Selective C–H bond functionalization with engineered heme proteins: new tools to generate complexity

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C–H functionalization is an attractive strategy to construct and diversify molecules. Heme proteins, predominantly cytochromes P450, are responsible for an array of C–H oxidations in biology. Recent work has coupled concepts from synthetic chemistry, computation, and natural product biosynthesis to engineer heme protein systems to deliver products with tailored oxidation patterns. Heme protein catalysis has been shown to go well beyond these native reactions and now accesses new-to-nature C–H transformations, including C–N and C–C bond forming processes. Emerging work with these systems moves us along the ambitious path of building complexity from the ubiquitous C–H bond.

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Introduction

Selective replacement of the ubiquitous carbon–hydrogen (C–H) bond with a carbon–heteroatom or a carbon–carbon bond is an outstanding synthetic chemistry challenge to which engineered enzymes are starting to make important contributions. Collectively termed C–H functionalization, this set of reactions has the immense potential to change the logic of chemical synthesis [1,2]. Though its development in synthetic chemistry has mainly been realized in the last few decades, nature has utilized a C–H functionalization approach to diversify molecules for eons. Most biological C–H functionalization reactions are catalyzed by cytochromes P450, a superfamily of heme-thiolate monooxygenases [3]. Inspired by the heme cofactor, early work with small-molecule transition metal catalysts on C–H hydroxylation employed the porphyrin scaffold [4]. It has since been demonstrated that porphyrin is a versatile scaffold for diverse C–H functionalization reactions (Figure 1a) [5,6].

We believe that nature's heme proteins have great potential for C–H functionalization, including catalyzing reactions with no biological counterparts. Enzymes could conceivably offer catalyst-controlled selectivity, high turnover numbers, or eliminate dependence on toxic noble metals, which are desirable advances for the field of C–H functionalization [1,2]. While protein engineering has historically focused on improving the known function of an enzyme [7], a recent paradigm shift has expanded this vision. The initial focus is now on the target reaction, such as a C–H functionalization transformation, and the necessary elements to achieve that chemistry, such as the ability to bind porphyrin. Protein engineers then search through the vast collection of existing proteins, select those which have the necessary parts (it can also be beneficial to introduce artificial cofactors or computationally design a suitable protein [8]), and test for the desired activity. Once even a low level of the activity has been found, a new enzyme can be created by directed evolution, a protein engineering strategy which uses iterative cycles of mutagenesis and screening to accumulate beneficial mutations that enhance catalyst performance (Figure 1b). This approach has generated porphyrin-containing enzymes which oxidize C–H bonds with tailored site-selectivity and perform new-to-nature C–H amination and C–C bond forming reactions.

In this short opinion piece, we survey C–H functionalization transformations catalyzed by engineered heme proteins and the methods used to introduce or optimize these functions. These efforts are compelling precedents for expanding the chemistry accessible to proteins, and we anticipate that they will inform and inspire exploration of other protein systems for new catalytic functions.

Oxidative transformations catalyzed by engineered cytochromes P450

Cytochromes P450 are nature's most prevalent catalysts for C–H functionalization [9]. Enzymes of this vast family directly activate inert C–H bonds for a broad spectrum of oxidative transformations such as hydroxylation, desaturation, decarboxylation, and carbon-skeleton rearrangement [10,11]. The exceptional activities of P450s have

Figure 1
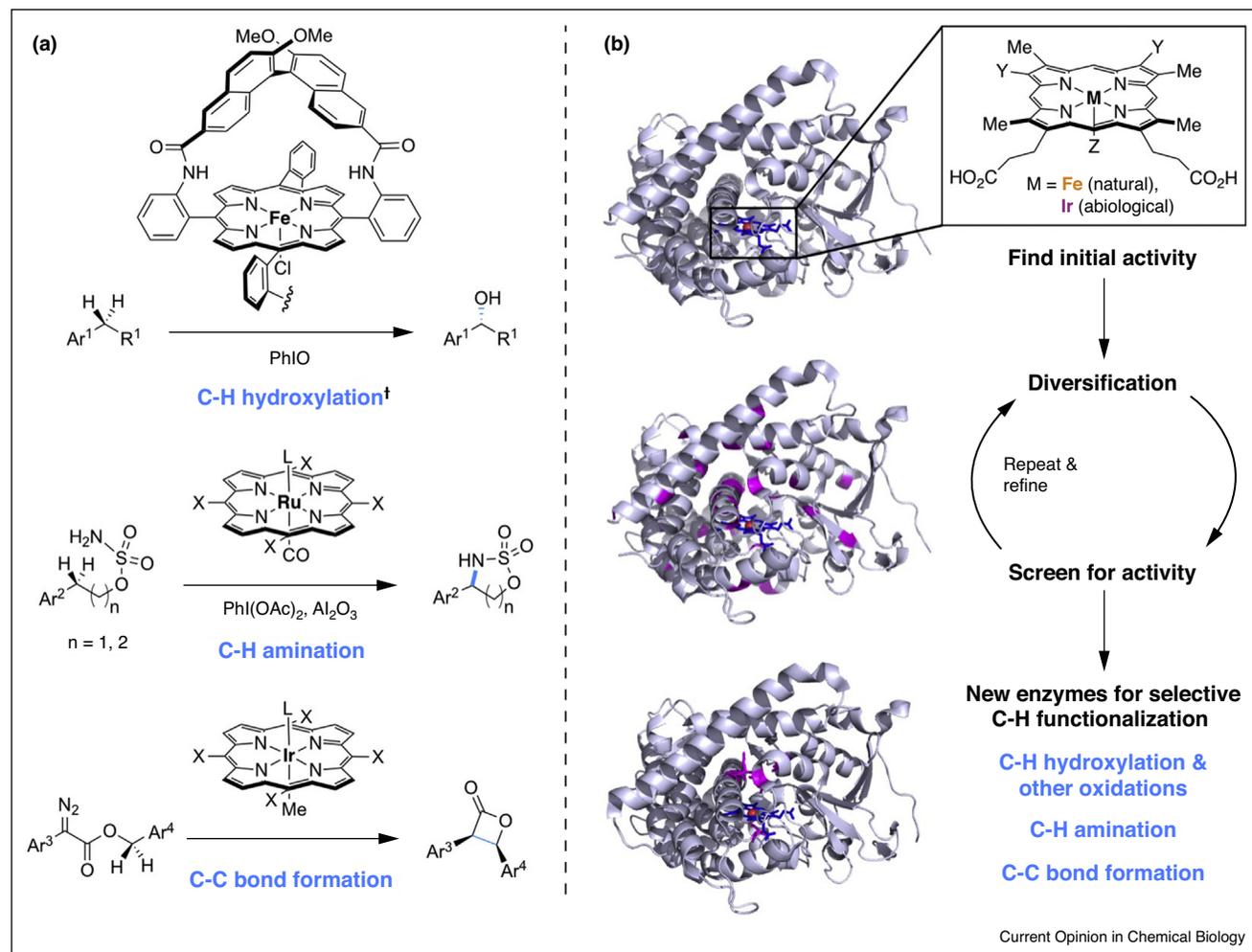
Porphyrin is a versatile scaffold for C–H bond functionalization. **(a)** Porphyrin-based transition metal catalysts which functionalize C–H bonds. Examples are from Refs. [4–6]. X, (1S,4R,5R,8S)-1,2,3,4,5,6,7,8-octahydro-1,4:5,8-dimethanoanthracene-9-yl. †Corresponding ketones were also formed; ketone formation is not due to further oxidation of alcohol products [4]. **(b)** Proteins which contain a porphyrin group have been engineered by directed evolution to perform C–H oxidation reactions with increased activity or tailored site-selectivity, C–H amination, and carbene C–H insertion. Structural models are *Bacillus megaterium* cytochrome P450$_{BM3}$ (PDB 1JPZ, top and middle) and an engineered C–H amination enzyme derived from P450$_{BM3}$ (PDB 5UCW, bottom); Y, vinyl or ethyl; Z, amino acid or organic functional group.

driven interest in utilizing them for diverse synthetic purposes [12]. With their remarkable capability for site-selective catalysis, P450s can be useful for late-stage molecular diversification. The use of a directing group to effect site-selectivity, a concept commonly used in small-molecule catalysis, has been combined with P450-catalysis using PikC from *Streptomyces venezuelae* [13]. The natural substrates of PikC are macrolides, macrocyclic lactones with a desosamine sugar; the desosamine sugar acts as an anchoring group and the *N,N*-dimethylamino moiety forms a salt bridge with a protein glutamate residue [14]. From substrate engineering studies, it was found that various synthetic *N,N*-dimethylamino and *N,N*-diethylamino groups are also suitable anchoring groups and affect site-selectivity in PikC catalysis [15,16••]. In a

powerful illustration of molecular diversification by enzymatic C–H functionalization, the merger of nickel and PikC catalysis accessed five different hydroxylated macrocyclic lactone products from a common linear intermediate (Figure 2a) [16••]. In this reaction, nickel-catalyzed regiodivergent cyclization converted the linear intermediate into 11-membered and 12-membered macrocycles; after appending anchoring groups, a single enzyme variant acted on each substrate to hydroxylate at a different position with good regioselectivity. Complementary to a substrate engineering approach, the application of homologous P450s from different natural product pathways can also access diverse outcomes available to a molecule. Starting from one intermediate, a combination of polyketide synthase (PKS) modules, *in vivo* glycosylation, and

Figure 2
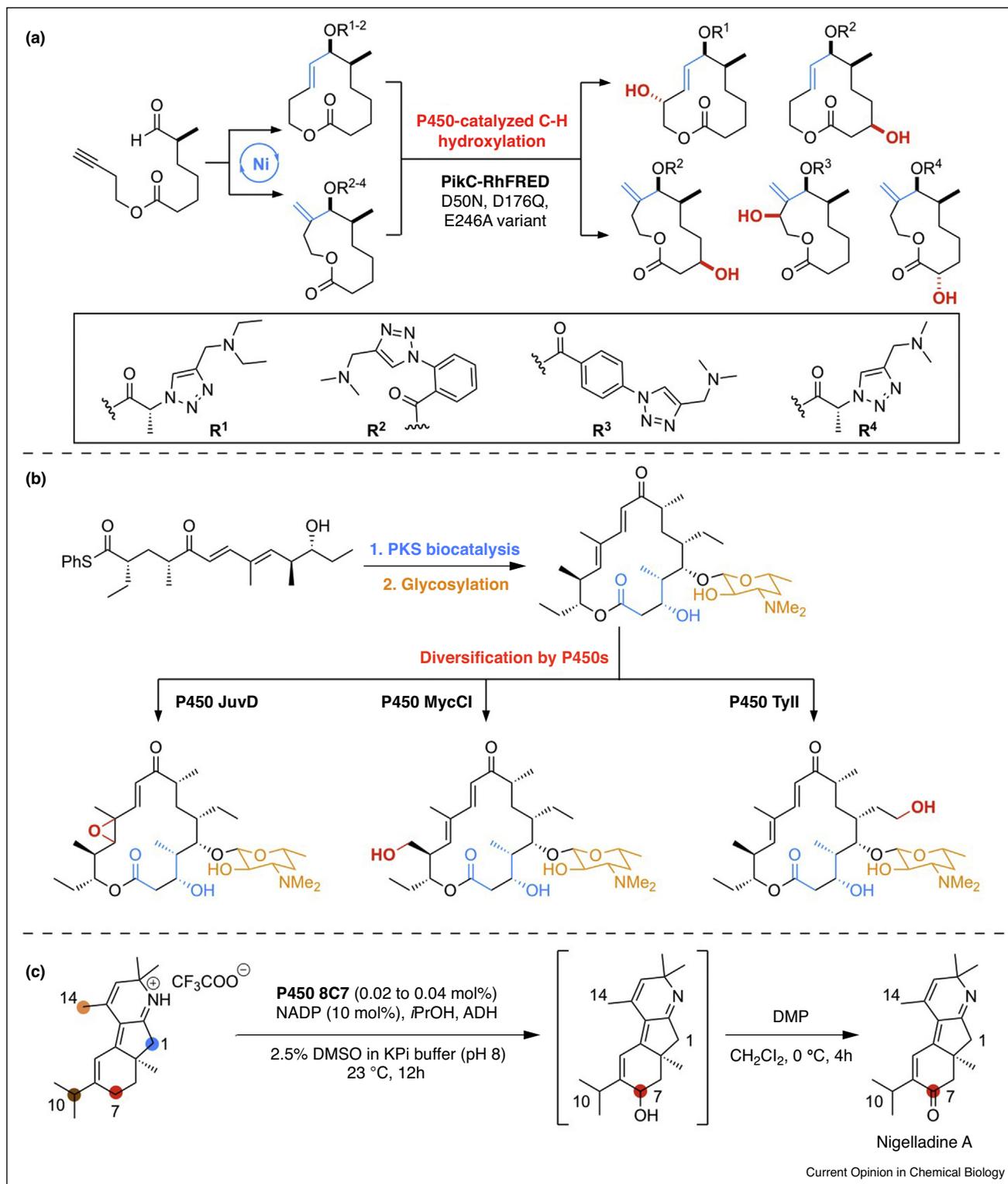
Cytochromes P450 catalyze diverse selective oxidative transformations. **(a)** Synthesis of macrocyclic lactones by merging nickel-catalyzed cyclization with P450-catalyzed C–H hydroxylation [16••]. R[1–4], shown in the box, are different anchoring groups employed to control the site-selectivity of PikC-catalyzed hydroxylation. **(b)** PKS catalysis followed by glycosylation and P450-catalyzed oxidation affords tylactone-based macrolides [17•]. **(c)** Site-selective oxidation by P450 8C7 at the C7 position of an advanced intermediate in the total synthesis of nigelladine A [19••]. ADH, alcohol dehydrogenase; KPi, potassium phosphate; DMP, Dess–Martin periodinane.

three different P450s delivered several tylactone-based macrolide antibiotics with varied oxygenation patterns (Figure 2b) [17•].

P450s have also inspired new strategies for complex molecule synthesis by offering catalysts that address challenging selectivity issues [18]. A compelling example is the first enantioselective total synthesis of nigelladine A [19••]. In this synthesis, a site-selective allylic oxidation of a tricyclic intermediate at the C7 position is required. However, the presence of multiple reactive allylic C–H bonds in this intermediate significantly complicated the seemingly straightforward transformation. Indeed, a survey of a broad range of chemical oxidation methods only led to mixtures of inseparable mono-oxidation and over-oxidation products. This synthetic challenge was solved by P450$_{BM3}$ variant 8C7, which was identified through screening a small set of P450$_{BM3}$ variants originally engineered for oxidation of large substrates with privileged scaffolds [20]. P450 8C7 efficiently catalyzed the desired C7 oxidation with up to 1700 total turnovers and enabled a concise synthesis of nigelladine A (Figure 2c).

Computational methods such as molecular dynamics (MD) simulations have emerged as powerful tools to facilitate the laboratory evolution/engineering of P450s [21]. These *in silico* methods can unveil key residues involved in important dynamic interactions that are not revealed by static structural data. Employing such a computation-driven approach, Narayan *et al.* expanded the scope of P450 PikC to include six-membered small ring systems [22•]. In another demonstration, by combining large-scale MD simulations with site-saturation mutagenesis, Dodani *et al.* identified several mutations (His176Phe/Tyr/Trp) that completely redirect the regioselectivity of P450 TxtE-catalyzed nitration from the C4 to the C5 position of L-tryptophan [23•]. Additionally, there are also several studies that employ docking and MD simulation to identify important mutational hotspots for improving selectivity or substrate specificity of P450 hydroxylation [24–26].

Common to the many thousands of P450s is the conserved cysteine residue that acts as an axial ligand to the heme iron. This axial cysteine is crucial for the C–H activation activity of P450 compound I [27,28]. In a study of the thermostable *Sulfolobus acidocaldarius* CYP119, mutation of the axial cysteine ligand to all other canonical amino acids created mutants which still folded and incorporated heme [29]. The crystal structure of a histidine-ligated CYP119 variant exhibited a tilted heme accompanied by a large rearrangement of the protein structure. While mutation of the axial ligand typically results in diminished or abolished hydroxylation activity, Green *et al.* discovered that the hydroxylation activity of CYP119 compound I could be enhanced by mutation of the axial cysteine to selenocysteine [30••]. This finding may open a new

avenue for the development of robust P450 catalysts for additional challenging transformations.

Advancing biocatalytic C–H amination using directed evolution

The frequent presence of nitrogen in natural products and drug molecules drives the search for methods to form C–N bonds. Biological systems typically rely on enzymatic functional group manipulation of pre-oxidized substrates to forge this bond. This approach has been applied in the design of multi-enzyme biocatalytic cascades for formal C–H amination (Figure 3a) [31,32]. As a complement to nature's biosynthetic logic, recent work with engineered heme proteins has identified enzymes which directly install a C–N bond in place of an sp^3-hydridized C–H bond.

In a study to produce metabolites of drug molecules, mutants of cytochrome P450$_{BM3}$ were discovered to perform an unusual cyclization reaction on lidocaine (Figure 3b) [33]. The cyclization, an intramolecular C–H amination reaction, competes with *N*-dealkylation, a known reaction of P450s. The distribution of the two products is entirely controlled by the protein scaffold: two variants with divergent selectivity for *N*-dealkylation and cyclization differed by a single amino acid. Further work created a set of P450$_{BM3}$ variants which performed α-functionalization of diverse 2-aminoacetamides and thioamides [34•]. In the proposed mechanism, P450 compound I is involved in the formation of an iminium species which subsequently undergoes cyclization. Examples of this transformation are limited to functionalization of α-amino C–H bonds, in agreement with the proposed mechanism. Wild-type P450$_{BM3}$ did not catalyze the cyclization reaction or only gave low conversion (<5%) on model substrates, demonstrating that the discovered mutations promote the chemistry [34•].

Heme proteins are not limited to the reactions of iron-oxo intermediates. When given the opportunity to interact with certain nitrogen-containing substrates, heme proteins can putatively form iron-nitrene species, which can then perform C–H amination. Though first demonstrated nearly 35 years ago [35], it was only with the advent of modern directed evolution techniques that variants of P450$_{BM3}$ adopted the C–H amination function with synthetically useful levels of activity [36,37]. Remarkably, these enzymes can be engineered to alter the regioselectivity of amination in an intramolecular system and override substrate reactivity patterns [38]. The creation of an intramolecular C–H amination enzyme does not necessitate use of an enzyme scaffold: *Physeter catodon* myoglobin, which has no known natural catalytic function, was also engineered to perform this chemistry [39]. Additional testing of engineered heme proteins [40] and directed evolution of heme protein-derived catalysts, including a CYP119 derivative in which the heme group was replaced

Figure 3
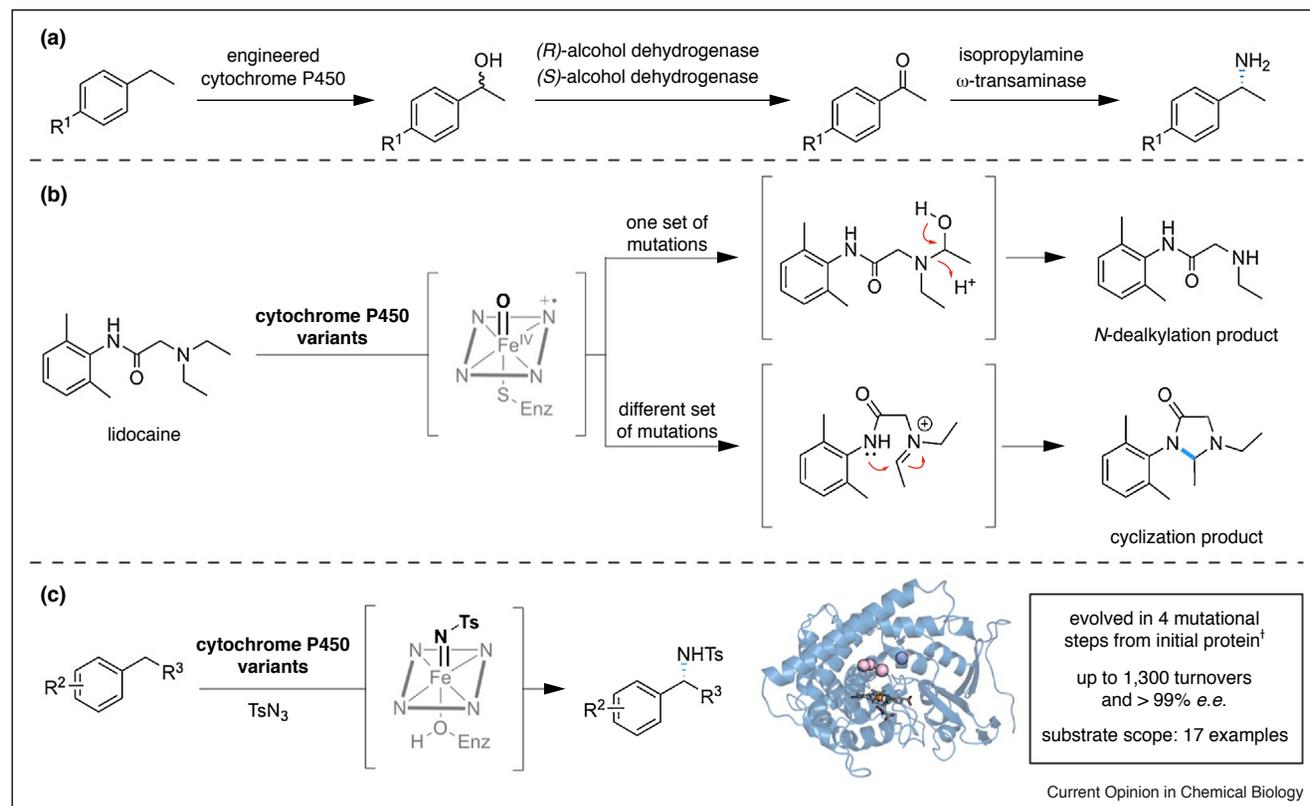
Representative examples of engineered heme proteins used for C–H amination. **(a)** Biocatalytic cascade for formal C–H amination [31]. **(b)** Cytochrome P450-catalyzed reactions of lidocaine [33]. Distribution between *N*-dealkylation and cyclization products is controlled by mutations to the protein scaffold. **(c)** Intermolecular C–H amination catalyzed by an engineered cytochrome P450 [43••]. This reaction proceeds through a putative iron-nitrene intermediate. Four beneficial mutations, whose positions are shown as spheres in the structural model (PDB 5UCW), were accumulated in the directed evolution of a C–H amination enzyme. †The initial protein was an engineered variant of P450$_{BM3}$ which differs from wild-type by seventeen mutations.

with Ir(Me)-porphyrin [41], further expanded the scope of enzymatic C–H amination.

Intermolecular C–H amination affords increased synthetic flexibility. However, changing from an intramolecular to an intermolecular reaction is a fundamental challenge in catalysis [42], and to date there is only one report of heme protein-catalyzed intermolecular C–H amination [43••]. In the intermolecular reaction, C–H insertion must compete with iron-nitrene decomposition pathways without the implicit proximity advantage of an intramolecular arrangement. Nonetheless, directed evolution found a protein scaffold which overcame these challenges: a serine-ligated P450$_{BM3}$ variant delivered seventeen different chiral amine compounds with good turnovers and high enantioselectivity (Figure 3c).

Previously thought to be absent from natural enzyme mechanisms, an iron-nitrene has been put forth as a possible intermediate for recently discovered cytochrome P450 BezE involved in benzastatin biosynthesis

[44•]. This is an excellent demonstration of how findings from biocatalysis can inform mechanistic possibilities for enzymes in complex biosynthetic pathways. At the same time, the discovery of new enzymes provides biocatalysis with an increasing repertoire of starting points for the implementation of new chemistry.

# Engineering heme proteins for C–C bond formation

A prevalent belief in biocatalysis is that proteins cannot access the diversity of chemical transformations available to synthetic chemistry. Some of this comes from the observation that biological systems appear to use just a small set of elements from the periodic table; for instance, known natural enzymes access their powerful and varied chemistry using predominantly earth-abundant first-row transition metals. In contrast, small-molecule catalysts for certain classes of reactions, such as sp^3 C–H functionalization, commonly employ noble metals [1,2].

The creation of artificial metalloenzymes which contain noble metal complexes [45,46], including the replacement of the heme group in heme proteins with porphyrins containing alternative metals, is one strategy to expand the scope of reactions accessible to enzymes. Replacing the iron-porphyrin cofactor with an iridium-porphyrin creates artificial metalloenzymes which install a new C–C bond in place of an sp^3 C–H bond (Figure 4a). First demonstrated using *P. catodon* myoglobin, protein variants containing the Ir(Me)-porphyrin complex and engineered by directed evolution catalyze enantioselective intramolecular carbene C–H insertion to deliver cyclic ether products [47••]. In agreement with previously observed reactivity patterns [6], the free Ir(Me)-porphyrin complex catalyzes the model reaction with a higher reaction rate than an Ir(Me)-porphyrin myoglobin enzyme. Changing the protein scaffold to apo-CYP119 and subsequent directed evolution, however, delivered an artificial metalloenzyme with 23-fold higher turnover frequency compared with the free cofactor [48•]. Intermolecular C–H functionalization of phthalan was accomplished using evolved variants of Ir(Me)-porphyrin CYP119 [48•] and variants of *P. catodon* apo-myoglobin equipped with porphyrins containing alternative metals, including iridium [49]. In contrast, the alkylation of sp^2-hybridized C–H bonds of unprotected indole substrates

has been achieved using variants of myoglobin which retain their native heme group. Chemoselective for C3 functionalization, the alkylation occurs through electrophilic aromatic substitution rather than a C–H insertion mechanism which is expected for sp^3 C–H functionalization [50•].

The described non-natural C–C bond-forming reactions proceed via the intermediacy of an electrophilic metal-carbene species. Remarkably, iron porphyrin carbene (IPC) intermediates in two engineered proteins have now been captured by X-ray crystallography and studied spectroscopically. Two recent reports show that the IPC can exist as either an end-on adduct [51•], which is responsible for carbene transfer activity, or have a Fe–C–N(pyrrole) bridging configuration, which (if formed) is in equilibrium with the end-on adduct (Figure 4b) [52•]. These reports illustrate that the protein scaffold affects bonding and the electronic state of the IPC, and can even dictate the orientation of this intermediate [51•]. Together with knowledge gained from quantum chemical calculations on iron-porphyrin carbene systems [53,54] and experimental work with small-molecule systems [55], these studies can inform new approaches to expand the limits of heme protein-catalyzed C–H functionalization.

Figure 4
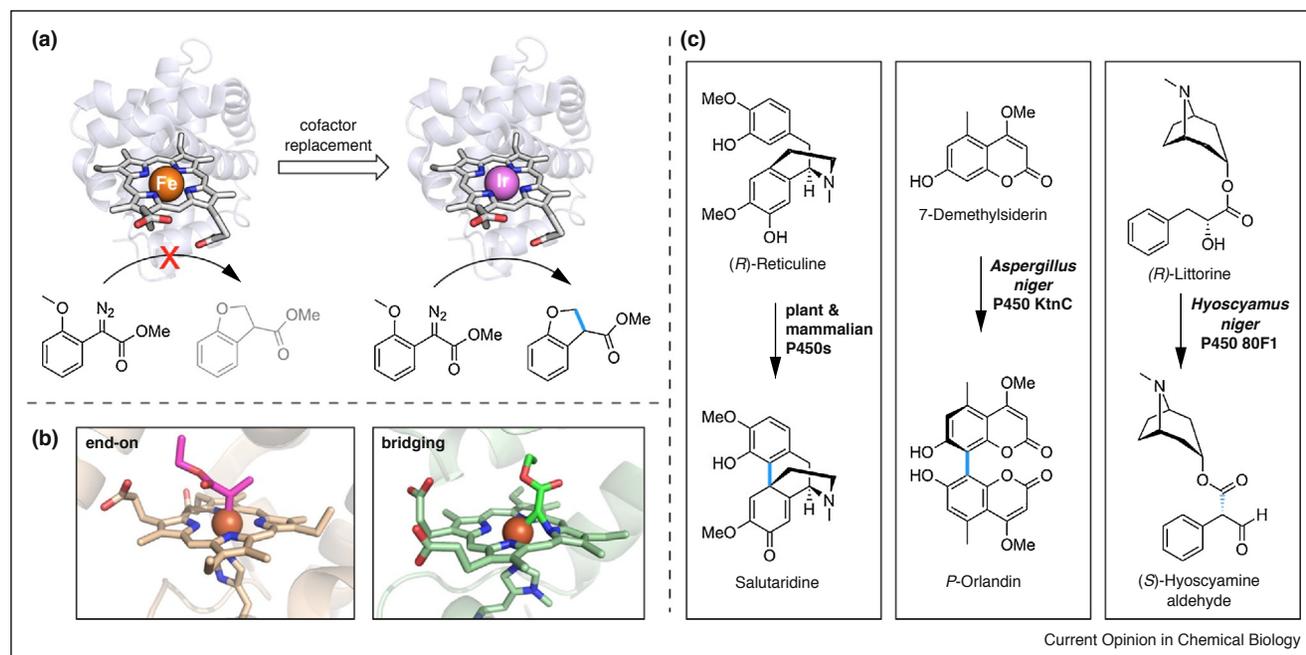
C–H to C–C bond transformations catalyzed by artificial metalloenzymes and cytochromes P450. **(a)** Replacement of heme in myoglobin with Ir (Me)-mesoporphyrin IX results in an artificial metalloenzyme which catalyzes carbene C–H insertion, a reaction that the iron-based enzyme does not catalyze [47••]. Cartoons were created using PDB 1MBN; porphyrin cofactors have been enlarged (not to scale). **(b)** The IPC intermediate has been captured by X-ray crystallography in two poses, end-on (left, PDB 6CUN) and Fe–C–N(pyrrole) bridging (right, PDB 6G5B), in engineered cytochrome *c* and myoglobin carbene transferases, respectively [51•,52•]. **(c)** P450 catalyzed C–C bond forming reactions in natural product biosynthesis. Representative examples are from Refs. [10], [56], and [57] (left to right).

Natural heme proteins have their own strategies to mediate C–H to C–C bond conversions [10]. Many of these transformations are catalyzed by cytochromes P450 and result from radical coupling (e.g. synthesis of salutaridine from reticuline in morphine biosynthesis [10], phenol coupling in fungal natural products [56]) or rearrangements of substrate radicals followed by quenching (e.g. transformation littorine to hyoscyamine aldehyde in tropane alkaloid biosynthesis [57]) (Figure 4c). The potentials of these enzymes for novel reactions have yet to be explored.

Outlook

New enzymes (some even with new cofactors [58]) are being discovered every day. Considering only cytochromes P450, there are more than 206 000 genes known in 2018 [10] versus only 18 000 just five years ago [59]. The considerable diversity of heme proteins and their proven evolvability supply a fertile landscape for the discovery and optimization of new reactions. Although new-to-nature C–N and C–C bond-forming processes have not yet been applied in the context of complex molecule synthesis or diversification, the achievements of P450-catalyzed site-selective hydroxylation provide a roadmap. Looking forward, the successes of engineering heme proteins for diverse chemistry should stimulate experimentation with other groups of proteins for non-native catalysis [60]. Approaching nature's protein diversity with a new chemical perspective and a powerful set of protein engineering tools, we see a bright future for creating new enzymes for selective C–H bond functionalization.

Conflict of interest statement

Nothing declared.

Acknowledgements

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- •• of outstanding interest

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