytochrome \(c\) (\textit{Rma cyt c}) readily promoted the desired B—H insertion reaction in living \textit{E. coli} cells, furnishing the corresponding enantioenriched organoborane product in 70% ee and 120 TTN. Directed evolution by targeting residues near the heme cofactor furnished \textit{Rma cyt c} \(V75R\ M100D\ M103T\) (BORR1), which provided the organoboron product in 2490 TTN and 95% ee.

A notable feature of this biocatalytic B—H insertion lies in its compatibility with an exceptionally broad range of \(\alpha,\alpha\)-disubstituted diazo compounds (Scheme 17).\textsuperscript{75,76} Using iterative SSM and screening, the BOR variants could be evolved quickly for the customized synthesis of diverse enantioenriched organoboron products that are versatile building blocks in organic synthesis. Flexible loop engineering\textsuperscript{7} proved particularly fruitful in the directed evolution of \textit{Rma cyt c} for borylation reactions. \(\alpha\)-Trifluoromethyl-substituted organoboranes\textsuperscript{76} and \(\alpha\)-boryl lactones\textsuperscript{77} could be synthesized with good enantioselectivity using BOR-CF\textsubscript{3}\textsuperscript{76} and BORLAC,\textsuperscript{77} respectively.

Collectively, the successful development of non-natural Si—H and B—H insertion reactions demonstrates a new strategy to unveil and augment the catalytic capability of proteins. Furthermore, the integration of enzymatic Si—H and B—H insertion into metabolic pathways in living cells could
potentially enable the production of unnatural organosilicon and organoboron compounds \textit{in vivo}, thus bringing new opportunities to synthetic biology.

\section*{Biocatalytic C–H Insertion}

From a reactivity perspective, the insertion of a metal carbenoid into C–H bonds is much more challenging than insertion into Si–H and B–H bonds. Due to the ubiquity of C–H bonds in organic molecules, site- and stereoselective C–H insertion reactions hold the promise to significantly simplify the syntheses of organic compounds. Unlike other late transition metals such as Rh, Cu, Ru, Ag, and Co, Fe catalysts displayed low activities for the catalytic insertion of carbenoids into C–H bonds in previous work.\textsuperscript{78,79} Prior to our work, a generally applicable Fe catalyst for the intermolecular C–H insertion was not available. Furthermore, iron-catalyzed enantioselective C–H insertion was recognized as a formidable challenge.

In our heme protein collections of P450s, P411s, cytochromes c, and globins, we found only two variants, P-4 A82L and Rma\_NOD Y32G, with measurable activity for the intermolecular C–H insertion reaction (TTN = 13 and 7, respectively), which underscored the challenges this transformation presents.\textsuperscript{3} Ultimately, however, directed evolution over 14 rounds of mutagenesis and screening culminated in the highly active variant P411CHF for C–H alkylation (Scheme 18). P411CHF differs from wild-type P450BM3 by 23 amino acids and lacks the FAD domain of the reductase domain. This truncated P411 variant catalyzed the carbene insertion into a variety of C(sp\textsuperscript{3})–H bonds, including benzylic, allylic, propargylic, and heteroatom-stabilized ones, with excellent enantioselectivity (Scheme 19). The use of acceptor carbenes for asymmetric C–H insertion is relatively underdeveloped with rhodium catalysts. Thus, these engineered P411s provided a new solution to enantioselective C–H insertion using acceptor carbenes. Furthermore, variants in the P411CHF lineage permitted the stereodivergent C–H insertion with \textit{N}-methyl tetrahydroquinoline, thus allowing for the formal synthesis of both enantiomeric forms of natural product cuspareine.

The biocatalytic enantioselective C–H insertion nicely illustrates the power of directed evolution in solving challenging problems in catalysis. Recently, we were able to engage $\alpha$-trifluoromethyl- and perfluoroalkyl-substituted diazo compounds\textsuperscript{10} in C–H alkylation (Scheme 20).\textsuperscript{51} A set of stereocomplementary P411 catalysts was engineered to access either enantiomeric trifluoroethylation product.
Mutation L401P dramatically enhanced the catalytic activity and the enantioselectivity of P411-PFA (1510 TTN, +88% ee → 4070 TTN, +98% ee). The L401 residue is located next to S400 bound to the heme cofactor. Previous study on another P450 enzyme, P450cam, showed that the leucine-to-proline mutation of the residue next to the heme-binding cysteine disrupted the hydrogen bonding network of the thiolate ligand, thus enhancing the donor strength of the proximal thiolate.82−84 Our finding showcased the importance of this hydrogen bonding network of the Fe-binding residue in abiological transformations.

Recently, Fasan,85 Koenigs,86 and our group87 all described biocatalytic methods for the C(sp2)−H functionalization of electron-rich heterocycles including indoles and pyrroles. We found that a range of indole substrates could be alkylated at the 3-position using evolved P411-HF (Scheme 21). When MeEDA was used as the diazo source, P411 biocatalysts could be evolved to furnish the alkylation product in an enantioselective fashion. This is a rare example of an evolved P411 enzyme showing promising stereocontrol for α,α-disubstituted diazo compounds. Prior to this, engineered Rma cyt c was the only protein class exhibiting good stereocontrol for these substrates.

Moreover, when pyrrole was used as the substrate, the site selectivity could be easily tuned using the P411 catalyst, leading to the C2 or C3 alkylation product with good regiocontrol.

\[ \text{BIOCATALYTIC C}−\text{H AMIDATION: INITIAL WORK} \]

Enantiopure amines represent key pharmacophores in small-molecule therapeutics.88 Consequently, general catalytic methods for stereoselective assembly of amines have long been sought after.89 In this context, catalytic nitrene transfer, i.e., the delivery of a nitrene fragment (R−N=) to organic substrates, is a powerful technique for the synthesis of valuable amines from readily available alkanes and alkenes. In a pioneering study published in 1985, Dawson and Breslow demonstrated that mammalian microsomal P450s could catalyze C−H amidation with a turnover frequency (TOF) of ca. 2 min⁻¹ using iminiodinanes as the nitrene precursor.90 Unfortunately, the rapid solvolysis of iminiodinanes91 made it challenging to further optimize this system.

Almost 30 years later, concurrent to our investigations into carbene transfer, we revisited this nitrene transfer chemistry and engineered P450BM3 variants to enhance the activity for C−H amidation (Scheme 22).37 We found sulfonyl azides to be superior nitrene precursors for biocatalytic amination reactions, in part due to the excellent water stability of organic azides. Similar to what we observed in P450BM3-catalyzed cyclopropanation, the C−H amidation also starts with ferrous heme, and the T268A and C400S mutations were found to be beneficial. Whole-cell biotransformations employing P411-CIS T438S (variant “P”) provided the sultam products with improved activity and enantioselectivity compared to the in vitro reaction. Parallel to our investigation, in 2014, Fasan and
co-workers also accomplished intramolecular C–H amidation using wild-type and engineered P450BM3.92

In a subsequent study, we demonstrated that either the six- or
the five-membered amidation product could be prepared with
excellent site selectivity in an enzyme-controlled fashion (Scheme 23).93 Specifically, the I263F variant was found to
favor the homobenzylic amidation, whereas the P450BM3
T268A F87A variant was selective for benzylic amidation. This
regiodivergent amidation showcased the power of enzymes to
override the innate regiochemical preference through the
delicate control of substrate binding in the active site. It also
underscored the possibility of achieving site selectivities
previously inaccessible with existing small-molecule catalysts.

Furthermore, we expanded this biocatalytic enantioselective
C–H amidation to intermolecular processes.94 In contrast to
intramolecular nitrene transfer, only the serine-ligated P411
variants could promote the entropically demanding intermo-
lecular amidation. The P-4 A82L variant, a P411 previously
developed for sulfi de imidation, was critical to this development,
showing low levels of promiscuous activity for benzylic C–H
amidation (Scheme 24). Introduction of three active-site
mutations greatly improved catalytic efficiency. The final
P411CHA variant effectively transforms diverse benzylic sub-
strates into the corresponding sulfonamides with excellent
enantioselectivity (Scheme 25).

More recently, with a significantly expanded library of P411
variants, we were eager to address unmet challenges in the area
of enantioselective C–H functionalization using P411 nitrene
transferases (Scheme 26). In principle, three classes of
enantioselective C(sp3)–H functionalization reactions could
be developed. Starting from a substrate bearing two prochiral
secondary C(sp3)–H bonds, asymmetric C–H functionaliza-
tion would furnish products with a trisubstituted stereocenter.
Desymmetrization of geminal dimethyl-substituted substrates
via primary C(sp3)–H functionalization would generate a
quaternary stereocenter. Furthermore, enantioconvergent func-
tionalization of tertiary C(sp3)–H bonds would allow for the
formation of tetrasubstituted stereocenters. Such enantiocon-
vergent tertiary C–H functionalizations have long eluded
synthetic chemists, presumably due to the lack of suitable
mechanisms for stereoreconvergence as well as effective catalysts
to induce asymmetry.95

Using the intramolecular C–H amidation of sulfamoyl azide
as a model system, we demonstrated that all three modes of
enantioinduction could be achieved using P411 catalysts.96

Scheme 23. Enzyme-Controlled, Regiodivergent C–H
Amidation (2014)

Scheme 24. Directed Evolution of Intermolecular C–H
Amidase (2017)

Scheme 25. P411-Catalyzed Intermolecular Benzylic C–H
Amidation (2017)

Scheme 26. P411-Catalyzed Asymmetric Amidation of
Primary, Secondary, and Tertiary C(sp3)–H Bonds (2019)
Among all the heme proteins tested, variants from the P411_CHF carbene transferase lineage provided the most promising results for the amidation of secondary C(sp³)–H bonds. Active-site engineering led to P411_Diane2, a variant capable of transforming a range of benzylic substrates into 1,2-diamines with excellent enantioselectivity. In addition, unactivated secondary C(sp³)–H bonds were also amidated effectively. By using a set of P411s that only differed by four mutations, both enantiomers could be accessed with good stereocontrol. Furthermore, P411_Diane1 I327P allowed the primary C(sp³)–H amidation to occur with excellent activity.

We also developed enantioconvergent tertiary C–H amidations via a putative stereoablative hydrogen atom transfer (HAT)/stereoselective rebound mechanism. With evolved P411_Diane3 and P411_Diane4 tetrasubstituted stereocenters possessing a nitrogen group could be efficiently accessed. Notably, this biocatalytic platform could be engineered to construct “methyl–ethyl” stereocenters with excellent enantioselectivity (87% ee). Due to the minimal steric and electronic difference, catalytic asymmetric formation of “methyl–ethyl” stereocenters is a notoriously difficult problem. Our study demonstrated that engineered enzymes could overcome this long-standing obstacle in asymmetric catalysis.

**BIOCATALYTIC IMIDATION OF SULFIDES**

P450-catalyzed enantioselective oxidation of sulfides is a powerful method for the preparation of optically active sulfoxides. Early in our nitrene transfer study, we wondered whether we could develop an analogous nitrene transfer to sulfides to generate enantioenriched sulfinimides. Due to their attenuated nucleophilicity, electron-enriched and electroneutral styrenes. Due to their attenuated nucleophilicity, electron-deficient styrenes were less effective substrates under these conditions.

It was long believed that nitrene transfer to olefins has no counterpart in the biological world. However, in 2018, spurred by the report of P411-catalyzed nitrene transfer, the Ohnishi group found that, during the biosynthesis of benzastatin natural products in *Streptomyces* sp. RI18, a cytochrome P450 (BezE) is responsible for the intramolecular aziridination via a nitrene transfer mechanism using N-acetoxyaniline as the nitrene source (Scheme 28).99 Through directed evolution, the competitive azide reduction could be suppressed. The final variant P I263F A328V L437V allowed for the preparation of a range of enantioenriched aziridines from electron-rich and electroneutral styrenes. Due to their attenuated nucleophilicity, electron-deficient styrenes were less effective substrates under these conditions.

The spontaneous [2,3]-sigmatropic rearrangement of allylic sulfurimides leads to allylic amines. To reprogram this biocatalytic imidation for the asymmetric preparation of amines, we re-engineered our P411 nitrene transferases using phenyl allyl sulfide as the model substrate.97 Using variant “P” as the template, active-site engineering provided variants P-4 and P-4 A82I for the effective conversion of both the (E)- and the (Z)-substrates. Further investigation revealed that this sulfide amidation was highly enantioselective. Partial erosion of stereochemical purity of the resulting allylic amine occurred during the [2,3]-sigmatropic rearrangement presumably due to the competing endo and exo transition states.

**BIOCATALYTIC AZIRIDINATION OF OLEFINS**

Optically pure aziridines are versatile building blocks to a variety of nitrogen-containing compounds. We demonstrated that P411s could catalyze the asymmetric aziridination of styrene derivatives using tosyl azide as the nitrene source (Scheme 28).99 Through directed evolution, the competitive azide reduction could be suppressed. The final variant P I263F A328V L437V allowed for the preparation of a range of enantioenriched aziridines from electron-rich and electroneutral styrenes. Due to their attenuated nucleophilicity, electron-deficient styrenes were less effective substrates under these conditions.

It was long believed that nitrene transfer to olefins has no counterpart in the biological world. However, in 2018, spurred by the report of P411-catalyzed nitrene transfer, the Ohnishi group found that, during the biosynthesis of benzastatin natural products in *Streptomyces* sp. RI18, a cytochrome P450 (BezE) is responsible for the intramolecular aziridination via a nitrene transfer mechanism using N-acetoxyaniline as the nitrene source (Scheme 29).100 We were thrilled that mechanistic insights gained through unnatural reactions in turn informed the discovery of natural enzymes that operate through unconventional pathways in biosynthesis.

**Scheme 27. P411-Catalyzed Asymmetric Imidation of Sulfides (2014 and 2016)**

| Scheme 28. P411-Catalyzed Asymmetric Aziridination (2015) |
|----------------------------------------------------------|
| ![Scheme 28. P411-Catalyzed Asymmetric Aziridination (2015)](image) |

| Scheme 29. Bez-Catalyzed Nitrene Transfer in the Biosynthesis of Benzastatins (2018) | ![Scheme 29. Bez-Catalyzed Nitrene Transfer in the Biosynthesis of Benzastatins (2018)](image) |

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BIOCATALYTIC C–H AMINATION USING EQUIVALENTS OF UNPROTECTED NITRENE

In previously developed biocatalytic nitrene transfer reactions, sulfonyl azides were the privileged nitrene source. Unfortunately, the removal of the N-sulfonyl group requires harsh reaction conditions, thus limiting the synthetic utility of nitrene transfer products. To circumvent this problem, we initiated a program to explore alternative nitrene precursors with no protective groups attached to the nitrogen. We were particularly intrigued by N-hydroxylamine esters (RC(O)O−NH₂) as aminating reagents, because the transformation of these reagents would furnish unprotected primary amines. 101−103 The straightforward synthesis of unprotected, enantioenriched primary amines remains a challenging problem. The successful development of biocatalytic primary amination via a nitrene transfer mechanism would allow for the conversion of unfunctionalized alkanes and alkenes into enantioenriched primary amines, thereby complementing transaminase-catalyzed processes.

From an enzymologist’s perspective, the putative iron nitrene species “Fe==NH” invoked in these amination reactions represents the closest structural mimic of the Fe==O intermediate (compound I) in native P450 catalysis (Scheme 30). Thus, understanding the bonding and reactivity of this presumed “Fe==NH” intermediate is of considerable interest to the bioinorganic community.

We first targeted the amination of styrene derivatives (Scheme 31). Using wild-type Rma cytochrome c as the catalyst, we found that the transfer of unprotected nitrene to styrenes afforded aminoalcohols as the final product, presumably due to the rapid ring opening of the unprotected aziridine under the reaction conditions.186 Starting from Rma cyt c, active-site engineering furnished the “TQL” variant, a highly active biocatalyst for the asymmetric aminohydroxylation of diverse styrenes including electron-deficient ones. Further mechanistic study showed that the hydrolysis of the unprotected, enantiopure aziridines is enantiospecific. This finding suggested that enzymatically formed enantiopure aziridine is a possible precursor to the 1,2-aminoalcohol product.

We next explored C–H amination reactions using unprotected nitrene equivalents. Using a P411 variant from the P411,CHA lineage94 as the parent, we engineered P411BPA for the primary amination of benzylic C–H bonds with high activity and excellent enantiocontrol. Most notably, no small-molecule catalysts are known to promote the primary amination of C(sp³)−H bonds despite extensive previous efforts dedicated to the study of Fe-catalyzed amination.101−103 The successful development of this biocatalytic primary amination of C(sp³)−H bonds illustrates the enormous potential of enzymes to enable desirable reactivities elusive to small-molecule catalysts.

Even more exciting, P411 enzymes were found to catalyze intermolecular allylic C–H amination with excellent enantioselectivity (Scheme 32). Variant P411APA chemoselectively aminates the allylic C–H bond, leaving the olefin moiety completely untouched. This chemoselectivity is orthogonal to the Rma cyt c TQL variant previously engineered for olefin aminohydroxylation.104 With P411BPA and P411APA, an array of easily available hydrocarbon starting materials could be converted into value-added primary amines. Further expanding this intermolecular amination activity to unactivated C(sp³)−H bonds would furnish an unprecedented, enzymatic method for the amination of hydrocarbon feedstocks.

EVOLUTIONARY TRAJECTORY OF P450 CARBENE TRANSFERASES AND NITRENE TRANSFERASES

P450BM3 has been the primary workhorse for the new-to-nature reactions described in this Account. Starting from the wild-type enzyme, we have evolved a family of P450BM3...
variants to serve as highly active carbene and nitrene transferases. It is interesting to follow the evolutionary trajectories of these different enzymes, delineated in Figure 1. These enzymes are all very closely related: the amino acid substitutions in key P450 variants are summarized in Table 1.

As can be seen from Table 1, except for several early variants, the majority of our evolved P411 carbene and nitrene transferases share the same ancestor, P411-CIS. We often found that a newly engineered carbene transferase acquired promiscuous activity for previously elusive nitrene transfer reactions. Similarly, newly evolved nitrene transferases opened up exciting opportunities for challenging carbene transfer reactions. Thus, there is a great deal of functional overlap in these new enzymes, which provides starting points for evolution and optimization of yet more novel functions.

Active-site evolution by site-saturation mutagenesis and screening has proven fruitful for P450-catalyzed carbene and nitrene transfer. Perhaps this is not surprising, since these activities do not require the same finely tuned amino acid environment evolved for P450BM3’s native function. Upon a closer examination of the data in Table 1, it is evident that the following residues are important for tuning the enzyme activity and selectivity: (1) residues 70–87 in the B’ region; (2) residues 177–181 in the F helix; (3) residues 261–269 in the I helix; (4) residues 327–330 in the K/K’ loop; (5) residues 436–438 in the loop; and (6) the iron-binding residue 400. The ability to quickly improve an abiological reaction of interest by iteratively mutating a relatively small set of key residues makes this protein engineering approach very attractive for catalyst development. To help future engineering of P450BM3 biocatalysts for other transformations, we label privileged active-site residues of P450BM3 in Figure 2, whose mutation resulted in substantial improvements in previously developed carbene and nitrene transfer enzymes. Beneficial mutations will of course also lie at more distant residues, as is often found in directed evolution campaigns. Thus, further optimization of a new catalytic activity is possible if the screening capacity is sufficient to capture these rarer events.

Figure 1. Evolutionary trajectory of P450 carbene and nitrene transferases.
Table 1. Directed Evolution of P450BM3 as Carbene and Nitrene Transferases

| entry | variant | type | reaction | mutations relative to WT P450BM3 | parent | mutations relative to parent | ref |
|-------|---------|------|----------|----------------------------------|--------|------------------------------|-----|
| 1     | P450BM3 T268A | carbone | cyclopropanation | T268A | P450BM3 | T268A | 1 |
| 2     | P450BM3 T268A F87A | nitrene | C–H amimation | F87A, T268A | P450BM3 | T268A | 93 |
| 3     | P450 Hstar | carbone | cyclopropanation | V78M, L181V, T268A, C400H1, L437W | P450BM3 | T268A | 39, 52, 54 |
| 4     | P450BM3-CIS (PDB: 4H24) | carbone | cyclopropanation | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, E442K | P450BM3-CIS | | |
| 5     | P411BM3-CIS (PDB: 4H23) | carbone | cyclopropanation | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, E442K | P450BM3-CIS | | 50 |
| 6     | P411-VACaa | carbone | cyclopropanation | L575Y, V78A, F87V, P142S, T175I, L181R, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, E442K | P411BM3-CIS | | 55 |
| 7     | P411-VACcsm | carbone | cyclopropanation | V78A, F87L, P142S, T175I, L181R, A184V, S226R, H236Q, E252G, I263G, T268A, A290V, L353V, I366V, C400S, E442K | P411BM3-CIS | | 55 |
| 8     | P-4 (P411-CIS T438S) | nitrene | C–H amimation, sulfide imidation | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | P411BM3-CIS | | 37, 97 |
| 9     | P I263A | nitrene | sulfide imidation | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, I263A, T268A, A290V, L353V, I366V, C400S, T438S, E442K | "P" | I263A | 97 |
| 10    | P I263F (PDB: 4WG2) | nitrene | C–H amimation | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | "P" | I263F | 93 |
| 11    | P I263F A328V L437V | nitrene | aziridination | V78A, F87V, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | P I263F | | 99 |
| 12    | P-4 (P411-CIS T438S) | nitrene | sulfide imidation | V78A, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | P I263F | | 98 |
| 13    | P-4 (PDB: 4UCW) | nitrene | C–H amimation | A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | P-4 | | 94 |
| 14    | P411_A5A | nitrene | C–H amimation | A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438S, E442K | P-4 | | 94 |
| 15    | E10 V79F S438A | carbone | bicyclobutanation | V78F, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, L353V, I366V, C400S, T438A, E442K | V78F, S438A | E10 | 71 |
| 16    | P411–K10 | carbone | cyclopropanation | S72W, V78S, P142S, T175I, A184V, L188C, S226R, H236Q, E252G, F261Q, I263G, T268A, A290V, L353V, I366V, C400S, T438A, E442K | P411–K10 | | 71 |
| 17    | P411–C6 | carbone | cyclopropanation | V78W, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, F261Q, I263G, T268A, A290V, T327V, A328V, L353V, I366V, C400S, T438A, E442K | P411–C6 | | 71 |
| 18    | P411_CWF | carbone | C–H alklylation | N70E, A74P, V78L, A82L, F87A, P142S, T175I, M177L, A184V, S226R, H236Q, E252G, T268A, A290V, A330V, L353V, I366V, C400S, T438A, E442K | P411_CWF | | 3 |
| 19    | P411_PYA | carbone | C–H alklylation | N70E, A74P, V78L, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, T268A, A290V, T327V, A328V, L353V, I366V, C400S, I437V, E442K | P411_PYA | | 81 |
| 20    | P411_Daus | nitrene | C–H amimation | A74C, V78L, A82L, F87A, P142S, T175I, M177L, A184V, S226R, H236Q, E252G, T268A, A290V, A328V, L353V, I366V, C400S, I437V, E442K | P411_Daus | | 96 |
| 21    | P411_WIRF | carbone | cyclopropanation | N70E, S72F, A74G, V78L, A82L, F87A, M118S, P142S, F162L, T175I, M177L, A184V, S226R, H236Q, E252G, I263W, H266V, T268G, A290V, A328V, L353V, I366V, C400S, T438A, E442K | P411_WIRF | | 71 |
| 22    | P411_BPA | nitrene | C–H amination | V78M, A82L, F87A, P142S, T175I, A184V, S226R, H236Q, E252G, I263M, E267D, T268G, A290V, T327A, A328V, L353V, I366V, C400S, I437V, T438G, E442K | E10 L263M | V78M, T268G, T327A, I437F, T438G | 4 |
CONCLUDING REMARKS

Over the past eight years, the field of unnatural biocatalysis has witnessed an explosion, and a variety of challenging stereoselective C−C and C−heteroatom bond forming transformations have been realized through the directed evolution of heme proteins. However, from a synthetic chemist’s perspective, biocatalytic carbene and nitrene transfer is far from mature. Numerous processes of significant synthetic utility remain to be discovered or optimized. For example, by taking advantage of the precise site- and stereocontrol imposed by the enzyme, exciting new possibilities of selective C−H functionalization await development.

Developing new biocatalytic reactions with no natural counterparts was once considered very difficult. Thus, one might ask why these new-to-nature enzymatic carbene transfer and nitrene transfer activities were achieved so easily. In retrospect, several factors may have played important roles (in addition to the inherent catalytic promiscuity of heme and heme proteins, which enabled improvement using reliable directed evolution approaches). First, the generation of Fe carbene and Fe nitrene is highly exergonic. Unlike the generation of compound I in native oxygenation reactions, the formation of Fe carbene and Fe nitrene is likely kinetically facile and does not require complex interactions with the protein scaffold. Second, the nascent iron carbene and iron nitrene are highly active, allowing for both bimolecular and unimolecular reactions to occur rapidly at ambient temperature. Based on these considerations, we propose that reaction rate ($k_{cat}$) is more important than substrate binding ($K_M$) for the initial development of new-to-nature reactions.

Third, despite the highly active nature of iron carbene and nitrene intermediates, their synthetic precursors including diazo compounds and organic azides are relatively stable under physiological conditions, even in living cells. This biorthogonality limits the spontaneous decay of substrates and ensures the productivity of enzymatic chemistry. Fourth, the excellent evolvability of heme proteins dramatically accelerated engineering campaigns. Last but not least, numerous ideas taken straight from synthetic organic and organometallic chemistry guided our design of new-to-nature biocatalytic processes. Standing on the shoulders of giants, we saw great opportunities at the colliding fields of synthetic chemistry and protein engineering.

We anticipate that the strategies underlying the work detailed in this Account will lead to the development of yet more enzymes for challenging transformations. After all, the astonishing structural and functional diversity of naturally available enzymes, and other proteins, will continue to provide exciting opportunities for catalyst discovery. Using an interdisciplinary approach combining genome mining, enzymology, protein engineering, and synthetic organic chemistry, a broader range of new-to-nature biocatalytic transformations is yet to come.

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Notes

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Frances H. Arnold is the Linus Pauling Professor of Chemical Engineering, Bioengineering and Biochemistry at the California Institute of Technology. She received her Ph.D. in chemical engineering from UC Berkeley in 1985. She was awarded the 2018 Nobel Prize in Chemistry for her pioneering work in directed enzyme evolution.

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