Engineering New Catalytic Activities in Enzymes

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Abstract

The efficiency, selectivity and sustainability benefits offered by enzymes are enticing chemists to consider biocatalytic transformations to complement or even supplant more traditional synthetic routes. Increasing demands for efficient and versatile synthetic methods combined with powerful new discovery and engineering tools have prompted innovations in biocatalysis, especially the development of new enzymes for precise transformations, or 'molecular editing'. As a result, the past decade has witnessed impressive expansion of the catalytic repertoire of enzymes to include new and useful transformations not known (or relevant) in the biological world. In this review, we illustrate various ways in which researchers have approached using the catalytic machineries of enzymes for new-to-nature transformations. These efforts have identified genetically-encoded catalysts that can be tuned and diversified by engineering the protein sequence, especially by directed evolution. Discovery and improvement of these new enzyme activities is opening a floodgate that connects the chemistry of the biological world to that invented by humans over the last 100 years.

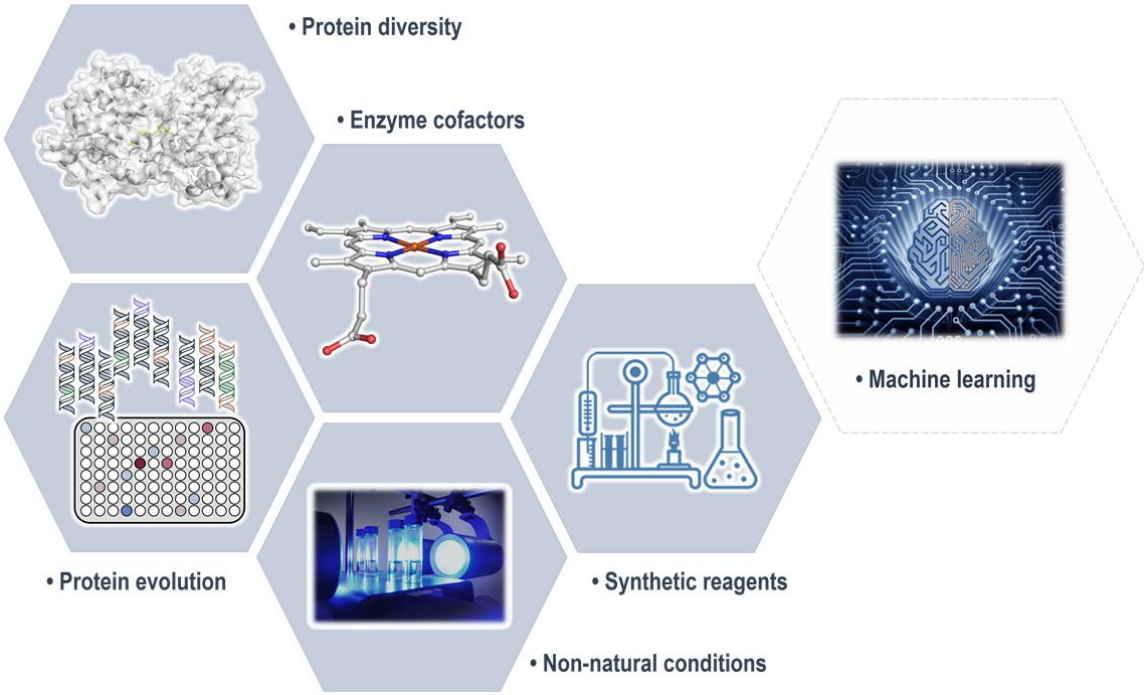
Introduction

Nature has evolved an astonishing array of enzymes to catalyze the chemical transformations that enable biological systems to eke out a living in diverse environments. Enzymes synthesize biological building blocks from available elemental resources, from which more enzymes go on to assemble new life, including essential biomolecules, complex natural products and macromolecular materials. Enzymes also break down these compounds into reusable fragments. While executing their biologically-relevant functions, enzymes can, when needed, exert precise control over reaction outcomes. The unique ability of enzymes to do this 'molecular editing' has prompted addition of some of nature's catalysts to the organic synthesis toolbox[1].

Nature's repertoire of enzyme functions is striking – from photosynthesis to nitrogen fixation, water splitting to aliphatic carbon assembly, there are still no human-made catalysts that can match these fundamental processes of life. On the other hand, the biological world has not followed the same path as human-invented chemistry, and many valuable transformations invented by synthetic chemists have no known enzyme-catalyzed counterparts. Among other reasons, nature does not use many of our favorite transformations because the products are not useful to living systems, the required reagents do not (stably) exist in nature, or because conditions to effect the reactions are not available.

To bridge nature's catalytic repertoire and the demands of synthetic chemistry, chemists and

biologists have started to import human-invented chemistry into enzymes. One approach researchers have tried is computational *de novo* enzyme design based on knowledge of the reaction transition-state structure[2,3]. Given our limited understanding of how enzymes function at an atomic level and how sequence encodes catalytic function in macromolecular design, however, an alternative avenue of engineering existing proteins has proven more successful, as least for now. Researchers are quickly unlocking new catalytic activities of existing enzymes simply by challenging and/or engineering them to work with non-natural reagents and in new environments. New activities can be released with relatively small modifications, such as introducing a different metal center or changing a few amino acids in an active site. (Perhaps this is not surprising, since this is also nature's innovation strategy.) Powerful molecular biology tools like directed evolution can then tune and diversify these new functions to provide catalysts that bring the benefits of nature's biosynthetic machinery to chemical synthesis. This review will cover these latter efforts to engineer new enzymes by starting from nature's designs (**Scheme 1**).



Scheme 1. Strategies in discovery of new enzyme functions. Discovery of new enzymes, engineering and diversification of proteins by directed evolution, exploitation of cofactors for new reactivities, and use of synthetic reagents and non-natural conditions have accelerated new enzyme activity development; other advances such as machine learning will expand biocatalysis capabilities by learning from growing gene/function databases.

A pivotal feature of enzymes, their promiscuity with respect to the substrates they accept and even the reactions they catalyze, has played a central role in the discovery and development of new biocatalytic functions. Enzymes can often accept various substrates in addition to their native one(s); they can even catalyze different transformations when offered the right reagents and environments[4]. These promiscuous activities may be left over from ancestral enzyme functions, or they may be activities that were never explored in the natural world and

come simply as a result of having catalytic machinery that exhibits its hidden capabilities when the environment changes[5]. There is immense potential in nature's vast repertoire of contemporary enzymes for us to discover and use, just as nature has done for more than three billion years. Early examples with hydrolytic enzymes showed, for instance, that enzymes whose native functions are amide or ester hydrolysis can also utilize their finely tuned networks of active-site residues for hydrolysis of other bonds or even formation of new bonds[6]. More recently, chemo-mimetic approaches developed by transferring human-invented chemistries to cofactor-dependent enzyme have significantly expanded the chemical space accessible to biocatalysis. Protein engineering, using non-physiological reaction conditions, and combination of chemo- and biocatalysis have further unveiled the potential for chemical innovation in existing enzymes.

Increasing demands for efficient, selective and versatile synthetic methods call for new enzymatic functions that may not be relevant in the biological world[7]. The challenges are daunting, requiring not only that enzymes take on new functions, but also that the newly developed biocatalysts exhibit activity and selectivity comparable to or better than current chemo-catalytic methods or that they fill gaps in synthetic chemistry. In this review, we summarize the current status of non-natural biocatalysis and describe how protein engineering integrated with chemical rationalization enables innovations that expand the chemical space accessible to enzymes. Creating abiological enzymatic functions represents a rising area of research that requires knowledge from different fields, including protein engineering, enzymology and synthetic chemistry. We hope this review will help chemists and biologists recognize, explore and use enzymes for new chemistry.

Main Text

• Section 1: Utilizing established active sites for new enzymatic functions

Early studies of enzyme promiscuity illustrated how enzyme active sites can catalyze physiologically irrelevant chemical transformations[8]. Although different in mechanism or path of bond formation and breakage, these non-native enzyme functions are typically enabled by the superior capacity of enzyme active sites to stabilize similar transition states and precisely control key intermediates, as exemplified by a large set of hydrolytic enzymes. Hydrolases usually feature an oxyanion hole, consisting of backbone amides or positively charged residues that stabilize the negative charge on a deprotonated oxygen or alkoxide in the transition state. Such structural properties allow the same hydrolases to catalyze diverse chemical reactions proceeding through oxyanionic intermediates, including aldol reactions, Michael additions, Mannich reations, and even peroxide-involved oxidative reactions[6]. For example, in 2003, Berglund and co-workers reported that *Candida antarctica* lipase B (CAL-B) catalyzes aldol reactions between aliphatic aldehydes; they used quantum molecular modeling to illustrate the importance of the oxyanion hole in stabilizing the enolate intermediate (Fig. 1a)[9]. Another class of hydrolase, glycosidase, was intensively investigated and engineered for promiscuous activities of glycoside synthesis: by mutating key residues such as catalytic acid/base pairs, Withers and co-workers converted a glycosidase into a glycosynthase[10] or a thioglycoligase[11] employing different α-glycosyl substrates and acceptor sugars.

The promiscuous functions of enzymes have been used for industrial production of valuable

compounds. One representative example is halohydrin dehalogenase (HHDH), which in nature catalyzes epoxide formation from corresponding substituted chloro- or bromohydrins[12]. Structural studies on various HHDHs revealed several highly conserved catalytic residues in the active sites that specifically bind epoxide and halide anion. A key tyrosine residue can act as a catalytic base for hydroxyl group deprotonation or a catalytic acid for epoxide protonation, which raised the possibility that it could catalyze the reverse reaction, epoxide ring opening. Acceptance of various nucleophiles, including azide, cyanide, nitrite, cyanate, thiocyanate and formate, and high enantioselectivity in the epoxide opening process, render HHDHs desirable catalysts for synthetic purposes, especially for preparation of enantio-enriched β-substituted alcohol and epoxide products. Among the biocatalytic applications of HHDH is the asymmetric synthesis of ethyl (*R*)-4-cyano-3-hydroxybutyrate, a precursor of atorvastatin, as reported by scientists at Codexis (Fig. 1b)[13]. Directed evolution was used to enhance the activity of *Agrobacterium radiobacter* HHDH, enabling production of the precursor with >99.9% *ee* based on a substrate loading of 130 $g \cdot L^{-1}$.

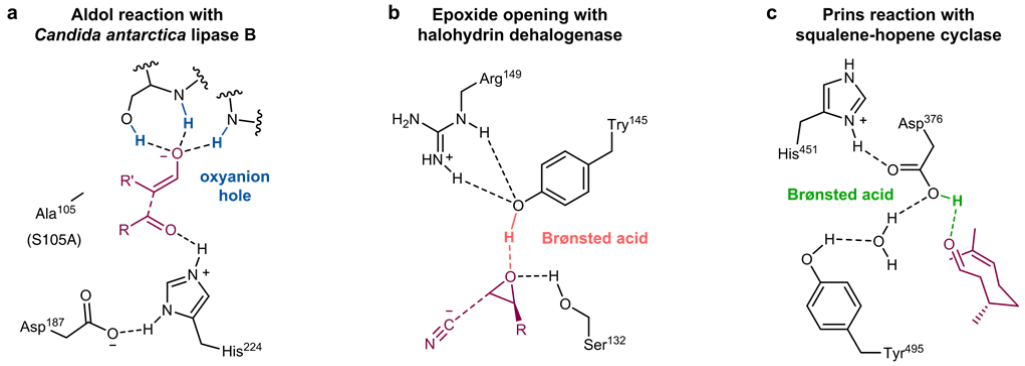


Fig. 1. Promiscuous functions enabled by versatile active sites. a, Aldol reaction with *Candida antarctica* lipase B (ref. [9]). **b**, Epoxide opening with halohydrin dehalogenase (ref. [13]). **c**, Prins reaction with squalene-hopene cyclase (ref. [16]).

The promiscuity of terpene cyclases has also attracted attention for abiological chemistry[14]. Terpene cyclases typically use acid/base catalytic residues for cationic cyclization of polyenes. Squalene-hopene cyclase (SHC), natively responsible for polycyclization of squalene to pentacyclic hopene and hopanol, has been explored as a promiscuous Brønsted-acid biocatalyst to harness a plethora of non-natural reactions driven by protonation processes. Hauer and co-workers employed SHC to construct abiological carbocyclic skeletons by using different internal nucleophilic terminators in the cyclization process[15]. Reshaping the active-site structure of SHC also allowed non-native acidic isomerizations of β-pinene, monocyclization of geraniol and Prins reaction of citronellal (Fig. 1c)[16]. For instance, a single amino acid mutation I261A in SHC from *A. acidocaldarius* (*Aac*SHC) improved activity 11-fold for a Prins reaction of (*S*)-citronellal to an (–)-iso-isopulegol isomer product (>99% *ee* and >99% *de*).

• Section 2: Exploiting the catalytic potentials of organo-cofactors

Cofactor-dependent enzymes are of particular interest, as many cofactors possess expansive catalytic potential for chemical transformations. This potential has been exploited in natural

evolution to create families of enzymes whose functions cover two-electron and single-electron redox/non-redox chemistries. The protein scaffold can direct the pathway through which a given reaction will proceed from among two or more possibilities. In this section, we will outline recent advances in realizing new chemistries with enzymes having organo-cofactors, including nicotinamide adenine dinucleotide (NADH or NADPH), flavin nucleotides (FMN or FAD), thiamine diphosphate (TDP) and pyridoxal phosphate (PLP) (Fig. 2). Reaction design and protein engineering have both promoted the discovery of new functions for these enzymes.

Nicotinamide adenine dinucleotide and its phosphate form (NADH or NADPH) act as biological reducing cofactors/co-substrates of numerous oxidoreductases. For example, keto-reductases (KREDs) reduce endogenous carbonyl compounds to alcohols. NADPH-dependent imine reductases (IREDs)[17] are also physiologically capable of reducing carbon–nitrogen double bonds. Recently, Turner and co-workers identified a subclass of IREDs which catalyze imine formation from ketones and amines prior to imine reduction and named them reductive aminases (RedAms)[18]. A RedAm from *Aspergillus oryzae* (*Asp*RedAm), an IRED homologue from a eukaryotic source, was discovered to accept a broad range of structurally diverse amines and ketones, which provides an attractive biocatalytic route to secondary/tertiary amines[19].

NAD(P)H is generally recognized as a hydride donor in catalysis. But NAD(P)H was recently found to be able to implement single-electron-transfer chemistry as well. Hyster and co-workers reported an abiological asymmetric radical dehalogenation of α-bromo-α-aryl/alkyl lactones with KREDs enabled by a photo-induced electron-transfer strategy[20]. The NAD(P)H cofactor can be excited by blue light to a triplet state, which serves as a potent single-electron reductant. A single electron transfer forms a substrate radical anion, which undergoes heterolytic cleavage of a C–Br bond to generate an alkyl radical. Then $NAD(P)H^{+\bullet}$ serves as a hydrogen atom donor to afford the dehalogenated lactone product. Several KREDs were found to catalyze dehalogenation of α-bromo γ/δ-lactones in good yields and enantioselectivities. Another system based on the combination of a NADPH-dependent double bond reductase (DBR) and the photocatalyst Rose Bengal (RB) enables enantioselective radical deacetoxylation of α-acetoxyl ketones, where the enzyme plays a role in activating the substrate for electron transfer followed by deacetoxylation and hydrogen atom transfer from NADPH[21].

Flavin cofactor natively appears in the form of flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) and is exceptionally versatile in enzymes, mediating a plethora of oxidative and reductive activities[22]. Flavin can exist in multiple redox states, including flavin-N_5-oxide (FMN or FAD oxide), oxidized flavin (quinone, FMN or FAD), flavin semiquinone ($FMNH^{\bullet}$ or $FADH^{\bullet}$), reduced flavin (hydroquinone, $FMNH_2$ or $FADH_2$), as well as flavin-C_4-peroxide (FMNOOH or FADOOH) when engaging with molecular oxygen. The protein scaffold and reaction conditions determine which states of the cofactor are accessible, leading to the diverse redox chemistry of natural flavoenzymes.

Compared to two-electron processes catalyzed by flavoenzymes, biological reactions with a single-electron mechanism involving the semiquinone state are rare. The Hyster lab discovered a promiscuous radical dehalogenation of α-bromo esters with flavin-dependent 'ene'-reductases (EREDs) by making use of the semiquinone state[23]. Interestingly, mutation

Y177F in ERED from *G. oxydans* abolishes the native 'ene'-reduction function in the absence of a proton donor, but significantly improves this non-native function, which further supports the radical mechanism for dehalogenation. Recently, Hyster and his team further expanded the capacity of EREDs to perform photo-induced radical cyclization to make various lactams (Fig. 2a)[24]. They reasoned that hydroquinone in the excited state can act as a single-electron reductant strong enough to activate α-chloro acetamide and generate the α-acetamide radical, which would cyclize to an *endo*-double bond and then abstract a hydrogen atom from semiquinone to form the desired lactam product. Lactam products with ring sizes ranging from 5 to 8 members were accessible via *endo*- or *exo*-cyclization processes in this system. A naturally-occurring flavin-dependent photodecarboxylase from *Chlorella variabilis* NC64A (*Cv*FAP) was discovered recently by Beisson and his team to employ a semiquinone state for a light-induced radical decarboxylation of fatty acids to alkanes or alkenes[25,26]. These newly demonstrated photoenzymatic platforms have revealed previously unknown catalytic potentials of the flavin cofactor.

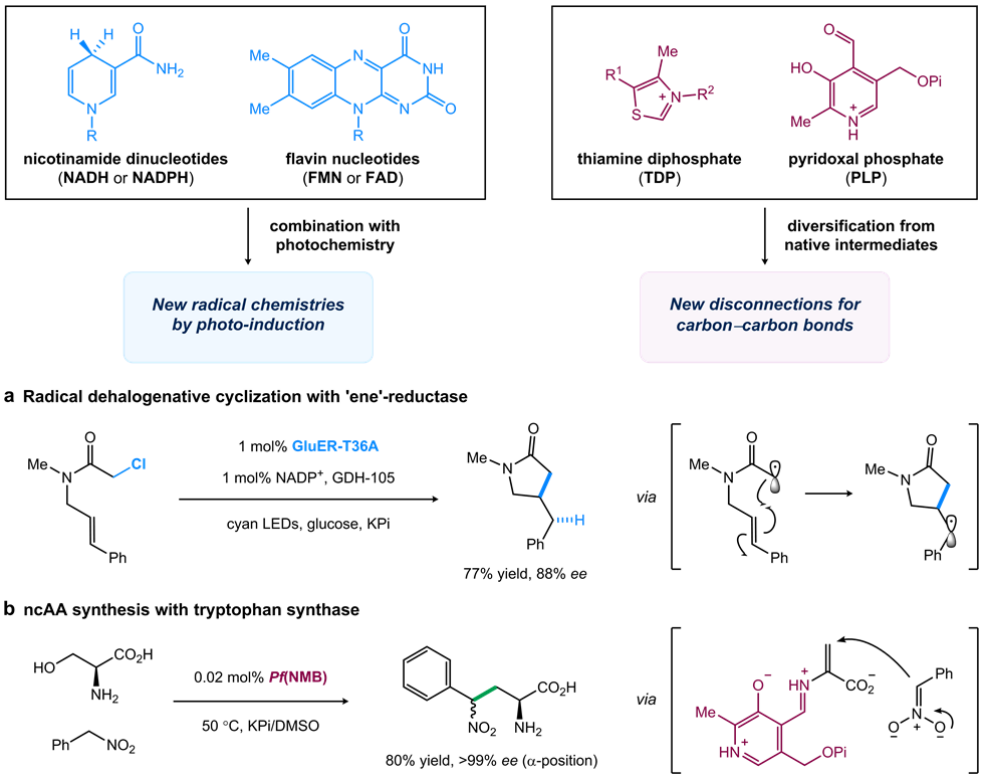


Fig. 2. New chemistries with cofactor-dependent enzymes. a, Radical dehalogenative cyclization with 'ene'-reductase (ref. [24]). **b**, Non-canonical amino acid synthesis with engineered tryptophan synthase (ref. [42]).

The thiamine-dependent enzymes offer another good example of how promiscuous catalytic functions can be exploited, in nature and by chemists. The TDP cofactor comprises a thiazolium core, an aminopyrimidine group and a diphosphate moiety. The aminopyrimidine group acts a key base for deprotonation of the C_2 position in the thiazolium ring, which leads to the formation of a nucleophilic thiazolium carbene and initiates all types of thiamine catalysis in nature[27]. The thiazolium carbene is a superior nucleophile for addition to carbonyl

groups, resulting in an enaminol species, known as the Breslow intermediate, for a variety of nucleophilic reactions[28]. In such a way, thiamine enzymes can take electrophilic aldehydes or other carbonyl substrates, turn them into a nucleophilic form and further enable desired bond constructions.

Thiamine-dependent enzymes have been explored for abiological asymmetric C–C bond-forming reactions that take advantage of the nucleophilic feature of the Breslow intermediate. A cross-benzoin condensation between acetaldehyde (after decarboxylation of pyruvate) and benzaldehyde was achieved by Müller and co-workers using the cyclohexane-1,2-dione hydrolase (CDH) from *Azoarcus sp.* to produce chiral α-hydroxy ketone products in high enantioselectivity[29]. CDH was also engineered to accept ketones as electrophiles[30,31]. The cross-benzoin reaction between two aromatic aldehydes is particularly challenging due to the chemo-selectivity problem arising from homo-couplings and mixed cross-couplings[32]. However, benzaldehyde lyase (BAL) from *Pseudomonas fluorescens* and a variant of benzoylformate decarboxylase (BFD) from *Pseudomonas putida* were found to catalyze this reaction, where the steric control from the *ortho*-substituted aldehyde as the electrophile substrate is key to high chemoselectivity[33]. Instead of aldehydes or ketones, α,β-unsaturated carbonyl substrates have also been investigated for this nucleophilic addition of the Breslow intermediate in a 1,4-conjugate manner (the Stetter reaction). A thiamine enzyme, PigD from *Serratia marcescens*, catalyzes acetaldehyde addition to α,β-unsaturated ketones with high enantioselectivity[34].

Pyridoxal phosphate (PLP) is another highly versatile enzyme cofactor. Taking advantage of the aldimine intermediate formed through the condensation between the aldehyde group of PLP and amino group from a substrate, PLP-dependent enzymes catalyze transamination, amino acid decarboxylation, deamination, racemization, and more. *O*-Acetylserine sulfhydrylase (OASS) is a PLP-dependent enzyme used for cysteine biosynthesis. It forms a key aminoacrylate intermediate through the loss of an acetate from the aldimine between *O*-acetylserine and PLP, and nucleophilic addition of H_2S to the aminoacrylate gives *L*-cysteine[35]. Early work reported that a variety of heteroatom-based nucleophiles could also be used by OASS to synthesize non-canonical β-substituted alanine derivatives[36].

Similar to OASS in mechanism, tryptophan synthase (TrpS) catalyzes the formation of tryptophan through addition of indole to the aminoacrylate electrophile formed with serine and PLP in its β-subunit (TrpB). Early work demonstrated that TrpS could accept some indole derivatives or other heterocyclic nucleophiles for the synthesis of tryptophan analogues[37]. Buller and co-workers engineered the β-subunit of tryptophan synthase from *Pyrococcus furiosus* (*Pf*TrpB) to serve as a stand-alone enzyme for non-canonical amino acid (ncAA) synthesis with different nucleophiles[38,39]. Further engineering of *Pf*TrpB expanded the scope of the serine electrophile to include threonine and other β-alkyl serine derivatives for production of β-substituted tryptophan analogues[40,41]. Recently, Romney and co-workers reported that an evolved *Pf*TrpB can accommodate nitroalkane nucleophiles structurally distinct from the indole analogues, amines and thiols that were demonstrated previously (Fig. 2b)[42]. Because the nitro group can serve as a handle for further modification, this biocatalytic strategy provides a convenient route to diverse ncAAs with aryl and alkyl side-chains.

Compared to the diversity of small-molecule catalyst scaffolds invented by chemists, nature uses a more limited set of organo-cofactors for catalysis. However, the catalytic potential of

these cofactors is still far from fully discovered or explored. Different protein structures or reaction conditions may completely alter the properties of the cofactors, a feature that natural evolution has exploited to create functionally diverse enzyme families. This chemical flexibility provides opportunities to use the diverse electronic and photo-chemical properties of cofactors and develop new reaction pathways that have not been explored by nature. Taking inspiration from the studies described here, we imagine that future efforts to the cofactors described here and others such as tetrahydrobiopterin (THB)[43], 4-methylideneimidazole-5-one (MIO)[44] and prenylated flavin (prFMN)[45], will lead to the discovery of yet more functionally diverse enzymes.

• Section 3: Taming metalloenzymes for non-native reactions

Natural enzymes also use metal ions or metal-based cofactors to implement diverse, challenging transformations, as exemplified by nitrogenase for nitrogen fixation. The versatility of transition metal electronic states and coordination modes lays the foundation for transition metal catalysis in chemistry. This versatility also provides opportunities to develop new chemistries starting from nature's vast collection of metalloproteins.

A given metalloenzyme family can encompass diverse functions, but usually their reactions proceed via a specific type of metallo-intermediate. For instance, iron(II)- and α-ketoglutarate-dependent (Fe/αKG) enzymes employ a high-valent iron-oxo (Fe^{IV}) intermediate for C–H hydroxylation, desaturation of aliphatic hydrocarbons, epoxidation of olefins, epimerization of sp^3-hybridized carbon centers and others. SyrB2, an Fe/αKG enzyme from the syringomycin biosynthetic pathway of *Pseudomonas syringae* B301D, is responsible for C–H halogenation of the side-chain methyl group of a threonine moiety tethered with its carrier protein SyrB1[46,47]. Mechanistically, a homolytic coupling between a halogen ligand of iron and a carbon-centered radical formed through a hydrogen-atom abstraction process results in the carbon–halogen bond formation. This halogenation activity is thought to originate from the hydroxylation activity of Fe/αKG enzyme homologues. Based on further mechanistic study, Bollinger and co-workers discovered that incorporation of a non-oxygen ligand at the iron center could lead to new enzymatic functions. Azide or nitrite anions can bind to the iron center of SyrB2, thus proceeding through radical azidation and nitration with different amino acid-based substrates[48]. These reactions still require carrier protein-appended substrates and can only occur in modest yields under single-turnover conditions, but such activity provides an unprecedented enzymatic route to C–N bond formation with aliphatic C–H bonds. Future work to identify other homologues and enzyme engineering may further expand the C–H functionalization chemistries of Fe/αKG enzymes and also allow these enzymes to accept diverse substrates for synthetic purposes.

Iron-oxo-mediated alkene epoxidation via oxo transfer to C–C double bonds is a well-established transformation with iron-based oxygenases, such as Fe/αKG or cytochrome P450 enzymes. Epoxidation typically follows a concerted [2+1] cycloaddition pathway with a low energy barrier. With a particular cytochrome P450, however, aldehydes were observed as side-products of this epoxidation reaction[49]. Hammer *et al.* hypothesized that this promiscuous activity resulted from a step-wise pathway of radical addition of iron-oxo to the alkene substrate, single electron transfer and a subsequent 1,2-hydride migration to deliver the *anti*-Markovnikov oxidation product[50]. Hammer then engineered this P450 from the

rhodobacterium *Labrenzia aggregata* ($P450_{LA1}$) to divert the iron-oxo intermediate into this *anti*-Markovnikov oxidation over the kinetically favored epoxidation. Accumulation of mutations in $P450_{LA1}$ led to a variant, aMOx, that catalyzes the *anti*-Markovnikov oxidation of styrene to phenylacetaldehyde with 3800 TTN and 81% selectivity. By providing a chiral environment in the enzyme's active site for this step-wise oxo-transfer pathway, the first example of *enantioselective anti*-Markovnikov oxidation was demonstrated with a prochiral α-methylstyrene substrate, giving 82% *ee*.

The above examples demonstrate the feasibility of hijacking key intermediates in the catalytic cycles of metalloenzymes into different reaction pathways in order to access new enzymatic activities. Alternatively, one could also enable new chemistries by introducing new reactive intermediates that mimic the native intermediates, structurally and functionally[51]. Metalloporphyrin complexes have been investigated for more than half century as structural analogues to heme cofactors in proteins. Many metalloporphyrins were developed in an effort to mimic the oxo-transfer activities of cytochrome P450s through the formation of high-valent metal-oxo intermediates[52]. Meanwhile, intermediates analogous to metal-oxo species, typically metallo-carbenes and metallo-nitrenes, could also be formed by the metalloporphyrins and used for carbene and nitrene transfer reactions[53,54]. For instance, a precursor such as a diazo compound can react with the transition metal center (*e.g.*, iron, cobalt, rhodium, ruthenium, iridium, osmium, *etc.*) in metalloporphyrin complexes to generate a metal-carbenoid intermediate, which can undergo transfer to organic molecules[55,56]. Reasoning that translating the non-natural activities of metalloporphyrin catalysts to the corresponding heme-dependent enzymes would be a promising way to access new enzymatic activities, Coelho and co-workers described the first 'carbene transferase' enzyme in 2013 (Fig. 3)[57].

Cytochrome P450 from *Bacillus megaterium* ($P450_{BM3}$) catalyzes alkene cyclopropanation via an abiological iron-carbenoid intermediate[5757]. A diazo reagent, ethyl diazoacetate (EDA), reacts with the cytochrome P450 in the Fe^{II} state and yields an iron-carbenoid intermediate; subsequent carbene transfer to styrene substrates led to the corresponding cyclopropane products. A cysteine-to-serine mutation at the heme-ligating residue furnished a new set of enzymes, designated P411s; this mutation increased the reduction potential of the ferric state of the iron center, allowing *endo*-cellular reductant NADPH to reduce the ferric state and thus conferred carbene transfer activity *in vivo*[58,59]. Histidine ligation was also structurally tolerated by the P450, where it accelerated cyclopropanation of acrylamide substrates[60]. Nature offers a diversity of heme proteins, and our group and the Fasan group have demonstrated that various small hemeproteins, including protoglobin, nitric oxide dioxygenase[61] and myoglobin[62], could also be engineered to catalyze cyclopropanation reactions in high efficiency and stereoselectivity, even with electron-deficient and electron-neutral alkenes.

Chen and co-workers hypothesized that an enzyme could catalyze carbene transfer to alkynes to construct highly strained cyclopropenes and that a second carbene-transfer step would give even more-strained bicyclo[1.1.0]butane structures[63]. Despite very few precedents of bicyclobutane formation using a carbene transfer strategy, an evolved P411 variant, P411-**E10** A78F S438A, adopts this approach to synthesize bicyclobutanes via successive carbene addition to phenylacetylene substrates (Fig. 3a). With aliphatic alkyne substrates, two stereo-

complementary P411 variants were obtained for the enantio-divergent synthesis of cyclopropenes. Identity of the amino acid residue at position 87 determines the stereochemistry.

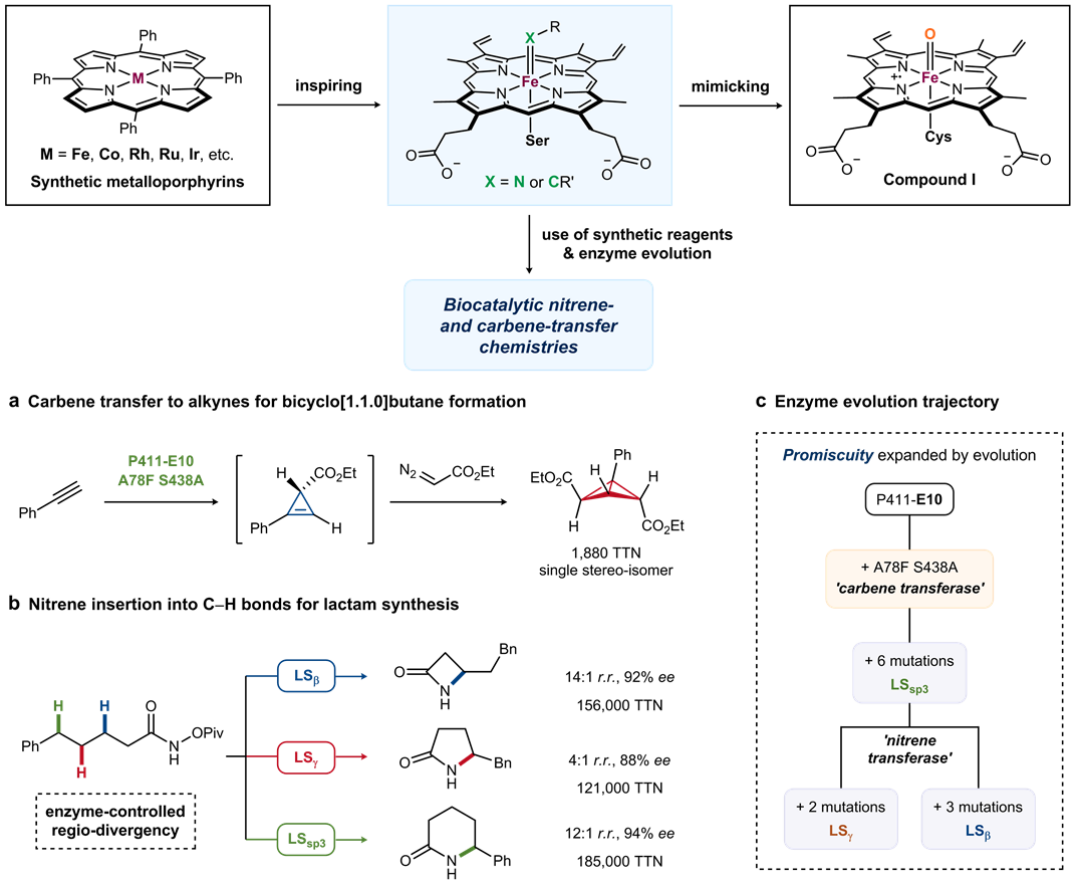


Fig. 3. Chemo-mimetic carbene- and nitrene-transfer chemistries with engineered hemeproteins. a, Carbene transfer to alkynes for bicyclo[1.1.0]butane formation (ref. [63]). **b**, Nitrene insertion into C–H bonds for diverse lactam synthesis (ref. [73]). **c**, Rapid expansion of P450s' promiscuity to carbene and nitrene transfers (in **3a** and **3b**) by laboratory evolution.

Aziridination via nitrene transfer to alkenes can also be achieved with P411 hemeproteins. Our group initially demonstrated aziridination of styrene-type substrates with engineered P411 variants using tosyl azide (TsN_3) as the reactive nitrene precursor[64]. Recently, cytochrome *c*, an electron-transfer protein, was shown to be capable of styrene aziridination, using *O*-pivaloyl hydroxylammonia triflate as the reagent to generate a putative unprotected nitrene species[65]. Under aqueous conditions, these aziridines are labile and undergo hydrolysis to afford unprotected chiral 1,2-amino alcohols.

In carbene- and nitrene-transfer chemistries, alkene cyclopropanation and aziridination are analogous to P450s' native epoxidation activities; the abiological counterparts to C–H hydroxylation are C–H alkylation and C–H amination. Early in the 1980s, Dawson and co-workers reported that a rabbit-liver cytochrome P450 catalyzed a nitrene C–H insertion reaction using an abiological iminoiodinane substrate as nitrene precursor with very limited

turnovers[66]. Inspired by this work, McIntosh *et al.* achieved intramolecular nitrene insertion into a proximal C(sp^3)–H bond using a P411 as a whole-cell catalyst, affording sultam products in decent yield and good enantioselectivity[67]. Fasan also showed that $P450_{BM3}$[68] or myoglobin[69] variants are capable of such intramolecular C–H amination reactions. In addition, the evolved P450 variants can also function with azidoformates for intramolecular C–N bond formation, providing oxazolidinones as the amination products[70].

Engineering site-selective C–H amination is of great interest, since regioselectivity with small-molecule catalysis is usually dominated by the inherent properties of the C–H bonds. Hyster and co-workers demonstrated that the active site of P450 can be reshaped to facilitate C–H amination in a regio-divergent manner[71]. With a sulfonylazide bearing two types of C(sp^3)–H bonds geometrically accessible to amination, two P411 variants were able to direct intramolecular nitrene insertion to benzylic and homo-benzylic C(sp^3)–H bonds, forming 5- and 6-membered sultam products.

After the work on abiological nitrene transfer was published, Ohnishi and co-workers disclosed that BezE, a cytochrome P450 in the biosynthetic pathway of benzastatin from Streptomyces sp. RI18, is responsible for an aziridination process via formation of an iron-nitrenoid intermediate with an *N*-acetoxy substrate and a subsequent nitrene transfer to a proximal double bond[72]. This is an excellent example of how non-natural biocatalysis can illuminate underexplored paths and lead to new discoveries in biological chemistry. In turn, the Ohnishi study inspired us to look at hydroxylamine-type nitrene precursors, which can be more accessible and have fewer stability issues compared to the azide compounds.

Recently, Cho *et al.* employed *O*-acyl hydroxylamides, readily prepared from carboxylic acids, as nitrene precursors for the synthesis of a variety of lactam products through intramolecular C–H amidation[73]. This work further highlights the tunability of P411 enzymes in site-selective C–H functionalization. Starting with a P411 variant, P411-**E10** A78F S438A, directed evolution was carried out in parallel for synthesizing lactam products of different sizes. Four complementary variants, LS_{sp2}, LS_{sp3}, LS_{β} and LS_{γ} were obtained for selective C–H amidation targeting various sp^2- or sp^3-carbon positions (Fig. 3c). With a substrate featuring three different types of C–H bonds that could be targeted for amidation, different P411 variants could make each of the β-, γ- and δ-lactams selectively (Fig. 3b). Given the differences in bond dissociation energies of the C–H bonds (>10 kcal/mol difference) and the strain of lactam products, the distinct product outcomes show that properly engineering the active-site environment for catalysis can override the inherent reactivity of the C–H bonds and guide product formation along desired reaction pathways.

P411s were also engineered to catalyze intermolecular nitrene C–H insertion enantioselectively[74], a problem with only limited examples of solutions with small-molecule catalysts. Accumulation of active-site mutations in a P411 enzyme helps to precisely orient the substrate in the distal pocket and accelerate the desired C–H insertion. Using TsN_3 as the nitrene precursor, evolved $P411_{CHA}$ enantioselectively aminated benzylic C–H bonds of alkyl benzene substrates. More importantly, this $P411_{CHA}$ variant provided a versatile platform for evolving biocatalysts for diverse carbene- or nitrene-transfer reactions inaccessible to chemical catalysis, including above-mentioned bicyclobutane formation and lactam synthesis.

Carbene C–H insertion to install alkyl groups onto organic molecules is also feasible with cytochrome P411 variants[75,76]. A variant obtained in the evolutionary lineage of $P411_{CHA}$

displayed promiscuous activity for carbene insertion into C–H bonds and thus served as a parent for evolution of a powerful alkyl-transferase, $P411_{CHF}$, which can target benzylic, allylic, propargylic and α-amino C–H bonds for carbene insertion. Interestingly, $P411_{CHF}$ alkylates a benzylic C–H bond in a substrate bearing a terminal alkene moiety, while an early P411 variant, **P**-I263F only cyclopropanates the double bond in the same molecule. This demonstration of catalyst-controlled chemoselectivity, once again speaks to the high tunability of enzyme catalysis, which provides a promising solution for addressing long-standing selectivity challenges.

Analogous to heteroatom oxidation by cytochrome P450s, electrophilic nitrene or carbene intermediates formed with hemeproteins, such as P411 or myoglobin variants, can be intercepted by sulfides to furnish sulfimides[77] and sulfonium ylides[78]. Moreover, with prochiral allylic sulfides, the corresponding allylic sulfimide or sulfonium ylide products can further undergo [2,3]-sigmatropic rearrangement to yield chiral allylic amines[79] or chiral sulfides[78].

Another useful class of carbene-transfer reactions is X–H (X = heteroatom, including N, S, Si, B, P and others) bond insertion. Our group showed that $P450_{BM3}$ variants can catalyze *N*-alkylation of aniline substrates via a formal carbene N–H insertion process[80]; the Fasan lab also described N–H insertion[81] and S–H insertion[82] reactions with engineered myoglobins. Recently, our lab reported a P450-catalyzed enantioselective S–H insertion reaction using a lactone-derived carbene which proceeds through a radical mechanism[83].

Carbene Si–H or B–H insertion reactions provide efficient routes for building C–Si and C–B bonds not found in biological systems, but useful and important in human-made products. Hemeproteins are capable of forming these bonds with much higher activities than reported for transition metal catalysts. Wild-type cytochrome *c* from *Rhodothermus marinus* (*Rma* cyt *c*) was discovered to catalyze carbene Si–H insertion using ethyl 2-diazopropanoate (Me-EDA) and phenyldimethylsilane as substrates with a modest turnover (44 TTN) but good enantioselectivity (97% *ee*)[84]. Introduction of three active-site mutations improved catalytic efficiency by over 30-fold. Evaluation of silane scope established that the evolved *Rma* cyt *c* is particularly selective for the desired silylation even with substrates bearing other functionalities that can participate in carbene-transfer chemistry. *Rma* cyt *c* was also engineered for enantioselective carbene B–H insertion reactions using *N*-heterocyclic carbene-stabilized boranes[85-87]. By modifying the active-site structure of *Rma* cyt *c*, a variety of structurally different carbenes can be accommodated for this B–H insertion reaction. With the established carbene chemistries of *Rma* cyt *c*, Lewis was able to capture in a crystal structure the carbene intermediate bound to the iron center of an *Rma* cyt *c* mutant[88], which allowed us to investigate the iron-carbenoid species and provided insight into how protein structure enables the desired chemistries.

These newly-discovered carbene- and nitrene-transfer activities of hemeproteins expand nature's catalytic repertoire to include many transformations which are not biologically relevant but are highly useful for chemical synthesis. For most of the reactions described here, the free heme cofactor catalyzes the reaction not at all or only with very poor efficiency, highlighting the contribution of the protein to enabling and facilitating these transformations. Although small-molecule catalysts have been developed for most of these chemistries, hemeproteins stand out as competent catalysts with high catalytic efficiency and readily

tunable stereo-/regio-/chemoselectivities. With the help of strategies to discover and improve new carbene and nitrene transferases, we foresee that hemeprotein biocatalysts will address more challenging problems in synthetic chemistry and will move to wider use at scale.

• Section 4: Developing new enzymes with artificial cofactors

The above examples show how small-molecule catalysis can inspire discovery of new enzyme functions. However, there are still many synthetically important reactions carried out with human-invented catalysts for which enzyme candidates have not yet been identified. Chemists have been trying to fill some of this large gap between classical catalysis and biocatalysis by incorporating catalytically competent artificial cofactors into proteins. Artificial metalloenzymes (ArMs), for example, can be traced back to the late 1970's when Wilson and Whitesides assembled an artificial metallo-hydrogenase for hydrogenation of α-acetamidoacrylic acid by introducing a biotin-tethered diphosphine-rhodium(I) complex to avidin[89]. Most early studies focused on proving that new enzymes with human-invented metallocatalysts could be made, and many functions performed by these ArMs, such as ester/amide hydrolysis, alcohol/olefin oxidation and ketone/imine/acrylate reduction, were already well-known for enzymes, despite mechanistic differences. More recently, the development of ArMs has been greatly accelerated by advances in organometallics and protein engineering. A broad range of ArMs have been created for important transformations in synthetic chemistry. A recent review from the Ward and Lewis groups comprehensively summarizes work in the field of ArMs[90]. Here we will focus only on representative ArMs with abiological functions.

The biotin-(strept)avidin system has been used extensively to construct ArMs. Avidin and streptavidin (Sav) feature deep binding pockets for biotin and provide a chiral environment for the catalytic center. Ward and co-workers applied this to the creation of palladium-ArMs for Suzuki-cross coupling[91] and allylic alkylation[92] and rhodium-ArMs for C–H activation/annulation reactions[93]. Based on their early work on ruthenium-ArMs for olefin metathesis[94], the team developed a system for selective assembly of artificial 'metathases' within the periplasm of *E. coli* cells through the fusion of Sav with the signal peptide OmpA[95]. This *in vivo* construction strategy substantially expedited application of directed evolution, leading to an evolved metathase with higher activity than the free 2nd-generation Grubbs catalyst towards a ring-closing metathesis reaction. Recently, the Rovis lab also reported a monomeric streptavidin (mSav) Rh(III)-ArM for enantioselective C–H activation/annulation with acrylamide hydroxamate esters and styrenes for the synthesis of a variety of substituted δ-lactams (Fig. 4a) [96].

ArMs can also be assembled through covalent linkages of metallo-cofactors to the protein. The thiol group on cysteine is typically used as a handle for covalent assembly via nucleophilic substitution or conjugate addition, as demonstrated for Ru-based olefin metathases by Hilvert[97] and Rh(I)-based olefin hydroformylase by Jarvis and Kamer[98]. However, selective ArM assembly with this thiol-linkage strategy is difficult when the protein has multiple accessible cysteine residues. Lewis and co-workers thus used a genetically-encoded azidophenylalanine (pN$_3$Phe) for specific coupling with strained alkyne-modified metallocofactors through "click chemistry"[99]. They selected a prolyl oligopeptidase (POP) featuring a large internal cavity as the protein scaffold and constructed diRh(II)-ArMs

through the cycloaddition between a strained alkyne pre-installed on a dirhodium catalyst and a pN_3Phe residue in the active site of POP[100]. This rapid and selective assembly system allowed them to develop a practical platform to evolve diRh(II)-ArMs for stereo-selective cyclopropanation of styrenes with donor-acceptor diazos using random mutagenesis and screening (Fig. 4b)[101].

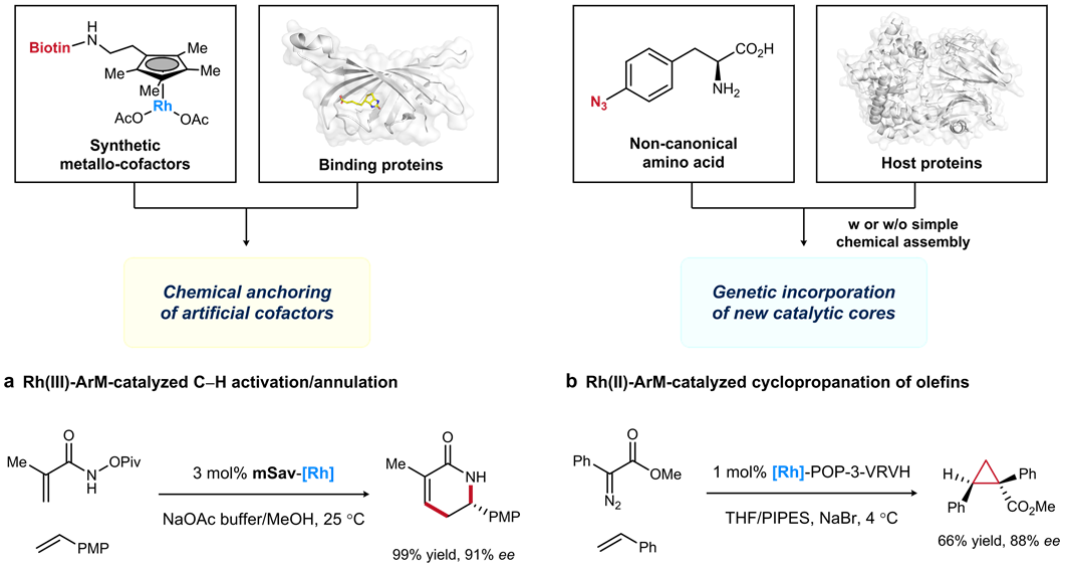


Fig. 4. Different strategies for artificial enzyme construction. a, Artificial Rh(III)-enzyme-catalyzed C–H activation/annulation (ref. [96]). **b**, Artificial diRh(II)-enzyme-catalyzed olefin cyclopropanation (ref. [101]).

Binding of metals or metal complexes with coordinating residues represents an alternative strategy for constructing ArMs. Ueno, Watanabe and co-workers reported a 'Suzukiase' enabled by ligation of a $[Pd(allyl)Cl]_2$ complex in apo-ferritin[102]. Metalation of native carbonic anhydrase (hCA) with a $[Rh(acac)(CO)_2]$ complex generated a hydroformylase, and further protein engineering helped to improve the overall enzyme activity and the selectivity of linear aldehyde product over the branched one[103]. The Roelfes group has also utilized coordinating ncAAs to bind metal ions for new catalytic functions, as exemplified by the Cu(II)-ArMs for Friedel–Crafts alkylation of indoles[104] and hydration with α,β-unsaturated alkenes[105]. A cofactor switch strategy has also been realized to generate ArMs. Hartwig, Clark and co-workers explored a set of iridium-substituted ArMs generated from myoglobin[106] and cytochrome P450[107] for carbene C–H insertion reactions. Engineered Ir-ArMs are also able to carry out cyclopropanation of unactivated or internal alkenes[108] and intramolecular nitrene C–H insertion[109]. It is interesting to note that most of these functions have also been demonstrated with engineered iron-hemeproteins that are fully genetically encoded.

Artificial enzymes with non-natural catalytic centers can also be constituted in a fully genetically-encoded scenario using non-canonical amino acid (ncAA) incorporation. A wide range of ncAAs can now be genetically encoded and incorporated into protein scaffolds, which allows introduction of unnatural cofactors for new catalytic functions. A recent review by Budisa summarizes the development of biocatalysts using this genetic strategy[110]. Most

examples only employ ncAAs to tune the properties of enzyme active sites or natural cofactors and to enhance the native catalytic functions. Until now, there are only a few examples of catalytically functional ncAAs for new enzymatic activity, among which is the use of *p*-aminophenylalanine (*p*AF) for catalytic condensation of carbonyls with hydrazines and hydroxylamines by the Roelfes' group[111,112].

Reaction design based on the selection of suitable catalytic scaffolds together with protein engineering has produced artificial enzymes for synthetically important chemical transformations. However, compared to natural enzymes, most artificial enzymes still exhibit low catalytic efficiency with limited turnovers and are usually not as versatile as the best small-molecule catalysts for the same type of reactions. Preparation of most ArMs still requires tedious processes, including chemical synthesis of specific metal cofactors, purification of the apo-proteins followed by assembly steps, and sometimes removal of excess cofactor from the system, which render tuning by directed evolution difficult. Therefore, developing versatile, amenable, readily evolvable systems is a challenge for future research.

Conclusion

The third wave of biocatalysis, starting in the early 1990s and empowered by directed evolution and other methods, saw solutions to many practical problems in enzyme catalysis, including enzyme stability issues, limitations in substrate breadth, efficiency, selectivity, and others[113]. These developments laid solid foundations for widespread adoption of biocatalysis for industrial applications in pharmaceuticals, fine chemicals, agriculture, materials and more[114]. In a recent perspective, Bornscheuer describes a fourth wave of biocatalysis[115] with discovery of new enzyme classes and development of non-natural activities as major new directions. These efforts lead not only to a broader appreciation of enzymes' capabilities but also fulfill the demand for new, sustainable methods in organic synthesis[116].

We predict that enzymes invented in the laboratory will become powerful complements and alternatives to synthetic catalysts. For example, engineered hemeprotein carbene and nitrene transferases, unknown less than ten years ago, are capable of catalyzing diverse transformations which are also accessible with synthetic catalysts based on rhodium, iridium and other transition metals. However, directed evolution has enabled the enzymes to display orders of magnitude higher turnovers, using an earth-abundant iron center; the enzymes also have selectivities that none of the small-molecule catalyst can offer. Perhaps most exciting, the enzymes can make molecules that are completely inaccessible to small-molecule catalysts (e.g. pure stereoisomers of bicyclobutanes[63]). It is thrilling to realize that the ability of enzymes to control reaction intermediates and specifically accelerate a desired reaction can now be used to control chemistry invented by us!

On the other hand, many of the new enzymes described here still exhibit low catalytic efficiencies, limited substrate ranges and moderate selectivities. It is reasonable to think that a natural enzyme co-opted for new chemistry or an artificial enzyme assembled from a protein scaffold and a synthetic cofactor does not provide an active site optimal for entire mechanistic pathway of the new reaction. Directed evolution can step in to reorganize active-site structures for non-natural catalysis, but this requires systems for mutagenesis and screening to identify beneficial changes in the protein sequence. We can anticipate that

improved rational protein design as well as new protein engineering methods based on machine learning will help navigate the landscape of enzyme activities and protein sequence to guide further engineering with reduced experimental effort[117].

Other challenges include the fact that the catalytic repertoire of enzymes is still very restricted compared to synthetic methods. For example, organofluorine moieties are particularly important in medicinal chemistry, but until now only one class of enzyme, fluorinase, is known to catalyze a C–F bond-forming reaction with high substrate specificity[118]. Bimolecular cycloadditions, developed by synthetic chemists to build various ring structures in a modular way, are barely utilized by natural enzymes[119]. Furthermore, most newly identified enzymes are not robust enough for synthetic or industrial application. Compared to transaminases, for example, which natively catalyze C–N bond formation and are also used widely in the pharmaceutical industry[120], the recent enzymatic C–H amination strategy using nitrene transfer to a C–H bond provides a straightforward way to get to the same targets without needing a pre-installed carbonyl functionality. However, utility for synthetic purposes necessitates further improvements in the enzyme and reaction engineering; the enzymes also have to be made broadly available to users. In principle, genetically encoded catalysts are available to anyone with access to the sequence. In practice, however, few synthetic laboratories have the expertise and equipment to exploit them.

Overall, we see a bright future for enzymes in a world that needs clean, efficient catalysts. New activities will be discovered at an ever faster pace as chemists look at enzymes with their goals in mind. The current challenges are worthy targets for creative problem solvers.

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Author contributions

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Competing interests

The authors declare no competing interests.

Additional information

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