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Perspective

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Chemomimetic Biocatalysis: Exploiting the Synthetic Potential of Cofactor-Dependent Enzymes To Create New Catalysts

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ABSTRACT: Despite the astonishing breadth of enzymes in nature, no enzymes are known for many of the valuable catalytic transformations discovered by chemists. Recent work in enzyme design and evolution, however, gives us good reason to think that this will change. We describe a chemomimetic biocatalysis approach that draws from small-molecule catalysis and synthetic chemistry, enzymology, and molecular evolution to discover or create enzymes with non-natural reactivities. We illustrate how cofactor-dependent enzymes can be exploited to promote reactions first established with related chemical catalysts. The cofactors can be biological, or they can be non-biological to further expand catalytic possibilities. The ability of enzymes to amplify and precisely control the reactivity of their cofactors together with the ability to optimize non-natural reactivity by directed evolution promises to yield exceptional catalysts for challenging transformations that have no biological counterparts.

INTRODUCTION

Challenges in catalysis demand the creation of enzymes with activities not yet found in the biological world. Nature has evolved a certain set of synthetic strategies and uses an impressive array of enzyme catalysts to construct everything from simple metabolites to complex natural products. For the production of medicinal compounds, fuels, materials, or chemicals,[1] however, nature's synthetic strategies may not be ideal or even appropriate. Toward this end, one might desire enzymes that act on nonbiological functional groups or promote non-natural bond constructions while still capitalizing on enzymes' extraordinary powers of rate acceleration and selectivity. Developing genetically encoded catalysts for non-natural chemical transformations will expand the reach of biocatalysis and facilitate construction of biocatalytic routes for the synthesis of valuable chemical products *in vitro* and *in vivo*.[2]

Enzymes constructed only of the 20 canonical amino acids catalyze a remarkable range of chemistries. To achieve certain types of activity, however, proteins are often augmented with organic metabolites or metal ions known as cofactors; these species have functional groups and properties that enable the protein−cofactor complex to catalyze reactions that the protein alone cannot.[3] In turn, the protein sequence plays a critical role in controlling and amplifying the reactivity of the cofactor, enabling the ensemble to effect transformations that the cofactor often cannot perform alone or dictating the regio-, diastereo-, or enantioselectivity of those transformations. Furthermore, a given cofactor can often catalyze a multitude of chemically diverse transformations, and the protein structure acts to guide reactivity down one out of many possible pathways.

Many cofactor-dependent enzymes have been studied in depth with regard to their reaction mechanisms and the complex interactions between protein and cofactor that promote catalysis. The enzymologists carrying out these studies almost always focus on the natural function and substrate(s). At the same time, synthetic chemists have developed catalysts for a broad range of reactions that are completely absent in biology, either because nature has not found it advantageous to use them or because they require reagents not normally found in biology. In many cases, the small-molecule catalysts resemble natural cofactors; sometimes their creation was inspired by enzymes, in a biomimetic chemistry approach to catalyst design.[4] Similarities between many chemical catalysts and natural cofactors, both structural and functional, raise the possibility that the non-natural activities of small-molecule catalysts can be translated back into the corresponding cofactor-dependent enzymes. As proteins can provide exquisite control over reaction pathways, this chemomimetic biology strategy can improve on the efficiencies and selectivities of small-molecule catalysts just as natural enzymes improve on the activities and selectivities of their cofactors (Figure 1).

This Perspective will demonstrate how a chemomimetic approach can generate new biocatalysts from existing cofactor-dependent enzymes. Other approaches, including catalytic antibodies[5] and *de novo* designed enzymes,[6] have also delivered biocatalysts that catalyze reactions not known in nature. However, despite extensive efforts, their reactions have been limited to a relatively narrow set of transformations, and most of the new enzymes do not catalyze reactions at useful rates. In contrast, by repurposing existing cofactor-dependent enzymes for new chemistry, protein engineers and chemists have created enzymes that execute a diverse range of synthetically challenging nonbiological reactions. Cofactors enable the generation of unique reactive intermediates in enzyme active sites, whereas synthetic chemistry serves as a guide for the types of activity that can be achieved with a given reactive motif, even if they have not been observed in nature. This Perspective is not intended as an extensive review of the literature but, rather, a discussion of case studies that illustrate how new activities may be introduced into existing enzymes. We will discuss novel activities for enzymes that use thiamine and heme cofactors as well as proteins that use natural amino acids for non-native aminocatalysis.[7] We will also briefly consider the introduction

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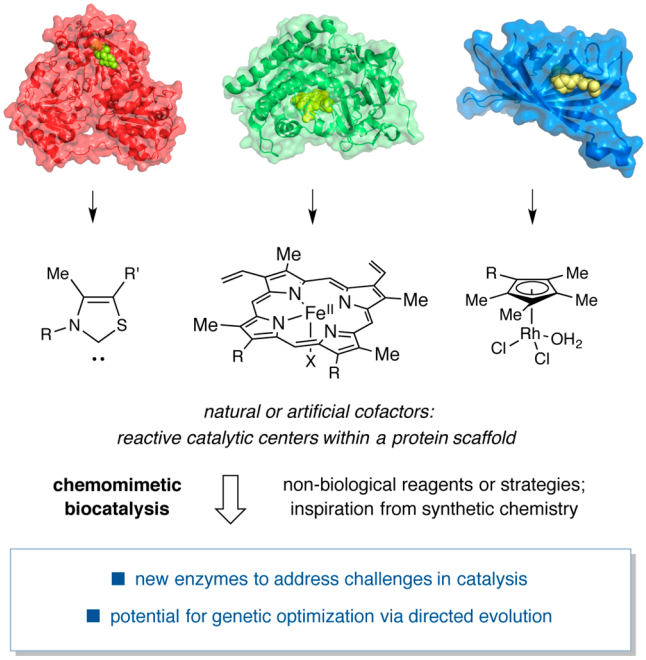


Figure 1. Chemomimetic strategies guide the creation of biocatalysts for reactions not known in nature.

of artificial cofactors into proteins to create new enzymes. Throughout, we will focus on reactions that are not biological rather than on creating new catalysts for transformations that are already known in nature, for which there is ample precedent.[8]

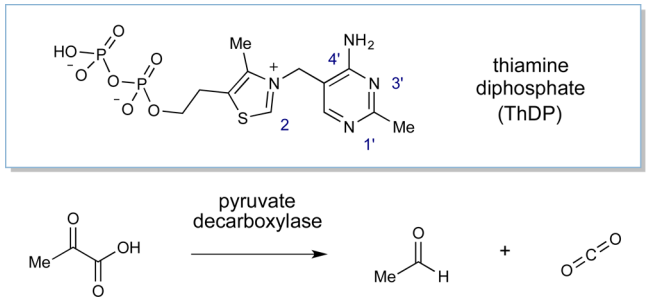
Interest in engineering non-natural enzyme activity is growing rapidly. Apart from better understanding of enzyme structures and mechanisms, one key driving force has been ready access to powerful methods of protein engineering. As natural enzymes are rarely proficient at performing non-natural chemistries, optimization of the protein structure is required to access synthetically useful catalysts; this is now possible and is in fact relatively straightforward. A hallmark of enzymes is that they can evolve and adapt under selective pressure, and this evolvability can be exploited in the laboratory to optimize enzymes via an iterative process of mutagenesis and screening for a desired outcome. This engineering approach, known as directed evolution, enables rapid tuning of key catalyst features such as selectivity, activity, and stability and circumvents our still poor understanding of how sequence affects enzyme function.[9] Thus, once a small amount of activity for a given transformation is discovered, the activity can often be greatly improved by introducing one or a few mutations at a time. Although mutagenesis guided by mechanistic understanding can sometimes be a successful approach to improving enzyme activities, the creation of exceptional catalysts almost always relies (at least in part) on a wider exploration of protein sequence space. In the realm of cofactor-dependent enzymes, the manner in which protein structure impacts the inherent reactivity of the cofactor provides fertile ground for protein engineers to alter the course of chemistry just by mutation of the protein sequence. In the world of chemical catalysis, there is no general strategy equivalent to evolution for optimizing catalyst structure. Catalyst modification often requires laborious resynthesis (as opposed to relatively straightforward gene modification), and subtle beneficial structural mutations rarely accumulate over generations of small-molecule catalyst optimization.

The creation of enzymes for nonbiological processes is an emerging field full of promise at the interface between chemistry and biology. Opportunities abound for protein engineers to exploit the wealth of knowledge gained from mechanistic enzymology and synthetic chemistry. Our goal here is to introduce concepts in this field and point to some of the opportunities; we encourage chemists to look at enzymes in a new way and contribute their intuition and insights to creating enzymes with new, synthetically useful activities.

THIAMINE-DEPENDENT ENZYMES

Chemists and protein engineers have developed new reactions using enzymes dependent on thiamine diphosphate (ThDP) by taking advantage of the unusual catalytic mechanisms enabled by this cofactor. Thiamine diphosphate comprises an *N*-alkyl thiazolium core, a tethered pyrimidine ring, and a diphosphate-terminated side chain (Scheme 1). The thiamine cofactor is

Scheme 1. Thiamine Diphosphate and the Native Activity of Thiamine-Dependent Pyruvate Decarboxylase (PDC)[10]



noncovalently bound to the enzyme, with the diphosphate group binding a second metal cofactor (typically, magnesium). Enzymes containing ThDP possess the unique ability to forge or break C−C bonds between two oxidized carbon centers; these enzymes catalyze decarboxylations (as in pyruvate decarboxylase), carboligations (as in transketolases), and oxidative transformations (as in pyruvate dehydrogenase).[10] Pyruvate decarboxylase (PDC), a particularly well-studied thiamine-dependent enzyme, catalyzes the conversion of pyruvate to acetaldehyde and carbon dioxide (Scheme 1).

Early studies demonstrated that ThDP can perform certain functions of thiamine-dependent enzymes, indicating that the cofactor itself contains all of the functionality required for catalysis.[11] It was not until 1957, however, that the now-accepted mechanism of thiamine catalysis was put forward by Breslow, who provided evidence for a mechanism involving deprotonation of the thiazolium ring at C2 ($pK_a \approx 18$)[12] to give the thiazolium ylide **1** (Scheme 2).[13] A resonance form of this ylide is the nucleophilic carbene **2**, in which a carbon atom bearing a sextet of electrons is stabilized by σ-electron withdrawal and π-electron donation from the adjacent heteroatoms. In the mechanism of pyruvate decarboxylation by PDC, addition of the nucleophilic carbene carbon (C2) to the pyruvate keto-group gives the covalent adduct **3**. The electron-withdrawing nature of the thiazolium ring then facilitates decarboxylation to yield the enaminol moiety **4**, known as the Breslow intermediate. This species is strongly nucleophilic; it may undergo protonation to provide thiazolium

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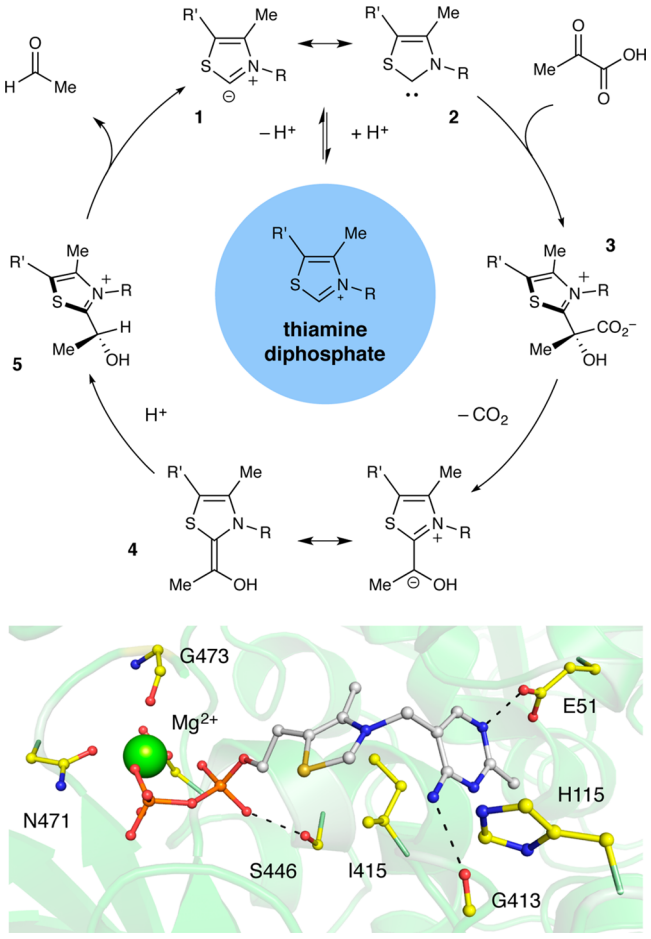
Scheme 2. Mechanism of Thiamine Catalysis in Pyruvate Decarboxylase and Active Site Architecture of Pyruvate Decarboxylase from *Saccharomyces cerevisiae*[10,a]



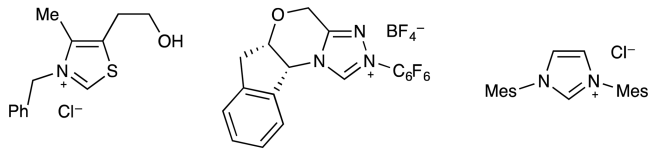
[a]ThDP is shown in gray, active site residues are in yellow, and magnesium is in green; PDB: 1PVD.[18]

5, which then collapses to release the product acetaldehyde and regenerate the active carbene form of the cofactor.

Whereas the cofactor alone decarboxylates pyruvate, the protein dramatically accelerates catalysis: by up to a factor of 10^{12} for yeast PDC.[14] Various mechanisms have been implicated in this rate acceleration. Conserved polar interactions with the pyrimidine ring (provided by E51 and G413 in PDC) increase the basicity of the 4′-nitrogen, whereas a conserved hydrophobic residue (I415 in yeast PDC) acts as a fulcrum between the two heteroaromatic rings, enforcing a V-shaped conformation of the cofactor and positioning the 4′-nitrogen in a favorable orientation to perform an intramolecular deprotonation of C2 (Scheme 2).[15] These active site features effectively lower the pK_a at C2 and accelerate deprotonation of the thiazolium.[16] Next, upon formation of the covalent adduct **3**, studies on other ThDP-dependent enzymes suggest that the protein promotes decarboxylation by enforcing maximal orbital overlap between the scissile C−C bond and the thiazolium π-system in the decarboxylation transition state.[17] Finally, specific residues have been implicated in both protonation of the Breslow intermediate and deprotonation of the alcoholic proton in the acetaldehyde-forming step.[10] The protein sequence of PDC thus functions to accelerate many steps throughout the catalytic cycle.

Concurrent with the enzymology studies delineating the mode of action of ThDP-dependent enzymes, chemists examining small molecules related to thiamine found that a broad range of heterocyclic structures (such as those shown in Scheme 3) undergo deprotonation to yield nucleophilic

Scheme 3. Small-Molecule Thiamine Equivalents (N-Heterocyclic Carbene Precursors) Used To Catalyze Diverse Organic Transformations[19]



carbenes analogous to intermediate **2**. These species, termed N-heterocyclic carbenes (NHCs), have found broad application in catalysis; similar to the mechanism of thiamine catalysis, these carbenes condense with carbonyl compounds to give Breslow intermediates.[19] This activation mode is particularly useful, as it reverses the typical reactivity of a carbonyl group: carbonyl carbon atoms are typically electrophilic, but formation of the Breslow intermediate (an acyl anion equivalent) renders the carbonyl carbon nucleophilic, enabling unique bond constructions. Using this catalytic manifold, chemists have accomplished many reactions that are not known to be catalyzed by thiamine-dependent enzymes in nature; these include benzoin and aza-benzoin condensations as well as Stetter, hydroacylation, and various annulation reactions.[19] Of the various catalyst systems that have been developed, triazolium-derived N-heterocyclic carbenes have been found to be especially useful for achieving enantioselective transformations.[20]

Recognizing the similarities between enzyme and NHC catalysts, biochemists and protein engineers have sought to use thiamine-dependent enzymes to perform some of these nonbiological synthetic transformations.[21] Many ThDP-dependent enzymes have been found to promote benzoin-type condensations between two aldehydes; in the catalytic mechanism, generation of the Breslow intermediate **6** is achieved via α-deprotonation of the initial covalent adduct rather than α-decarboxylation, as in the mechanism of PDC (Scheme 4A).[22] Nucleophilic addition to a second equivalent of aldehyde followed by expulsion of thiamine then gives the α-hydroxyketone **7**. Various other enzymatic heterocouplings between aromatic and aliphatic aldehydes have also been established.[23]

Particularly notable applications of ThDP-dependent enzymes have been found for reactions that have historically proven to be challenging for their small-molecule equivalents. In one such transformation, the asymmetric cross-benzoin reaction, one aldehyde must exclusively react with the carbene while a second (chemically very similar) aldehyde must function only as an acceptor. Due to this chemoselectivity problem, small-molecule methods are typically limited to aldehyde homocoupling. Müller and co-workers identified two thiamine-dependent enzymes, benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* and a variant of benzoylformate decarboxylase (BFD) from *Pseudomonas putida*, that successfully execute the reaction.[24] Several aldehydes bearing ortho-substituents (such as 2-chlorobenzaldehyde, **8**) were selected as

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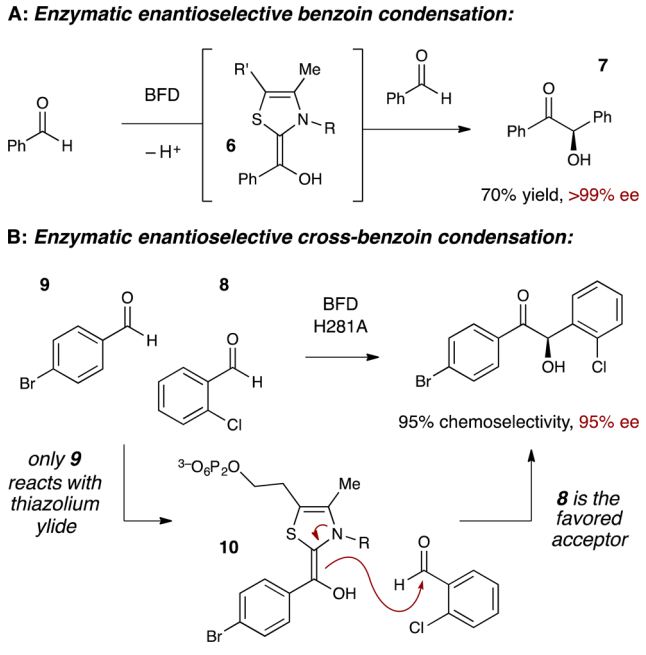
Scheme 4. Benzoin (A) and Cross-Benzoin (B) Reactions Promoted by Thiamine-Dependent Benzoylformate Decarboxylase (BFD)[22−24]

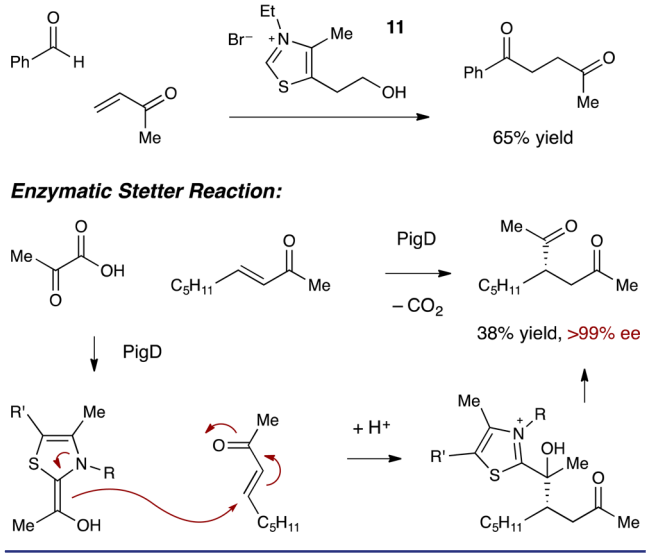


acceptor substrates because they do not undergo enzyme-catalyzed homocoupling, suggesting that their condensation with the thiamine cofactor is not possible. Thus, for the example shown in Scheme 4, only benzaldehyde **9** reacts with the carbene to give the Breslow intermediate **10**; subsequent addition to the favored acceptor **8** yields the cross-benzoin adduct with high chemo- and enantioselectivity. Using 2-chlorobenzaldehyde as the acceptor, selective cross-benzoin couplings may be achieved with a range of electronically diverse donor aldehydes.

Directed evolution of thiamine-dependent enzymes has been performed, enabled by a colorimetric high-throughput screen for the detection of α-hydroxyketone products. These efforts have enhanced the activities and enantioselectivities, as well as expanded the substrate scope, of enzymes performing benzoin-type condensations.[25]

Instead of adding to carbonyls in a 1,2-fashion, the Breslow intermediate may also add in a 1,4-conjugate fashion to α,β-unsaturated carbonyl compounds; the resulting transformation is known as the Stetter reaction. Some of the earliest reported Stetter reactions were actually achieved using thiazolium **11**, which features the core of the thiamine cofactor (Scheme 5).[26] However, achieving intermolecular, enantioselective Stetter reactions has been very challenging for small-molecule NHC catalysts; although examples of this activity have been developed, significant limitations remain with regard to scope and enantioselectivity.[27] Dresen et al. demonstrated that the thiamine-dependent enzyme PigD from *Serratia marcescens* performs the intermolecular Stetter coupling of an acetaldehyde unit (derived from pyruvate) with enones (Scheme 5).[28] This enzyme was postulated to perform a Stetter reaction as its native function in the biosynthesis of prodigiosin,[29] but experiments conducted with purified PigD and the proposed enal substrates for such reactions provided only the products of 1,2-addition. Upon evaluation of enone substrates, however, Stetter activity was observed and no 1,2-addition could be

Scheme 5. Thiamine-Dependent Enzyme PigD Performs the Intermolecular Stetter Reaction[28]



detected. A variety of enones having aliphatic, aromatic, and heteroaromatic functionalities at the 4-position undergo the PigD-catalyzed Stetter reaction, in many cases with excellent enantioselectivity.[30] Subsequent studies identified two PigD homologues that also display "Stetterase" activity.[31]

Benzaldehyde lyase (BAL) has also been engineered to perform the formose reaction, in which dihydroxyacetone is produced from the condensation of three equivalents of formaldehyde. Whereas chemical catalysts, including thiazolium salts, are known to perform this transformation, the reaction is not known in biology. Siegel et al. used computational design and directed evolution to identify a variant having seven mutations and 100-fold improved "formolase" activity relative to that of BAL.[32] This variant was used in a biosynthetic pathway for the conversion of formate into three-carbon metabolites.

In all of these examples, a mechanistic understanding of thiamine catalysis combined with experience from the synthetic chemistry of small-molecule thiamine analogues guided the discovery of new enzyme activities. By mimicking reactions first achieved with chemical catalysts related to the thiamine cofactor, native enzymes could be used to perform desired non-natural functions and, notably, even provide solutions to long-standing synthetic challenges. These activities proceed via the same key intermediate (the Breslow intermediate) as that in the natural enzyme transformations, but they access or utilize the intermediate in a nonbiological manner via selection of appropriate chemical reagents.

■ HEME-DEPENDENT ENZYMES

Heme-containing enzymes have been useful starting points for new enzyme activities. Particularly versatile are the cytochrome P450s (CYPs), a remarkable class of iron porphyrin-dependent enzymes that participate in xenobiotic metabolism and natural product biosynthesis.[33] These enzymes activate dioxygen, in a process requiring two electrons from NAD(P)H, to perform a multitude of oxygenation reactions including C−H hydroxylation, epoxidation, sulfoxidation, and heteroatom dealkylation. The mechanism of P450-catalyzed hydroxylation proceeds via a series of distinct iron intermediates to achieve

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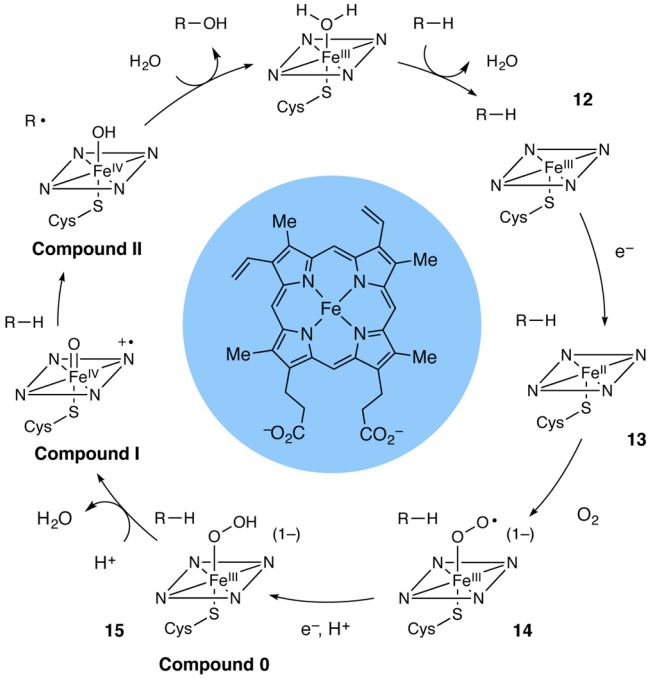
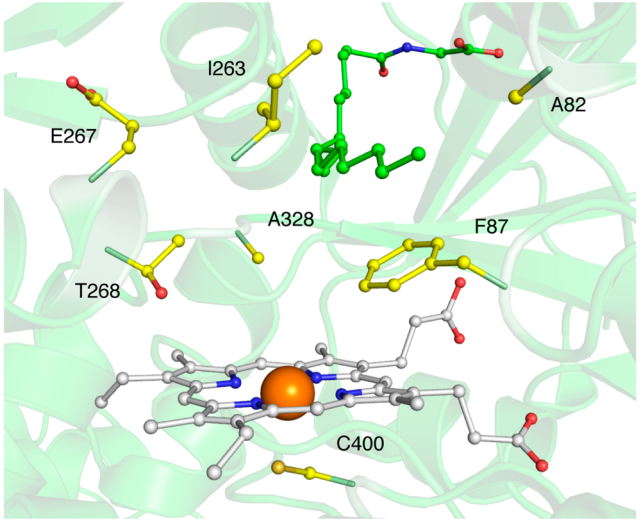
the insertion of an atom from dioxygen into a C−H bond.[34] First, substrate binding displaces a water ligand of the resting ferric state of the cofactor, giving the penta-coordinate intermediate **12** (Scheme 6). This event induces a transition

Scheme 6. Mechanism of Monooxygenation Catalyzed by Cytochrome P450s and Active Site Structure of Cytochrome $P450_{BM3}$ Bound to *N*-Palmitoylglycine[34,a]





[a]The heme is shown in gray, *N*-palmitoylglycine is in green, active site residues are in yellow, and iron is in orange; PDB: 1JPZ.[39]

of the heme iron from low to high spin, increasing its reduction potential (by 140 mV for $P450_{BM3}$)[35] and triggering electron transfer from a reductase partner. Upon reduction of the cofactor to its ferrous state (**13**), molecular oxygen binds to give a ferric−superoxide complex **14**; a subsequent second electron transfer followed by protonation then delivers the iron−hydroperoxy species **15**. This species, termed Compound 0, is protonated to release water and form a key iron(IV)−oxo porphyrin radical cation intermediate termed Compound I. This intermediate is the species that performs most of the oxygenation chemistry characteristic of P450s; in hydroxylation, Compound I abstracts a hydrogen atom from the substrate to generate an organic radical as well as the iron(IV)−hydroxyl species, Compound II. Radical rebound delivers the oxygenated product and returns the cofactor to its ferric resting state. Protein engineers have targeted a range of P450s for applications in biocatalysis, but one of the most widely used is $P450_{BM3}$ from *Bacillus megaterium* (CYP102A1). This soluble protein contains heme and diflavin reductase domains fused in a single polypeptide chain and naturally performs the subterminal hydroxylation of long chain fatty acids.[36]

Although all of the catalytic intermediates in the cytochrome P450 monooxygenation cycle are heme-bound, the protein's primary sequence makes critical contributions to catalysis. In all P450s, the heme iron is ligated by an axial cysteine thiolate residue (C400 in $P450_{BM3}$). Coordination by an electron-rich ligand decreases the reduction potential of the cofactor, preventing initiation of the catalytic cycle in the absence of substrate. The thiolate ligand is also postulated to promote heterolytic cleavage of the O−O bond in the iron−hydroperoxy intermediate **15**.[34] Furthermore, as demonstrated by recent studies,[37] thiolate ligation increases the basicity (pK_a) of Compound II, causing Compound I to favor abstraction of a hydrogen atom from the substrate over single-electron oxidation events that would be destructive to the protein. Another highly conserved residue is an active-site threonine (T268 in $P450_{BM3}$) that has been implicated in protonation and stabilization of heme-bound intermediates through active-site water molecules.[38] Protonation of the iron−hydroperoxy intermediate **15** mediated by this threonine likely promotes heterolytic O−O bond scission in the generation of Compound I.

Synthetic chemists have long sought to replicate the remarkable reactivity of cytochrome P450s, and many small molecules have been developed that mimic their oxene transfer activity; some of these catalysts are metalloporphyrin complexes structurally analogous to the native heme cofactor.[40] At the same time, many metalloporphyrins perform reactions unknown in biology; prominent among these is the transfer of carbenes and nitrenes to organic substrates.[41] In such reactions, an activated chemical precursor such as a diazo or azido species reacts with a transition metal (typically, Ru, Rh, Cu, Fe, Co, or Mn) to give a metal carbenoid or metal nitrenoid, respectively (Scheme 7). These electrophilic species, electronically analogous to the iron(IV)−oxo intermediate Compound I, may subsequently transfer the carbene or nitrene to an organic substrate. This mechanism has been employed to achieve a number of challenging non-natural transformations including cyclopropanation, C−H alkylation, and C−H amination.[42] Whereas porphyrin complexes are often highly active toward many of these reactions, they are typically not highly enantioselective. Instead, a number of nonporphyrin chiral scaffolds, such as copper bis(oxazolines) and dirhodium carboxylates or carboxamidates, have been more broadly useful for asymmetric catalysis (Scheme 7).

Several years ago, our group demonstrated that cytochrome $P450_{BM3}$ can, in fact, perform the cyclopropanation of styrenes via nonbiological metal carbenoid intermediates.[43] In this mode of catalysis, sodium dithionite ($Na_2S_2O_4$) first converts the resting ferric state of $P450_{BM3}$ to an active ferrous state (Scheme 8). Reaction with the reagent ethyl diazoacetate (EDA, **16**) then yields the iron carbenoid **17** with concomitant

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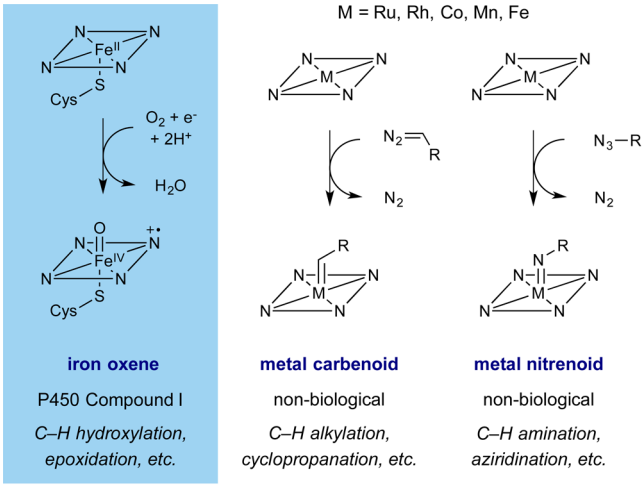
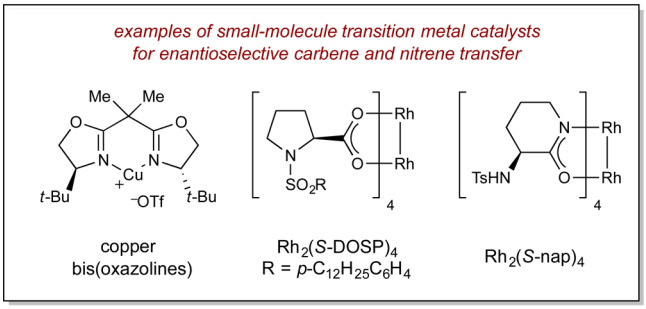
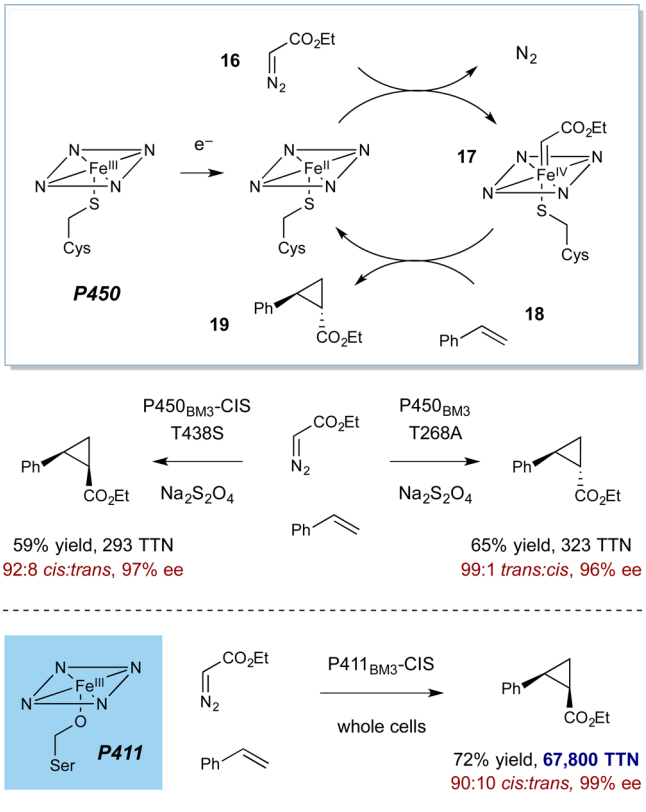
Scheme 7. Transition Metal Complexes Perform Catalysis via the Formation of Metal Carbenoid and Nitrenoid Intermediates[41,42]





Scheme 8. Variants of Cytochrome $P450_{BM3}$ Catalyze the Cyclopropanation of Styrenes via Carbene Transfer[43,44]



loss of dinitrogen. The electrophilic carbenoid intermediate reacts with styrene (**18**) to provide the cyclopropane product **19**; this carbene transfer step returns the cofactor to the catalytically active ferrous state. Wild-type $P450_{BM3}$ performs the cyclopropanation of styrene with very low efficiency (5 total turnovers) and with low levels of diastereo- and enantioselectivity (37:63 *cis*/*trans* and 27% ee for the *cis*-cyclopropane). However, mutating the highly conserved threonine 268 to alanine is strongly activating, providing an enzyme with over 60-fold improved cyclopropanation activity as well as excellent selectivity for a single enantiomer of the *trans*-cyclopropane (Scheme 8). Other variants were identified that preferentially deliver the *cis* isomer; the variant $P450_{BM3}$-CIS T438S contains 14 mutations relative to wild-type $P450_{BM3}$ and produces the *cis*-cyclopropane with excellent diastereo- and enantioselectivity. Thus, the inherent reactivity of the heme cofactor allows new reactions to be performed with a P450 enzyme, whereas changes to the protein sequence both enhance the activity and allow for exquisite control over the outcome of the new pathway.

Whereas axial ligation of the iron center by a cysteine residue is critical to the monooxygenation activity of cytochrome P450s, we found that mutation of the axial cysteine in $P450_{BM3}$ to serine (C400S), histidine, and even other amino acids is activating toward carbene transfer and enables catalysis in whole cells.[44−47] For cysteine-ligated P450s, a strong reductant such as dithionite ($E^{\circ\prime}$ = −660 mV vs the standard hydrogen electrode, SHE) is required to convert the resting ferric state to the ferrous state ($E^{\circ\prime}$ $Fe^{III/II}$ = −420 mV vs SHE for wild-type $P450_{BM3}$) in the absence of a substrate-induced spin shift. Mutation of the axial cysteine to serine, however, significantly increases the reduction potential of the ferric state (to $E^{\circ\prime}$ $Fe^{III/II}$ = −293 mV vs SHE).[44] The C400S and other mutations enable the cyclopropanation of styrene in whole cells, where the endogenous reductant NADPH ($E^{\circ\prime}$ = −320 mV vs SHE) is capable of performing the required initial electron transfer. As the Soret peak of the ferrous CO-bound enzyme is shifted from 450 to 411 nm in the serine-ligated variants, we termed these catalysts "cytochrome P411s". Furthermore, crystal structures show that the serine residue coordinates the iron center and does not cause significant structural changes to the protein.[44,48] Variant $P411_{BM3}$-CIS catalyzes the cyclopropanation of styrene with very high selectivity in whole cells and is capable of greater than 67 000 turnovers (Scheme 8). In addition, the C400S mutation abolishes the monooxygenation activity; this single mutation thus enhances a non-natural function at the expense of the native function. Mutation of the axial residue tunes the electronics of the iron center, adapting the enzyme to the demands of the non-native catalytic cycle. Other cytochrome P450s and other heme-containing proteins also catalyze cyclopropanation.[43,45]

Histidine-ligated cytochrome $P450_{BM3}$ mutants were evaluated toward the cyclopropanation of acrylamide **20** as the key step in a formal synthesis of the antidepressant levomilnacipran (Scheme 9).[46] $P450_{BM3}$ T268A C400H performs the carbene transfer reaction with good activity and selectivity for the desired diastereomer, but it does so with only moderate enantioselectivity. Directed evolution was performed by evaluating site-saturation libraries at active site residues for improvements in enantiomeric excess and accumulating the beneficial mutations. The catalyst identified by this approach, termed BM3-Hstar, contains an additional three mutations and provides the cyclopropane **21** with excellent selectivity. BM3-

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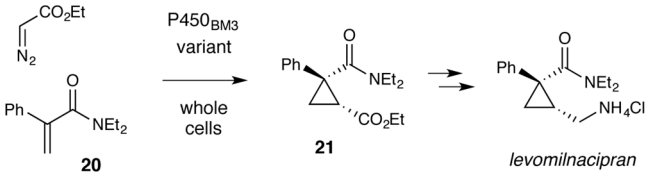
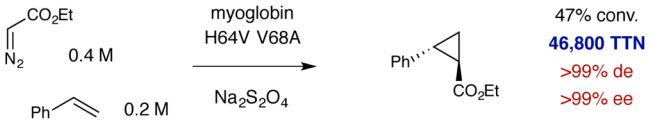
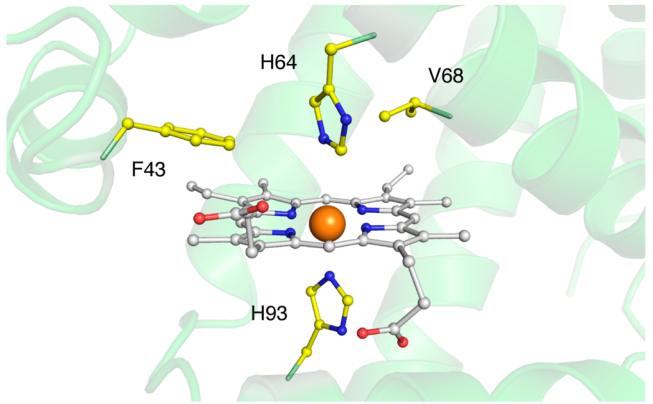
Scheme 9. Cyclopropanation by Heme-Containing Proteins Having Axial Histidine Ligation[46,a]





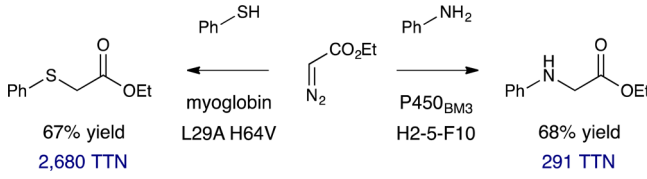


[a]The active site architecture of myoglobin was used by Fasan and co-workers to identify mutations at H64 and V68 that greatly enhance styrene cyclopropanation activity.[50] The heme is shown in gray, active site residues are in yellow, and iron is in orange; PDB: 1A6K.[51]

Hstar is also highly active, performing cyclopropanation with an initial rate of over 1000 turnovers per minute, and even maintains activity under aerobic conditions. It is notable that $P450_{BM3}$ tolerates the introduction of the large histidine side chain at the axial position; the crystal structure of a variant of the thermostable CYP119 from *Sulfolobus acidocaldarius* having the corresponding axial Cys-to-His mutation reveals dramatic structural rearrangements caused by non-native ligation.[49]

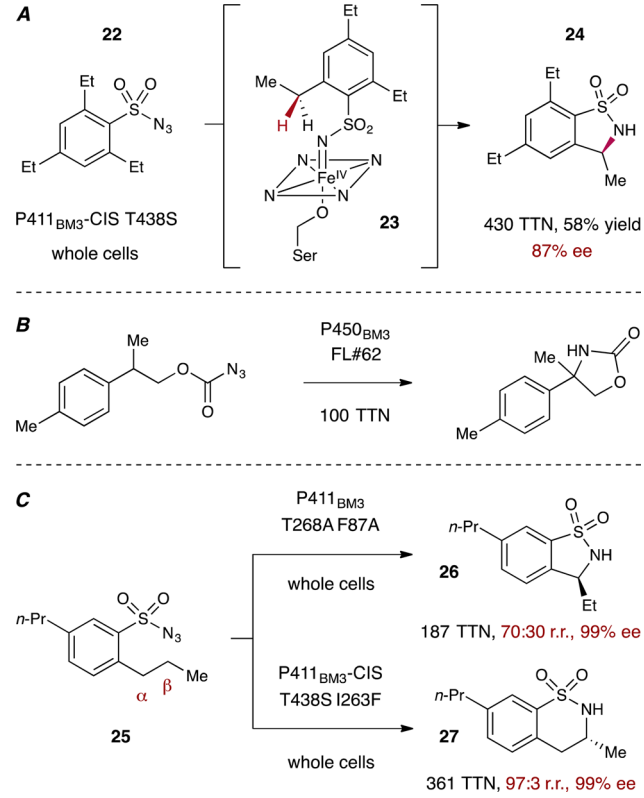
Fasan's group recently demonstrated that myoglobin (Mb), a heme-containing protein that naturally features an axial histidine ligand, also performs cyclopropanation efficiently via carbene transfer when a second, distal histidine is replaced with a smaller side chain.[50] Under reducing, anaerobic conditions, sperm whale Mb catalyzes the cyclopropanation of styrene with EDA with good diastereoselectivity for the *trans* isomer but minimal enantioselectivity (6% ee). However, introduction of mutations at H64 and V68, both located on the distal face of the heme, created a highly diastereo- and enantioselective catalyst capable of greater than 46 000 turnovers at high substrate concentrations (Scheme 9). The Mb variant is active toward cyclopropanation of a range of terminal styrenes. Finally, our group has shown that variants of $P450_{BM3}$ perform the N-alkylation of anilines via formal metal carbenoid insertion into N–H bonds,[52] and Fasan has described Mb-promoted N–H insertion[53] and S–H insertion reactions (Scheme 10).[54]

Scheme 10. N–H and S–H Insertion Reactions Catalyzed by Heme-Dependent Proteins[52−54]



Given that cytochrome P450s (and other heme proteins) readily adopt the ability to perform carbene transfer, one might anticipate that they could also display nitrene transfer activity. Indeed, in an early report, Dawson and Breslow showed that incubating rabbit liver microsomal P450s with iminoiodinanes yielded the products of nitrogen insertion into C–H bonds, presumably via a metal nitrenoid intermediate, with very low activity (2 turnovers).[55] We were inspired by this report to investigate sulfonylazides as nitrene precursors for catalysis with cytochrome $P450_{BM3}$.[56] Analogous to the mechanism of carbene formation from diazoesters, the sulfonylazide **22** may react with the ferrous state of the P450 via loss of dinitrogen to yield the iron nitrenoid **23** (Scheme 11A). Intramolecular

Scheme 11. Cytochrome P450s Catalyze C–H Amination Reactions via Nitrene Transfer[48,56,59]



insertion of the nitrenoid into one of the substrate's benzylic C–H bonds then generates the benzosultam **24**, while returning the cofactor to the ferrous state. Whereas wild-type $P450_{BM3}$ displayed low activity toward this transformation (TTN = 2), variants incorporating both the C400S and T268A mutations were much more efficient amination catalysts (120 TTN performed by $P411_{BM3}$ T268A *in vitro*). $P411_{BM3}$-CIS T438S, which incorporates both of these mutations, was an

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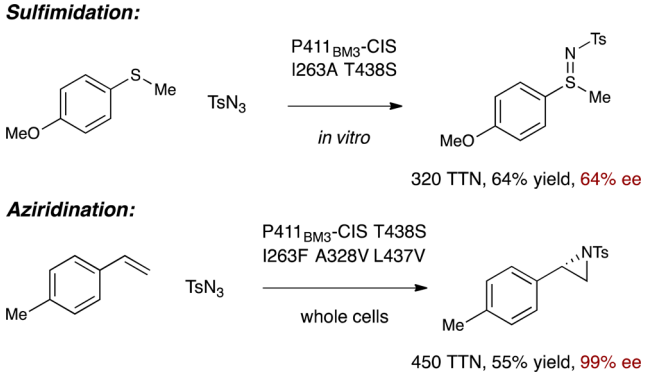
even better catalyst, affording sultam **24** in 58% yield and 87% ee with 430 total turnovers.

Fasan has demonstrated that other $P450_{BM3}$ variants, interestingly lacking either the C400S or T268A mutation, can also catalyze the C−H amination of sulfonylazides with up to 388 total turnovers.[57] Thus, a particular axial ligand is not strictly required to achieve hundreds of turnovers, and other mutations can generate the same (moderate) level of performance. Fasan has shown that Mb variants are also capable of performing the intramolecular C−H amination of sulfonylazides (with up to 200 turnovers)[58] and has demonstrated that azidoformates can function as nitrenoid precursors in P450-catalyzed C−H amination, providing access to oxazolidinone products (Scheme 11B).[59]

Whereas small-molecule metal complexes also enable enantioselective C−H amination, the regioselectivity of insertion is commonly dictated by the nature of the substrate.[41,42] In contrast, this laboratory recently demonstrated that the P450 active site can be engineered to promote regiodivergent outcomes in C−H amination.[48] The sulfonylazide substrate **25** features two potential sites for C−H insertion: amination at the benzylic (α) position leads to the five-membered sultam **26**, whereas amination at the homobenzylic (β) position leads to the six-membered sultam **27** (Scheme 11C). Although the C−H bonds at the β position are significantly stronger (BDE = ~98 kcal/mol vs ~85 kcal/mol at α), variant $P411_{BM3}$-CIS T438S I263F strongly favors C−H insertion at this site (97:3 **27**/**26**), demonstrating the ability of the protein sequence to override the inherent reactivity of the cofactor and control reaction outcomes. $P411_{BM3}$ T268A F87A, in contrast, preferentially catalyzes insertion at the benzylic position (70:30 **26**/**27**). The demonstration of catalyst-controlled regioselectivity in an insertion process highlights the ability of enzymes to address historical challenges for small-molecule catalysts.

In addition to these intramolecular C−H insertion reactions, heme-dependent enzymes also catalyze intermolecular nitrene transfer. Our group has shown that tosyl azide (TsN_3) serves as a suitable reagent for generation of an iron nitrenoid with various cytochrome P411s. This reactive intermediate may be intercepted by either sulfide or olefin nucleophiles to yield the products of sulfimidation[60] or aziridination,[61] respectively (Scheme 12). In the case of the intermolecular aziridination reaction, targeted mutagenesis and screening were performed to evolve the parent ($P411_{BM3}$-CIS T438S I263F, 40% yield, 55% ee in the aziridination of 4-methylstyrene) into a more active and much more selective nitrene transfer catalyst (55% yield, 99% ee). Notably, although the hemin cofactor alone displays some (typically low) activity toward other carbene and nitrene transfer reactions, it does not catalyze sulfimidation or aziridination. The creation of active P411 catalysts for these reactions highlights the ability of proteins to confer activity upon otherwise poorly active catalytic motifs.

Scheme 12. Intermolecular Nitrene Transfer in Sulfimidation and Aziridination Reactions[60,61]



Heme-dependent enzymes performing carbene and nitrene transfers in many cases perform as well as small-molecule catalysts developed for the same reactions with respect to turnover numbers. For instance, achieving tens of thousands of turnovers for cyclopropanation ranks these enzymes among the most efficient catalysts for enantioselective carbene transfer.[62] Transition metal catalysts developed for asymmetric nitrene transfer are typically capable of fewer than one hundred turnovers, making the enzymes comparable to the most active metal complexes that have been reported.[63] Enzymes compare unfavorably to the best small-molecule catalysts, however, on an activity per weight basis. This is balanced by the fact that enzymes are prepared simply by growing bacterial cells. Many of the reactions reported do not even require purification of the enzyme: the whole bacterial cells can be used for the biotransformation. Compared to small-molecule catalysts, the enzymes developed to date for this chemistry are active on a narrow range of substrates, and further engineering will be required to achieve broader activity profiles.

Most significantly, the development of chemomimetic carbene and nitrene transfers by heme-dependent enzymes illustrates the ability of existing enzymes to perform new transformations via the reactive intermediates that form when the biocatalyst is exposed to nonbiological reagents (diazoesters and azides). As suitable carbenoid and nitrenoid precursors are absent from the natural world, heme-containing proteins never had the opportunity to access these reaction manifolds and evolve these capabilities. This all changes in the laboratory: although heme proteins initially display, at best, only low levels of activity toward carbene and nitrene transfers, reaction efficiencies and selectivities are greatly improved by protein engineering and evolution. Altering the axial heme ligand tunes the nature of the reactive intermediate itself; that some heme proteins are remarkably tolerant of mutation at the axial ligand provides opportunities for new catalyst development.[49] Most other beneficial mutations that have been identified in P450s or Mb lie on the distal face of the heme, where they likely contribute to the binding and productive orientation of substrates, a hallmark of enzymatic catalysis.

Moreover, once an enzyme is identified for a given catalytic activation mode, synthetic chemistry precedent provides a range of different reactions that may be possible. Engineering an enzyme for improved activity toward a particular non-natural reaction often enhances its ability to execute other new reactions within that catalytic manifold, enabling protein engineers to evolve enzymes toward progressively more and more challenging reactions. In the context of carbene and nitrene transfer chemistries, cytochrome P450s seem to lie at the base of fitness peaks for these new functions, which are readily scaled by directed evolution. It will be interesting to see whether the smaller heme proteins such as Mb have similar inherent capacity for evolutionary tuning of selectivity and activity.

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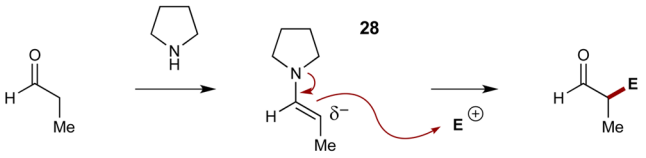
AMINE CATALYSIS

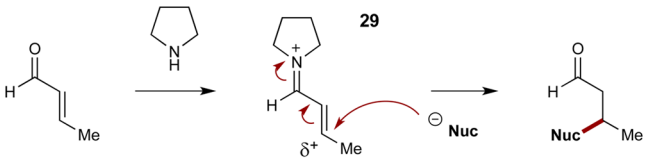
Enzymes may also catalyze nonbiological reactions using amines present in proteinogenic amino acids; although they are not cofactors, amino groups can be exploited to perform new reaction types just as thiamine and heme functionalities have been exploited in the studies described above. Aminocatalysis, the use of small-molecule amines to catalyze transformations of carbonyl compounds, has emerged relatively recently as a powerful method for asymmetric bond construction. Most commonly, these catalysts achieve substrate activation via the generation of either enamine or iminium ion intermediates. In enamine catalysis, condensation of an aldehyde or ketone substrate with the secondary amine catalyst followed by tautomerization yields an enamine intermediate (**28**), rendering the carbonyl α-position nucleophilic and facilitating reaction with a range of electrophiles (E^+) (Scheme 13A).[64] This enamine mechanism is also utilized in nature: type I adolases employ the ε-amino group of lysine as a nucleophilic amine catalyst to achieve aldol couplings.[65]

Scheme 13. Enamine (A) and Iminium (B) Activation Modes of Aminocatalysis[64,66,a]

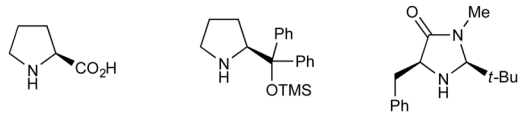
A. *Enamine catalysis:*



B. *Iminium catalysis:*



Common small molecules used for enamine/iminium catalysis:



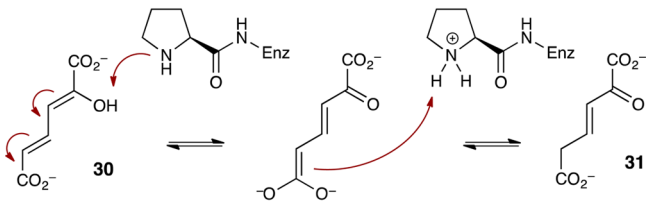
[a]E is a generic electrophile, and Nuc is a generic nucleophile.

Alternatively, in iminium catalysis, condensation of the amine with an α,β-unsaturated carbonyl compound provides an α,β-unsaturated iminium ion (**29**) in which the β-carbon is activated toward coupling with nucleophiles (Nuc^-) (Scheme 13B).[66] No biological examples of this β-activation strategy are known. Notably, the simple amino acid proline is a versatile organocatalyst, and the discoveries in 2000 that proline and its derivatives catalyze enantioselective aldol reactions (via an enamine mechanism)[67] as well as enantioselective Diels–Alder reactions (via an iminium mechanism)[68] initiated a period of intense research in aminocatalysis. These two activation modes have been exploited to achieve a large number of reactions that have no known biological equivalents, prompting researchers to examine amine-containing proteins as catalysts for these reaction types.

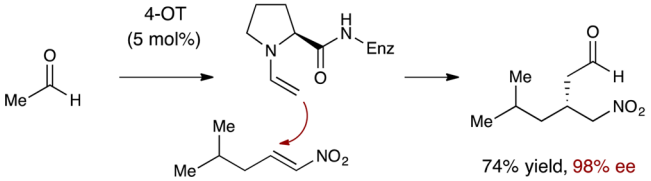
The homohexameric enzyme 4-oxalocrotonate tautomerase (4-OT) features a catalytic amino-terminal proline located in an active site where it has a $pK_b \approx 6.4$.[69] In the natural function of 4-OT, this residue (Pro1) acts as a general base to promote the conversion of 2-hydroxy-2,4-hexadienedioate (**30**) into 2-oxo-3-hexendioate (**31**) (Scheme 14A). Seeking to mimic the

Scheme 14. 4-Oxalocrotonate (4-OT) Performs Enamine Catalysis via an N-Terminal Proline[69–71]

A. *Native reaction of 4-oxalocrotonate tautomerase (4-OT):*



B. *Enzymatic enamine catalysis with 4-OT:*



structures and activities of proline-based organocatalysts, Poelarends and co-workers hypothesized that this residue might be capable of functioning as a nucleophilic catalyst to carry out enamine-mediated transformations. By exposing the enzyme to the appropriate reagents, they demonstrated that 4-OT can catalyze a non-natural Michael-type addition of aldehydes to nitroalkenes (Scheme 14B).[70] Evidence for enamine formation at Pro1 was provided by mutagenesis and covalent modification studies as well as crystallography.[71] Thus, a change in substrate is sufficient to alter the function of the N-terminal proline from acting as a general base catalyst to acting as a nucleophilic catalyst. 4-OT catalyzes the α-coupling of a range of alkyl aldehydes with diverse nitroalkene acceptors, often providing the product γ-nitroaldehydes with good to excellent enantioselectivity; the reaction may also be carried out in whole cells.[72] As proline residues at any position other than the N-terminus lack the ability to form enamine intermediates, Pro1 is cofactor-like in the sense that it is not widespread in proteins and must, in fact, be installed via a post-translational modification of the N-terminus.

Enamine-mediated Michael reactions have also been carried out using the catalytic antibody 38C2; this antibody was originally developed to catalyze aldol reactions via the ε-amino group of an active-site lysine residue in the manner of type I aldolases.[73] Weinstain et al. demonstrated that 38C2 is also capable of promoting asymmetric, intramolecular alkylation reactions via the Michael addition of an enamine intermediate **32** to a pendent enone (Scheme 15).[74] The reactions proceed with high enantioselectivity as well as good diastereoselectivity for the *cis*-cyclopentane product.

As noted above, iminium catalysis activates α,β-unsaturated carbonyl substrates toward the 1,4-conjugate addition of nucleophiles. In contrast, nature opts to activate electrophiles for conjugate addition via hydrogen bonding, as in the mechanism of enoyl-CoA hydratase,[75] or via pyridoxal phosphate (PLP)-dependent generation of α-aminoacrylate

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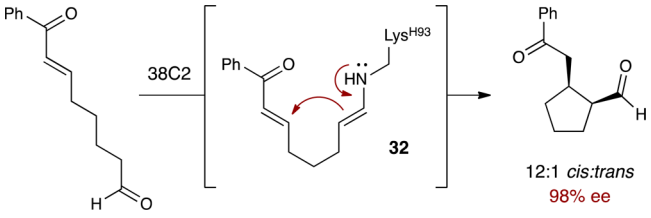
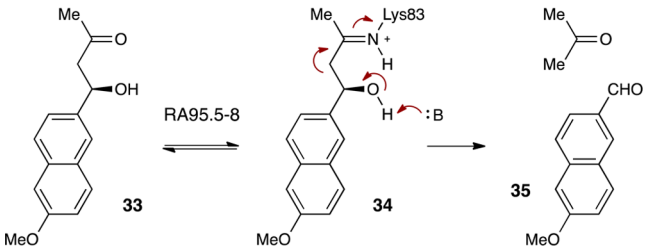
Scheme 15. Intramolecular Michael Addition Catalyzed by the Catalytic Antibody 38C2 via an Enamine Mechanism[74]



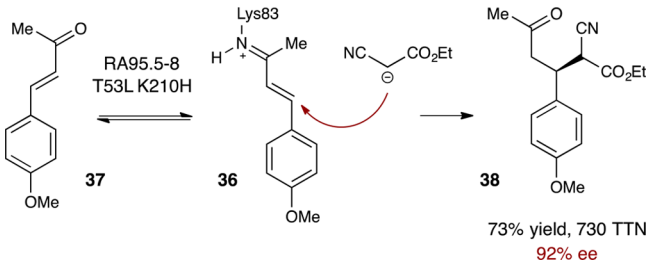
intermediates, as in the mechanism of tryptophan and cysteine synthases.[76] By analogy to small-molecule iminium catalysis, Hilvert and co-workers speculated that a computationally designed retro-aldolase containing a catalytic lysine residue could activate enones toward conjugate additions.[77] In earlier studies, artificial retro-aldolases were designed *in silico* to catalyze the degradation of (*S*)-methodol (**33**) via condensation of an active site lysine with the ketone functionality; formation of iminium **34** precipitates retro-aldol bond cleavage to give acetone and naphthaldehyde **35** (Scheme 16).[78] One of

Scheme 16. Enzymatic Asymmetric Michael Addition via Iminium Catalysis Performed by the Retro-Aldolase RA95.5-8[77]

Retro-aldolase activity of RA95.5-8:



Enantioselective Michael addition activity:



the experimentally active designs was subsequently optimized via directed evolution, delivering an improved retro-aldolase termed RA95.5-8.[79] To determine whether this enzyme could also catalyze 1,4-conjugate addition reactions via formation of an activated α,β-unsaturated iminium ion such as **36**, a variety of enone and nucleophilic reagents was surveyed. RA95.5-8, in fact, catalyzes the Michael addition of ethyl 2-cyanoacetate to enone **37**, furnishing the product β-alkyl ketone **38** in high yield and enantioselectivity (Scheme 16). The Michael activity of RA95.5-8 could be improved by evolution, with the incorporation of two mutations (T53L and K210H) providing a roughly 3-fold rate enhancement.

The examples of enamine and iminium catalyses discussed here demonstrate that enzymes are capable of performing unnatural aminocatalytic reactions. As in the carbene and nitrene transfer activities developed with heme-dependent enzymes, these functions were inspired by the activities of small-molecule catalysts and then achieved by combining appropriate enzymes with the required chemical reagents (aldehydes, nitroalkenes, or enones). In the three studies described, the parent enzyme (4-OT, 38C2, or RA95.5-8) was selected based on the knowledge that it performs catalysis using its amine functionality. Notably, the parent could be a natural enzyme, a catalytic antibody, or a computationally designed catalyst.

One drawback to catalysis by small-molecule amines is a need for high catalyst loadings; examples of either enamine or iminium catalysis proceeding with greater than 100 turnovers are rare.[80] In this context, Hilvert's development of an enzyme for iminium catalysis capable of greater than 700 turnovers is a notable accomplishment. Given the much greater molecular weight of enzymes compared to the simple architectures of many small-molecule organocatalysts, however, even greater efficiencies will be required for these enzymes to be competitive for synthetic purposes. Taking inspiration from these studies, other non-natural amine-catalyzed reactions will likely be accessible to enzymes, and further efforts may yield enzyme catalysts that exceed the capabilities of small molecules with regard to either activity or efficiency.

■ ARTIFICIAL COFACTORS

The availability of enzymes containing thiazolium, metalloporphyrin, and amine functionalities has enabled the introduction of functions first established with synthetic catalysts into biocatalysis. For many reaction classes accessible to synthetic catalysts, however, no obvious candidates exist in the enzyme world that may readily take on those functions. This activity gap has inspired chemists to introduce artificial cofactors into protein scaffolds, with the aim that a particular new functional group or metal will allow a desired reaction to take place, while the presence of a protein scaffold will enable control of the reaction pathway in ways not possible with small molecules.[81] By far, the majority of artificial cofactors that have been introduced into proteins are metal ions or metal complexes; when they are catalytically active, the resulting complexes are known as artificial metalloenzymes. Many of the artificial metalloenzymes that have been developed perform reactions that are catalyzed by existing enzymes;[82] in some cases, however, the installation of a metal has enabled functions that are absent in nature.

In the cofactor-dependent enzymes discussed above, highly specific interactions and recognition motifs have evolved to bind the cofactor to its cognate protein. In contrast, catalysis with an artificial cofactor (and, particularly, enantioselective catalysis) usually requires the installation of the cofactor at a specific site in a scaffold that has not evolved to bind it. One general strategy for the site-specific introduction of an artificial metal cofactor in a protein makes use of the extremely high affinity of the organic cofactor biotin for the proteins avidin and streptavidin (K_a = ~10^{14} M^{-1}). In a seminal report, Whitesides described the synthesis of the rhodium complex **39**, in which the metal center is tethered to the carboxylate side chain of biotin via a diphosphine motif (Scheme 17).[83] Modification of biotin with the metal complex does not impede binding to avidin, and simply combining these components generates a noncovalent artificial metalloenzyme adduct; this complex performs the hydrogenation of α-acetamidoacrylic acid (**40**) with moderate enantioselectivity (41% ee). Thus, binding of

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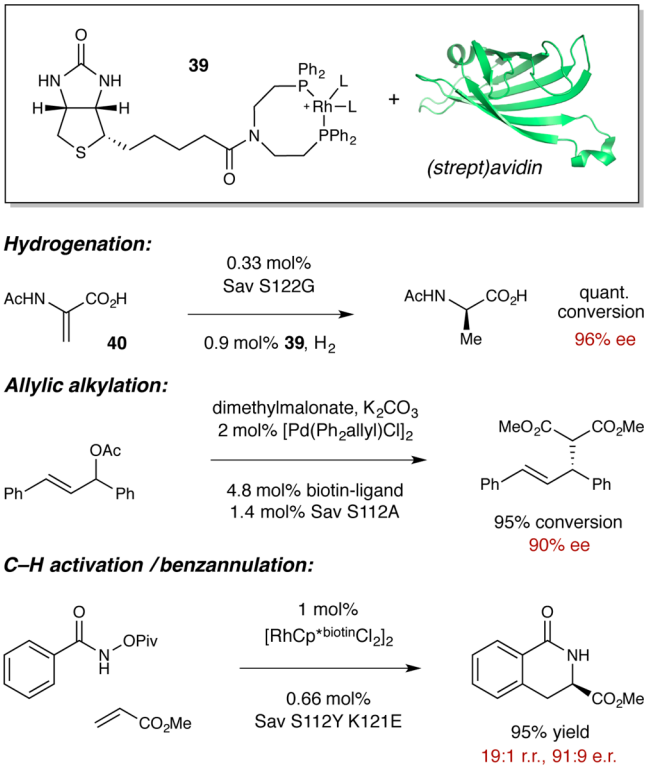
Scheme 17. Artificial Metalloenzymes Based on Binding of Biotin-Tethered Metal Complexes to (Strept)Avidin[83,85,88,89,a]



[a]Sav = streptavidin; PDB: 1STP.[92]

the modified biotin cofactor to avidin positions the achiral rhodium center in a chiral pocket that is capable of accommodating substrates as well as inducing asymmetry during catalysis.

Ward and co-workers have extensively investigated the applications of this metallobiotin concept.[84] First, Ward demonstrated that the metalloenzyme derived from streptavidin (Sav, which features a deeper biotin-binding pocket than avidin) is a more selective hydrogenation catalyst than the corresponding avidin complex.[85] In particular, the variant Sav S122G hydrogenates amidoacrylate **40** with excellent enantioselectivity (96% ee). Using different transition metals and ligand scaffolds, the biotin–(strept)avidin system has been further employed to achieve the transfer hydrogenation of ketones[86] and imines,[87] a palladium-catalyzed asymmetric allylic alkylation,[88] and an asymmetric rhodium-catalyzed C–H insertion/benzannulation reaction (Scheme 17).[89] Optimization of these transformations was performed via both alteration of the transition metal/biotin complex and genetic manipulation of the (strept)avidin scaffold.

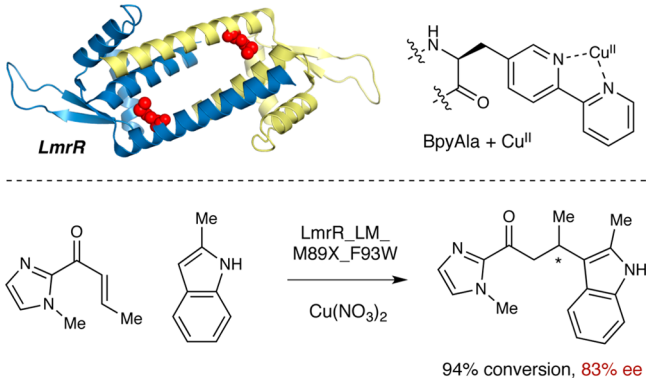
Directed evolution of the biotin–(strept)avidin system has been performed by Reetz, but these efforts were constrained by the requirement for protein purification prior to catalysis.[90] Ward and co-workers, therefore, developed a protocol for extraction of (strept)avidin from crude cell extracts by immobilization on biotinylated Sepharose; as avidin and streptavidin are homotetrameric, one subunit may bind to the solid support while leaving other subunits available for catalysis.[91] This protocol was used to screen moderately sized libraries, delivering streptavidin variants capable of performing the transfer hydrogenation of ketones with excellent selectivities (up to 96% ee).

In related work, Ward and co-workers have shown human carbonic anhydrase II to be a useful platform for metalloenzyme creation based on the high affinity of its zinc cofactor for aryl sulfonamide ligands; as in the biotin chemistry described above, the sulfonamide ligand may be linked to a metal complex.[93] In the case of one construct, computational design was employed to improve the binding of the artificial cofactor to the protein, resulting in an assembly with 4-fold improved activity for the transfer hydrogenation of imines.[94]

In contrast to the reactions above, which proceed via organometallic intermediates, metal ions may also act as Lewis acids. Roelfes and co-workers constructed enzymes for this mode of catalysis by covalently conjugating defined metal-binding sites, namely, a 2,2′-bipyridine or phenanthroline moiety, to cysteine residues introduced into the dimeric transcription factor LmrR (Lactococcal multidrug resistance regulator) from *Lactococcus lactis*.[95] This scaffold possesses a large hydrophobic pore at the interface between LmrR monomers, and the sites for cysteine conjugation were selected such that two metal-binding ligands might project into this dimer interface, one on each monomer. Conjugation followed by purification and treatment with a copper(II) salt then generated artificial metalloenzymes capable of functioning as Lewis acid catalysts; these constructs promote asymmetric Diels–Alder[95] and hydration reactions.[96]

Metal-binding sites may also be incorporated into proteins by genetically encoding noncanonical amino acids; for example, Schultz and co-workers achieved genetic incorporation of (2,2′-bipyridin-5-yl)alanine (BpyAla) via amber stop codon suppression.[97] Using this methodology, Roelfes introduced BpyAla into LmrR, thereby eliminating the need to perform a separate conjugation step to assemble the active catalyst (Scheme 18).[98,99] Installing the unnatural amino acid again at

Scheme 18. Friedel–Crafts Alkylation Enabled by Genetic Incorporation of a Metal-Binding Motif[98,a]



[a]LmrR is the Lactococcal multidrug resistance regulator; the two monomers are shown in blue and yellow and M89 is shown in red in the wild-type structure; PDB: 3F8B.[102]

the dimer interface and using copper(II) as the metal ion generated artificial metalloenzymes that catalyze the enantioselective Friedel–Crafts alkylation of indoles (Scheme 18). Selective catalysis required optimization of the site of unnatural amino acid incorporation (incorporation at M89 was found to give the best performance) as well as the introduction of an

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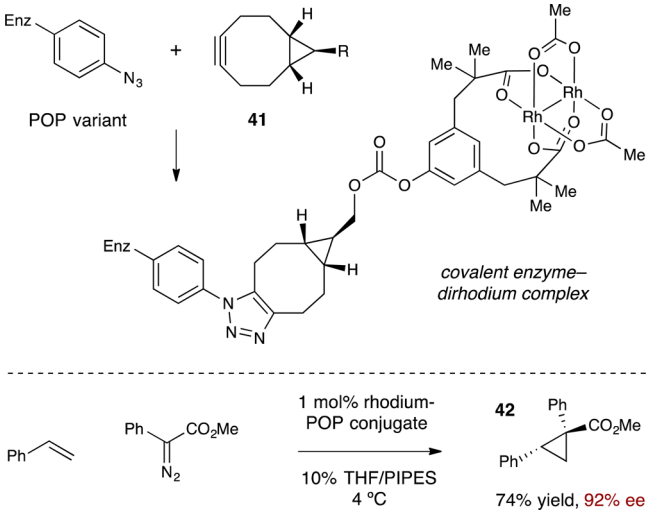
additional mutation (F93W) in the vicinity of the new catalytic center.

Notably, covalent attachment of the ligand is not actually required in this system: Roelfes also demonstrated that a phenanthroline–copper(II) complex binds noncovalently at the LmrR dimer interface, providing an artificial metalloenzyme competent at performing the Friedel–Crafts alkylation.[100] Reetz also demonstrated that noncovalent association between a phthalocyanine–copper complex and bovine serum albumin (BSA) is sufficient for enantioselective catalysis of Diels–Alder reactions.[101]

Lewis has also developed an approach to artificial metalloenzyme assembly based on unnatural amino acid (UAA) incorporation. Instead of binding the metal directly, however, the UAA contains an azide that undergoes strain-promoted azide–alkyne cycloaddition with a bicyclononyne-substituted metal complex.[103] The bioorthogonal azide–alkyne coupling reaction ensures specificity for conjugation of the metal complex at a desired site and avoids cross-reactivity with protein side chains or the components of cell lysate. In an application of this strategy, Lewis and co-workers incorporated a dirhodium tetracarboxylate complex into prolyl oligopeptidase (POP), a protease with a large internal cavity.[104] A single unnatural amino acid, L-4-azidophenylalanine, was incorporated in the interior of the protein in place of the protease's catalytic cysteine residue, and subsequent cycloaddition with bicyclononyne **41** delivered the covalent enzyme–dirhodium complex (Scheme 19). Notably, achieving conjugation required

Scheme 19. Artificial Dirhodium Metalloenzyme Constructed from Prolyl Oligopeptidase (POP) Catalyzes Enantioselective Styrene Cyclopropanation[104]



mutating four large residues on one face of the protein to alanine, thereby enlarging a pore and presumably allowing the large cofactor **41** to access the site of UAA incorporation. The resulting enzyme catalyzes the cyclopropanation of styrenes with donor–acceptor diazo compounds via a rhodium carbenoid, providing cyclopropanes such as **42.** Three further beneficial mutations were identified in the active site, providing a construct capable of performing cyclopropanation with good yields and high enantioselectivities as well as reducing the extent of a side reaction: carbenoid degradation via formal insertion into the O–H bond of water.

Work in the artificial metalloenzyme field has demonstrated that nonbiological reactive centers (including many metals that are rare or absent in biology, such as rhodium) can be introduced into proteins that have not evolved for any explicit metal-binding capability. These constructs have been shown to be selective catalysts for a number of synthetically useful transformations, validating their designs based on the mimicry of known chemical catalysts. The logic of chemomimetic biocatalysis is thus applicable to both naturally cofactor-dependent enzymes and artificial cofactor-containing constructs. However, the efficiency of these metalloenzymes is typically poor; many of the systems described above are not capable of more than 100 turnovers (although, in select cases, thousands of turnovers have been reported[87]). Most artificial metalloenzymes are thus not as efficient as the best small-molecule catalysts for the same reactions.[62,105] Furthermore, many of these systems are not amenable to directed evolution, typically because they require purification and catalyst assembly, because catalysis is not compatible with the components of cell lysate, or because the starting activities are simply too low for screening to find beneficial mutations. Developing systems that are amenable to high-throughput genetic optimization is a challenge to which the field is now responding in order to create exceptional catalysts.

■ CONCLUSIONS

We have described an approach to creating novel enzyme activity based on exploiting functionalities naturally present in enzymes for new purposes. As there is significant overlap between the properties of certain enzyme cofactors and small-molecule catalysts, synthetic chemistry can serve as an inspiration and blueprint for reaction pathways that can be introduced into biocatalysis (what we have termed a chemomimetic approach to biocatalysis). Thus, new activities may be discovered in or introduced into existing enzymes by exploiting the catalytic potential of their heme, thiamine, amine, and other functional groups. In addition, proteins can be augmented with artificial metal cofactors to perform non-natural metal-catalyzed transformations. Again, these processes draw inspiration from synthetic reactions and seek to mimic, in a protein environment, processes that were first established with small molecules.

It is interesting to note that protein engineers and synthetic chemists may find somewhat different solutions to the same problems; for instance, whereas rhodium complexes are generally the most versatile small-molecule catalysts for carbene and nitrene transfers, the examples discussed here show that iron-containing enzymes can, in some cases, perform comparably. Likewise, protein engineers have taken advantage of the natural cofactor thiamine to perform acyl anion chemistry, whereas chemists have found that triazolium catalysts are preferable for many applications. Thus, the optimal small-molecule and enzymatic catalysts for a given reaction might be structurally similar, but key features of the catalyst, such as the identity of the metal, need not be the same. These differences arise in part because proteins are especially good at modulating and contributing to the reactivity of their cofactors, creating a structural ensemble at the active site with properties distinct from those of either protein or cofactor alone. We anticipate that these advantages can also be exploited by metalloenzymes made from artificial cofactors.

Although protein engineers have exploited several classes of cofactor-dependent enzymes for creating new activities, the natural diversity of cofactor-dependent enzymes offers many

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further opportunities. Pyridoxal phosphate (PLP)-dependent enzymes,[106] for instance, have been targets of extensive protein engineering, but, to date, these efforts have largely focused on native-like reactivity. Other cofactor-dependent enzymes, such as those that utilize the 4-methylideneimidazole-5-one (MIO),[107] adenosylcobalamin,[108] and *S*-adenosyl methionine (SAM) cofactors,[109] promote remarkable synthetic transformations in nature but have not been widely explored for biocatalytic applications distant from their native chemistries. Given the unique mechanistic pathways enabled by these cofactors, these enzymes are promising candidates for introducing further new functions.

For many of the non-natural reactions that have been reported, the enzyme catalysts do not (yet) outperform small molecules in terms of total turnovers, reaction rates, or enantioselectivities. Creating useful enzymes will thus depend on directed evolution, for which cofactor-dependent enzymes should be well-suited. Although natural cofactors are often structurally complex molecules, they are supplied directly by metabolism and thus do not require independent preparation by chemical synthesis. Furthermore, their assembly into the enzyme is preprogrammed by the protein and the host cell, eliminating any need for protein purification and laborious catalyst assembly. Facile catalyst assembly and activity in complex environments is useful for the implementation of the high-throughput screening protocols often required for directed evolution.

We have discussed several examples in which the non-natural activities of thiamine-, heme-, and amine-dependent enzymes have been enhanced by directed evolution. In some cases, new activities could be improved via semirational approaches such as mutagenesis of key catalytic residues. In the majority of cases, however, extensive evolution has not been performed, either due to a lack of effective screening protocols or, as is typically the case for artificial metalloenzymes, the requirement for purification and/or *in vitro* assembly of the catalyst. Given that supporting entirely new mechanisms of catalysis likely requires significant structural reorganization of enzymes' active sites as well as tuning of global enzyme properties, we expect that the efficiencies reported in the literature for these systems are far removed from the capabilities that can be accessed by changes to the protein sequence. Evolution will also likely enable protein engineers to pursue more and more difficult reactions, as mutations that are generally activating toward a given non-natural catalytic manifold (such as nitrene transfer or iminium catalysis) are identified. We anticipate that it will be possible to evolve enzymes to perform non-natural reactions with high levels of efficiency and selectivity and even the ability to perform reactions that are currently inaccessible to chemocatalytic methods.

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Notes

The authors declare no competing financial interest.

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