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Exploiting and Engineering Hemoproteins for Abiological Carbene and Nitrene Transfer Reactions

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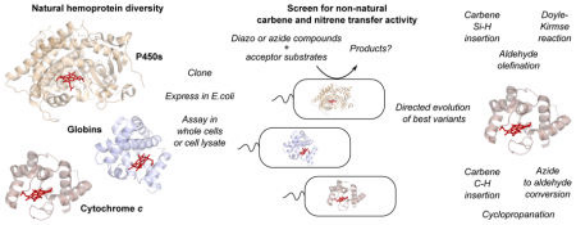
Abstract

The surge in reports of heme-dependent proteins as catalysts for abiotic, synthetically valuable carbene and nitrene transfer reactions dramatically illustrates the evolvability of the protein world and our nascent ability to exploit that for new enzyme chemistry. We highlight the latest additions to the hemoprotein-catalyzed reaction repertoire (including carbene Si–H and C–H insertions, Doyle-Kirmse reactions, aldehyde olefinations, azide-to-aldehyde conversions, and intermolecular nitrene C–H insertion) and show how different hemoprotein scaffolds offer varied reactivity and selectivity. Preparative-scale syntheses of pharmaceutically relevant compounds accomplished with these new catalysts are beginning to demonstrate their biotechnological relevance. Insights into the determinants of enzyme lifetime and product yield are providing generalizable cues for engineering heme-dependent proteins to further broaden the scope and utility of these non-natural activities.

Graphical Abstract



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Conflict of interest statement

The authors declare the following competing financial interest(s): Caltech and FHA have a financial ownership interest in Provivi, Inc., the company that sponsored a part of this research through the National Science Foundation STTR Program. FHA and Caltech may benefit financially from this interest if the company is successful in making product(s) that is/are related to this research. The terms of this arrangement have been reviewed and approved by Caltech in accordance with its conflict of interest policies.

Introduction

The rapidly growing implementation of biocatalysis for production of chemicals is driven by the ability of enzymes to operate under mild, aqueous reaction conditions with high rates and selectivity. The result can be a significant reduction in industrial waste and cost of production compared to stoichiometric syntheses and other catalytic processes. The importance of directed evolution and other protein engineering tools for tuning enzyme activity, selectivity or stability to meet industrial process requirements has been thoroughly established [1,2]. However, a significant hurdle is presented by the fact that a number of valuable chemical transformations, including many catalyzed by synthetic catalysts, do not have biocatalytic counterparts. The necessary enzymes simply do not exist. One effective approach to creating new enzymes has been to engineer existing proteins to exhibit new, synthetically useful reactivity, mainly by exploiting their metallo- and other cofactors for new chemistry and improving those activities by directed evolution [3–5]. The resulting fully genetically-encoded catalysts could, at least in principle, be incorporated into *in vivo* biosynthetic pathways to access new products or provide alternative routes to existing products. Repurposing existing proteins for new chemistry, a trick used by evolution for eons, has proven particularly fruitful with heme-dependent proteins. In this review we cover the most recent developments as well as some of the key limitations in this growing area.

In a seminal paper, Coelho and coworkers demonstrated that cytochrome $P450_{BM3}$ and other heme-containing proteins can catalyze the cyclopropanation of styrene using ethyl diazoacetate (EDA) as a carbene precursor [6]. Their work was inspired by the well-established capacity of ‘biomimetic’ metalloporphyrin complexes to catalyze carbene and nitrene transfer reactions, providing ample options for the introduction of C–C, C–N and other bonds into organic compounds. Coelho *et al.* further demonstrated that the activity and stereoselectivity of cytochrome $P450_{BM3}$ as a cyclopropanation biocatalyst could be improved by mutating the protein. The evolvability of $P450_{BM3}$ for this abiotic activity was underscored in subsequent reports showing that replacement of the conserved P450 axial cysteine residue with serine [7] or histidine [8] significantly boosts cyclopropanation activity. Furthermore, P450 variants catalyzed carbene N–H insertion to make new C–N bonds [9]. The new activities were not limited to carbene transfer reactions: P450 variants were shown to catalyze intramolecular nitrene C–H insertion reactions [10,11], and ‘P411s’ with an axial Cys-to-Ser mutation were shown to be competent catalysts for sulfimidation [12], aziridination [13], and intramolecular C–H amination [14,15] reactions. In each case, the observed activities were found to exceed those of previously-reported transition metal complexes.

Fasan and coworkers showed that these novel activities are not restricted to cytochrome P450s: the small, well-expressed, oxygen-transport hemoprotein myoglobin can become a powerful and selective cyclopropanation [16], carbene N–H and S–H insertion [17,18], and intramolecular C–H amination catalyst [19]. These contributions, including the underlying concepts of chemical intuition and enzyme promiscuity guiding the discovery and enhancement of these non-biological activities, have been reviewed in detail [5,20,21].

In this review, we focus on three recent, concurrent developments. First, we discuss how the earlier studies paved the way to the discovery of yet more non-natural carbene and nitrene transfer reactions mediated by engineered hemoproteins and how the cornucopia of existing hemoproteins is being leveraged to discover new, unprecedented reactivities. Second, we show how the field is transitioning from proof-of-concept studies towards implementation, with several studies demonstrating preparative-scale production of pharmaceutically relevant compounds. Third, we illustrate how the growing body of literature, including recent reports of mechanism-based enzyme inactivation, provide stimulus for further protein engineering to address what controls (and limits) these non-natural activities of hemoproteins.

New carbene transfer reactions catalyzed by engineered heme proteins

A compelling demonstration of the power of hemoprotein engineering to create a new enzyme is the recent report of biocatalytic carbon-silicon bond formation [22••], an activity not known in the biological world (Figure 1). Current rhodium, iridium and copper complexes developed to promote asymmetric carbene insertions into Si–H bonds of silanes all have limited catalytic efficiencies (<100 total turnovers (TTN)). Testing a variety of proteins for carbene insertion into the Si-H bond of phenyldimethylsilane, Kan *et al.* discovered that a variety of hemoproteins produce the organosilicon product with 10 to 50 TTN, although with low enantio-induction [22••]. A highly thermostable cytochrome *c* from the marine bacterium *Rhodothermus marinus* (Rma), however, catalyzed the transformation with excellent enantioselectivity (97% *ee*). Subsequent directed evolution by mutagenesis of active-site residues yielded an Rma cytochrome *c* triple variant (V75T,M100D,M103E), which provided 70% isolated product yield in preparative-scale (0.1 mmol) bacterial whole-cell reactions with 98% *ee* and 3,400 TTN. The enzyme exceeded the activity of the best-reported synthetic catalysts by up to 80-fold, while accepting a range of silane substrates and showing a high chemoselectivity for Si–H insertion over cyclopropanation or N–H insertion, a feature not exhibited by small-molecule transition metal catalysts.

Mechanistic investigations of myoglobin-catalyzed S–H insertion reactions [18] provided a basis for developing catalysts that promote the [2,3]-sigmatropic rearrangement of allylic and propargylic sulfides in the presence of α-diazoester reagents [23•]. While this Doyle-Kirmse reaction provides a powerful C–C bond forming strategy for the synthesis of compounds bearing a new stereogenic center, synthetic catalysts exhibit only modest catalytic efficiency and low enantioselectivity (<10–50% *ee*). In contrast, the myoglobin triple mutant Mb(L29S,H64V,V68F) showed high product conversions (57–99%), high total turnovers (1,000–8,800 TTN), and up to 71% *ee* across a range of substrates. The scope of the reaction could be extended to conversion of propargylic sulfides to give allenes, which are valuable synthetic intermediates. A representative [2,3]-sigmatropic rearrangement product (ethyl 2-(phenylthio)pent-4-enoate) was isolated in 84% yield.

In another study, Tyagi *et al.* engineered myoglobins for aldehyde olefination reactions [24], providing a convenient and mild alternative to the widely used Wittig reaction. Screening of Mb active-site variants identified Mb(F43V,V68F), which converts a broad range of aryl aldehydes and alkyl α-diazo acetates into the corresponding olefin products with high catalytic efficiency (1,000–4,900 TTN) and very good to excellent *trans* selectivity (91–99%

de). The TTNs reported for the Mb variant are 10- to 100-fold higher than those for similar transformations catalyzed by organometallic complexes working in organic solvents and at elevated temperature. Mb product conversion was found to not exceed 50%, however, due to inhibition by the phosphine (or arsine) oxide side product generated during the reaction.

Focusing on a similar reaction, Hauer and coworkers mined the *E. coli* proteome in search of enzymes capable of catalyzing the olefination of benzaldehyde in the presence of EDA [25]. The most promising biocatalyst they identified was YfeX, a heme-containing protein of unknown function. Under optimized conditions, this hemoprotein showed 92% selectivity for the *trans* olefin product, albeit at low product conversions (<5%) and with only 15–20 turnovers. Interestingly, YfeX-catalyzed benzaldehyde olefination was reported to take place in the absence of triphenylphosphine/arsine, suggesting that this transformation may involve a different mechanism than that proposed for myoglobin-catalyzed aldehyde olefination [24].

Progress in terms of substrate scope and synthetic utility is also being made with enzymatic cyclopropanation. Gober *et al.* recently reported cyclopropanation of dihydroalanine (DHA) residues contained in thiopeptides, a class of ribosomally synthesized peptides with a range of bioactivities, to yield 1-amino-2-cyclopropane carboxylic acid moieties [26]. Two P450 enzymes involved in monooxygenation reactions within the bacterial thiopeptide biogenesis pathway were engineered to harbor mutations previously found to be beneficial for promoting P450-mediated cyclopropanation [27•]. The resulting P450 variants were able to catalyze the cyclopropanation of DHA moieties in peptide model substrates and in complex linear and cyclic thiopeptide cores, offering new opportunities for thiopeptide functionalization. In another recent report, Tinoco *et al.* introduced a two-compartment, chemobiocatalytic strategy for realizing olefin cyclopropanation reactions in the presence of 2-diazo-1,1,1-trifluoroethane (CF_3CHN_2) as the carbene source. This transformation yields trifluoromethyl-substituted cyclopropanes, which are valuable building blocks for medicinal chemistry [28•]. In this system, gaseous CF_3CHN_2 was generated *ex situ* and subsequently utilized by whole-cell catalysts expressing engineered Mb cyclopropanation variants. This approach was applied to a range of vinylarene substrates to deliver the corresponding trifluoromethyl cyclopropanes in high yields and with excellent diastereo- and enantioselectivity.

A detailed account of artificial metalloenzymes containing non-native cofactors is beyond the scope of this short review, and this subject has been reviewed recently [4,29]. However, we will briefly describe how substitution of the heme cofactor with non- native metalloporphyrins is emerging as an alternative strategy for engineering biocatalysts for carbene and nitrene transfer reactions. Brustad and coworkers evolved variants of $P450_{BM3}$ that are capable of incorporating iron-deuteroporphyrin IX, demonstrating the feasibility of engineering cytochrome P450 variants with orthogonal cofactor specificity [30]. Bordeaux *et al.* demonstrated that Mb variants incorporating a non-natural Mn- or Co-porphyrin IX cofactor catalyze intramolecular C–H amination reactions with sulfonyl azides, with reactivities comparable to their iron-containing counterpart [19]. This work was recently expanded by Sreenilayam *et al.*, who reported the recombinant expression of Mb variants incorporating various first, second and third row transition metal cofactors (Mn, Co, Ru, Rh,

Ir) [31]. The resulting Mb variants were catalytically active in cyclopropanation, N-H and S-H insertion reactions albeit with generally reduced efficiency compared to the iron-containing counterparts. Notably, Mn- and Co-substituted Mb variants showed activity for an intermolecular carbene C–H insertion reaction with phthalan and EDA, a reaction not supported by the iron- containing myoglobins and previously achieved only using an Ir-containing biocatalyst (vide infra).

By replacing the heme in myoglobin and the thermostable P450 CYP119 with Ir(Me)-mesoporphyrin IX, Hartwig and coworkers expanded the reactivity of these hemoproteins to carbene C–H insertion [32••,33]. Most notably, Ir(Me)-PIX-substituted variants of CYP119 catalyzed intramolecular carbene C–H insertion of various ethyl 2- diazo-2-(2-methoxyphenyl)acetate derivatives with 120–600 TTN and 76–98% *ee,* and up to 35,000 TTN under very low catalyst loadings (0.00125 mol%). The CYP119- derived biocatalysts were also found to be active for the intermolecular carbene C–H insertion reaction with phthalan and EDA. Ir-substituted CYP119 variants were also shown to be active catalysts for the cyclopropanation of olefins currently not accepted by Fe-containing enzymes, including various internal, aliphatic and electron-deficient alkenes [34]. These artificial enzymes have the shortcomings of requiring a cofactor containing rare and expensive metal (Ir) and specialized *in vitro* reconstitution or expression protocols for their assembly. This may limit the range of hemoprotein scaffolds that can be used and potentially complicates any effort to couple these enzymes to other activities *in vivo*. Whether these limitations can be overcome or whether engineered hemoprotein variants containing the native iron heme cofactor can access such challenging reactions as carbene C–H insertion remains to be shown.

Nitrene transfer reactions catalyzed by engineered hemoproteins

Among the new, non-natural activities discovered for hemoproteins are a variety of nitrene transfer reactions previously reported using transition metal complexes and, in one early case, a cytochrome P450 [35] (Figure 2). Recent studies established that cytochrome P450s and other heme-dependent enzymes (horseradish peroxidase, catalase, chloroperoxidase) and proteins (myoglobin, hemoglobin) can activate arylsulfonyl azide substrates and catalyze their conversion into sultam products via an intramolecular C–H amination reaction [11,14,15,19]. Subsequent studies expanded the scope of P450-catalyzed intramolecular C–H aminations in the context of carbonazidates, enabling the synthesis of protected forms of 1,2-aminoalcohols, which are valuable synthetic intermediates [10]. Other studies revealed that P450 variants from the serine-ligated cytochrome ‘P411’ lineage in combination with tosyl azide reagents can catalyze intermolecular nitrene addition to sulfides (sulfimidation) and alkenes (aziridination) [12,13].

Additional synthetically useful nitrene-mediated transformations have appeared in the last two years. Prier *et al.* leveraged the high sulfimidation reactivity of P411 enzymes to catalyze the conversion of phenylallylsulfides into allylic amines [36•]. This reaction involves a spontaneous [2,3]-sigmatropic rearrangement of the sulfimidation product generated by the P411-catalyzed reaction between the allyl sulfide and tosyl azide. Carrying out these transformations in whole cells, the authors discovered that the intracellular environment results in loss of the thiophenol group, effectively leading to the formation of

protected allylic amines. Starting from an initial P411 variant catalyzing this reaction with only moderate activity (<5% conversion), a significantly more efficient biocatalyst (P-5) was obtained after several rounds of directed evolution (79% conversion; 2,000 TTN). Importantly, the evolved enzyme also featured high enantioselectivity in both the sulfimidation (88–98% *ee*) and the sulfimidation/sigmatropic rearrangement reactions (68–82% *ee*).

Giovani *et al.* have shown that engineered myoglobins can catalyze the oxidative deamination of benzylic azides to yield aldehydes [37•], providing a biocatalytic alternative for aldehyde synthesis by alcohol oxidation with chromium- or manganese-based reagents. Myoglobin variant Mb(H64V,V68A) converted a broad range of primary azides to the corresponding aldehydes with up to 89% yield, exhibiting between 100- and 1000-fold higher catalytic activity than previously reported synthetic catalysts. In an extension of this study, investigation of a broader panel of hemoproteins revealed that engineered P450s catalyze the same reaction with high efficiency and selectivity [38]. A $P450_{BM3}$ variant previously determined to be active in C–H amination reactions enabled the nearly quantitative conversion of benzyl azide to benzaldehyde at a catalyst loading of only 0.01 mol% (>10,000 TTN). This enhanced reactivity enabled the conversion of secondary azides to ketones (49–76%), a transformation that proceeds to only <2% conversion in the presence of Mb(H64V,V68A).

Interestingly, Carrera and coworkers recently reported the enzymatic transformation of benzyl azide to benzonitrile using a Rieske non-heme iron oxygenase (toluene dioxygenase) [39]. While not involving a heme enzyme, this reaction was proposed to involve catalytic intermediates that are reminiscent of those implicated in hemoprotein-catalyzed nitrene C–H insertion [11] and azide oxidation [37•].

Another noteworthy contribution relates to the recent application of engineered P450s for the synthesis of imidazolidin-4-ones via an intramolecular oxidative C–H amination [40]. While this reaction does not involve a nitrene-mediated process *per se*, it represents a previously undocumented enzymatic transformation useful for the creation of new C–N bonds, which further highlights the functional plasticity of cytochrome P450 enzymes.

Finally, Prier *et al.* recently demonstrated P450-mediated intermolecular amination of benzylic C–H bonds [41•]. The authors discovered that a P411 variant evolved for the imidation of allylic sulfides [36•] displays activity for the amination of 4-ethylanisole with tosyl azide. Subsequent rounds of directed evolution delivered variant $P411_{CHA}$, providing up to 1300 TTN and 99% *ee* for benzylic C–H amination of a range of alkylarene substrates. Engineering the enzyme scaffold endows the native iron heme cofactor, by itself an inactive C–H amination catalyst, with activity for a challenging non-natural reaction.

Preparative-scale synthesis of cyclopropane pharmaceutical intermediates

Efforts to utilize these new biocatalysts for the preparation of high-value compounds are already starting to appear. In a first example, Wang *et al.* demonstrated the utility of an engineered P450-based carbene transfer catalyst, $P450_{BM3}$ HStar, for producing the chiral

precursor to the antidepressant levomilnacipran in a single biocatalytic step [8]. Applying $P450_{BM3}$ HStar in whole-cell reactions, the cyclopropane core of levomilnacipran was obtained in 86% isolated yield, 96% *de* and 92% *ee* (Figure 3A). Another recent study used myoglobin-based cyclopropanation catalysts for preparative-scale synthesis: applying a structure-reactivity guided approach, Bajaj *et al.* engineered a panel of myoglobin variants to access *trans-(*1*R*,2*R)*-configured aryl-substituted cyclopropanes [42•], complementing the stereoselectivity of a previously developed *trans*-(1*S*,2*S*)-selective Mb(H64V,V68A) cyclopropanation catalyst [16]. Using these biocatalysts in whole-cell transformations yielded gram-scale amounts (up to 4.7 g) of the chiral cyclopropane cores of four drugs (tranylcypromine, tasimelteon, an investigational TRPV1 inhibitor, and ticagrelor) (Figure 3B–E). The desired *trans*-(1*S*,2*S*)- or *trans*-(1*R*,2*R*)-configured cyclopropane intermediates could be obtained in high yield (75–91%) and significantly higher diastereo- and enantioselectivity (98–99.9% *de*, 96–99.9% *ee*) than was possible with asymmetric cyclopropanation protocols adopted in the process-scale syntheses of these compounds. In the case of ticagrelor, the biocatalytic reaction offers a significantly shorter route (2 vs. 4–5 steps) to preparation of its chiral cyclopropane core compared to previously reported methods. In a parallel study, Hernandez and colleagues also reported the stereoselective synthesis of the ticagrelor cyclopropane (Figure 3B) using an engineered variant of group II truncated hemoglobin from *Bacillus subtilis.* Mutation of active-site residues in this protein led to an improved variant (Y25L,T45A,Q49A), which produced the desired enantiomer in 79% yield, 96% *de* and 99% *ee* in 0.4 mmol-scale reactions [43]. The above studies highlight the biotechnological relevance of these new enzymes and underscore the advantages of testing a variety of protein scaffolds to find the best starting points for achieving desirable transformations and selectivities.

Current limitations

P450 oxygenases, myoglobins, and other hemoproteins have evolved dedicated mechanisms to limit self-inactivation through oxidative damage by reactive oxygen species in P450s or oxidation to metmyoglobin. However, they have not had a chance to evolve comparable protection from the reactive intermediates produced in the active site during carbene and nitrene transfer reactions. This issue was recently addressed by Renata *et al.,* who showed that a proficient P450 cyclopropanation variant, $P450_{BM3}$ HStar, undergoes mechanism-based inactivation whereby the reactive carbene species generated in the active site is transferred to nucleophilic groups on the porphyrin ring and enzyme side chains [44•] (Figure 4A). These alkylation side reactions severely limit catalyst lifetime. Substitution of affected amino acid residues with non-nucleophilic residues resulted in a variant with ~2-fold improved activity (TTN). Nevertheless, the variant is still alkylated at various sites and has a short catalyst lifetime, highlighting significant room for further improvement.

Nitrene transfer catalysts suffer from a different problem. All hemoprotein-catalyzed nitrene transfer reactions reported to date are accompanied by unproductive consumption of the azide substrate to give a sulfonamide (or carbamate) byproduct [10–14,19,36•,41•]. This unproductive pathway has been ascribed to over-reduction and protonation of the putative heme-nitrene intermediate (Figure 4B) [11]. Consistent with this, mutation of a highly conserved residue (T268) implicated in proton transfer to the hydroxoferryl intermediate as

part of the native P450 catalytic cycle was found to improve the activity (TTN) of these enzymes as C–H amination catalysts [11,14]. The extent of this side reaction also depends on the nature of the substrate [11] and optimization of the enzyme active site for a target substrate was shown to improve desired product formation, possibly by facilitating productive interception of the nitrene [12]. More recently, heme substitution with an Ir-porphyrin provided another strategy to obtain P450-based catalysts capable of catalyzing the intramolecular C–H amination of arylsulfonyl azides with reduced propensity for azide reduction [45]. The TTN supported by these artificial enzymes, however, was comparable to those of previously reported hemoprotein-based catalysts, which suggests that other inactivating mechanisms may be at play. Another, currently unexplored strategy to overcome the problem associated with over-reduction of the iron-nitrene intermediate could be the engineering of P450 variants with altered (i.e., slower) rates of electron transfer [11].

Outlook - future opportunities for hemoprotein engineering

The preceding sections highlighted how diverse hemoprotein scaffolds can be adapted for various carbene and nitrene transfer reactions, with different scaffolds frequently showing altered reactivity or selectivity for a given transformation. Future efforts are anticipated to continue to take advantage of the wealth of natural hemoprotein scaffolds contained in genomic and structural databases (with >3900 hemoprotein structures currently deposited in the PDB) [46,47] to unveil catalysts with novel or superior activity or selectivity for abiological transformations. The ever-decreasing costs of DNA synthesis render it increasingly economical to obtain synthetic (hemoprotein) genes for rapid testing and engineering [48]. The nascent activities discovered in this vast collection can then be improved and tuned by directed evolution.

The studies reviewed here have begun to provide important clues and guidance for fine-tuning carbene and nitrene transfer reactivity in hemoproteins by protein engineering. Even non-enzyme hemoproteins like myoglobin or cytochrome *c* can be turned into proficient enzymes by “carving out” active sites through mutation of a few selected residues surrounding the heme pocket [16,22••,43]. In the context of P450s, Gober and Brustad showed that the T268A mutation, previously found to be beneficial for boosting cyclopropanation activity in $P450_{BM3}$ [6], can be transferred to other cytochrome P450s to improve cyclopropanation activity [27•]. McIntosh *et al.* showed that the thermostable P450 CYP119 can accommodate substitution of its axial Cys residue with His, a mutation that also enhances cyclopropanation activity in $P450_{BM3}$, by undergoing substantial structural rearrangement [49]. Intriguingly, soluble CYP119 enzymes with diverse spectroscopic characteristics were obtained for all 19 possible axial Cys substitutions, providing a small library of diverse potential starting points for further engineering.

Further guidance in hemoprotein engineering may come from computational approaches. Using model systems, recent DFT studies have investigated the nature of the putative iron carbenoid intermediate, shedding light on factors governing its formation and reactivity [50–52]. In particular, work by Sharon *et al.* on carbene N–H insertion [52] indicated that altering the basicity of the enzyme active site may allow access to aliphatic amines,

potentially extending the substrate scope from the aromatic amines reported to date [9,17]. This proposal has not yet been tested experimentally.

To date, none of these new catalysts have been utilized in enzymatic cascades or metabolic engineering to create new biosynthetic pathways. With most carbene and nitrene transfer enzymes showing similar or higher turnovers in whole-cell contexts compared to purified enzyme, integrating them into metabolic pathways to produce non-natural chemicals is certainly tempting. However, successful realization may require further efforts to engineer the enzymes to exhibit lifetimes and kinetic properties (e.g., K_m) that are more compatible with synthesis in living cells. A proof-of-concept for interfacing microbial metabolism with a non-natural reaction was recently published by Wallace and coworkers, who demonstrated styrene production from glucose in *E. coli* with subsequent cyclopropanation using a synthetic cyclopropanation catalyst [53•].

In conclusion, the field has come a long way since the initial report of hemoprotein-catalyzed olefin cyclopropanation just four years ago. The studies reviewed here provide ample inspiration for future protein engineering to discover yet more novel reactivities and to enhance the catalytic efficiencies of the current, nascent enzymes. Growing genomic databases and decreasing costs of DNA synthesis render it increasingly simple to sample natural hemoproteins for desired reactivities. Thus, propelled by the lack of biocatalytic alternatives for many synthetically useful reactions, we anticipate many more members to join the family of hemoproteins catalyzing non-natural reactions.

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Highlights

- Engineered hemoproteins expand the biocatalytic repertoire
- Different hemoprotein scaffolds offer diverse activities and selectivities
- New enzymes enable preparative-scale syntheses of pharmaceutical intermediates
- Insights into factors limiting enzyme lifetime offer new options for engineering

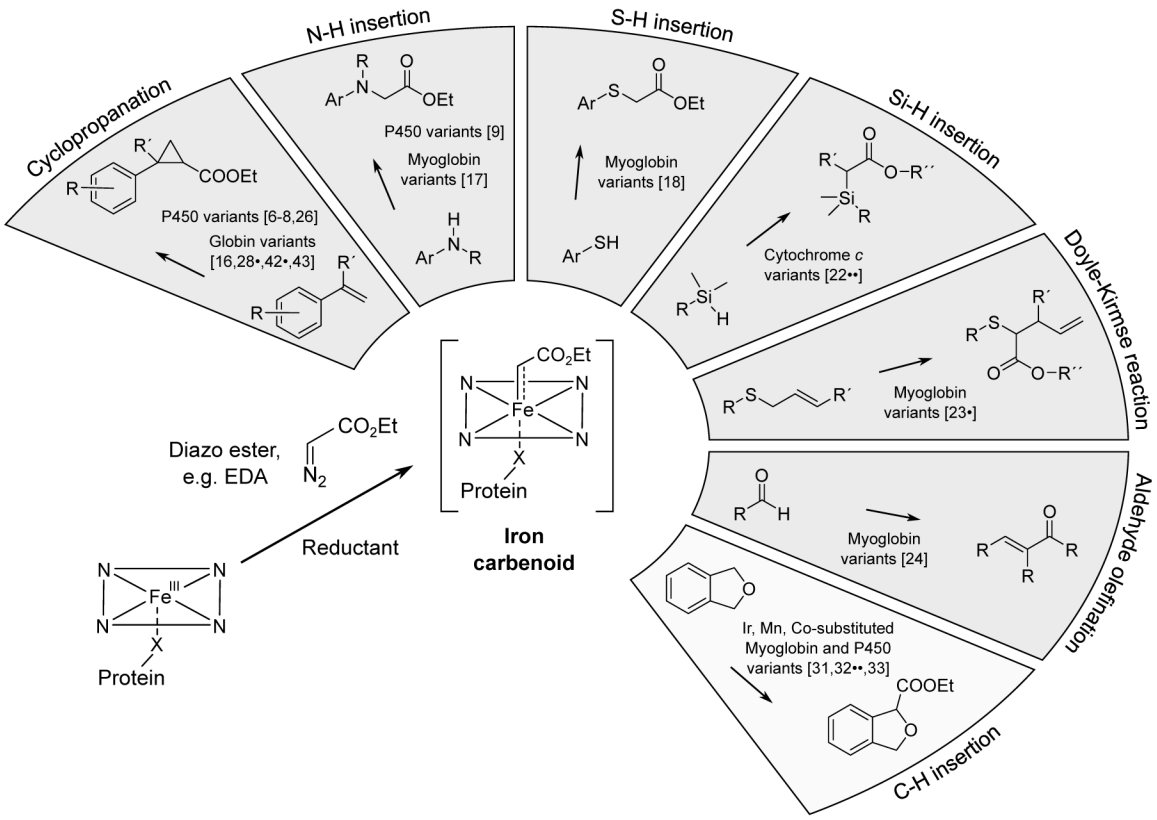

Figure 1. Carbene transfer reactions catalyzed by engineered hemoproteins

Carbene transfer by hemoproteins requires reduction of the heme iron from the Fe(III) to the catalytically active Fe(II) state. Subsequent encounter with a diazo compound results in release of N_2 (not shown) and formation of a putative iron carbenoid reactive intermediate. Iron carbenoid formation in the heme pocket has been exploited for a range of carbene transfer reactions. The iron heme is returned to the catalytically active Fe(II) state (not shown) following carbene transfer. While most reactions were achieved with the native iron heme cofactor, C–H insertion activity (shown in lighter gray) required replacement of the iron heme with an Ir, Mn or Co porphyrin cofactor.

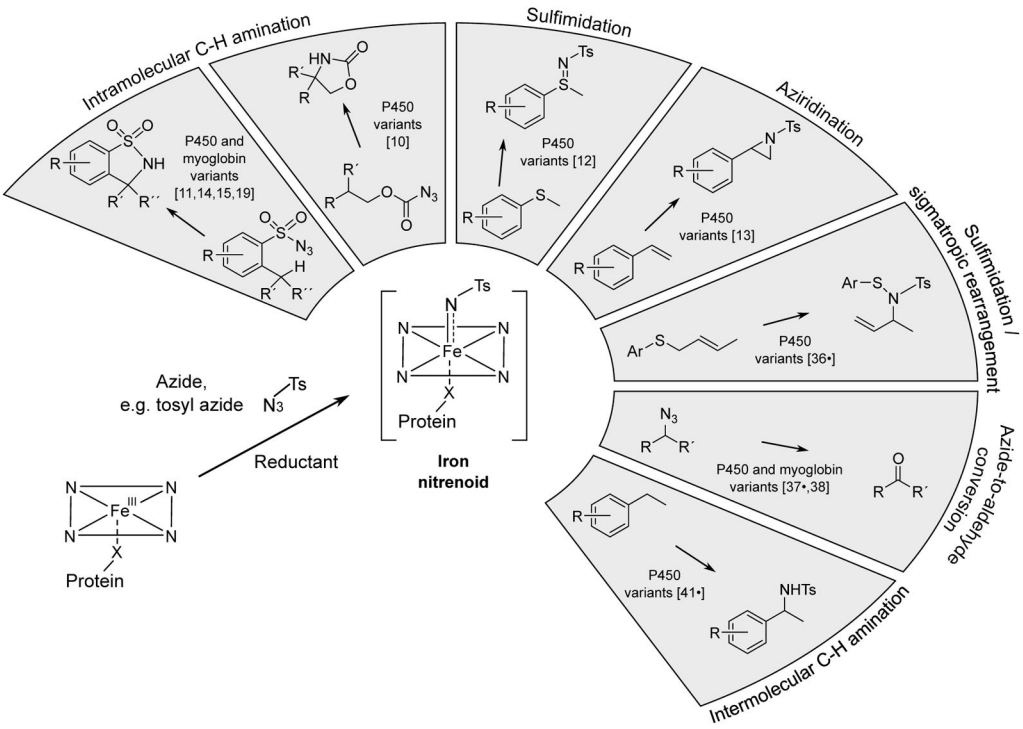

Figure 2. Nitrene transfer reactions catalyzed by engineered hemoproteins

Enzymatic nitrene transfer requires reduction of the iron heme from the Fe(III) to the catalytically active Fe(II) state. Encounter with azide substrates results in release of N_2 (not shown) and formation of a putative iron nitrenoid reactive intermediate. Iron nitrenoid formation by hemoproteins has been exploited in a range of biocatalytic nitrene transfer reactions. The iron heme is returned to the catalytically active Fe(II) state (not shown) following nitrene transfer.

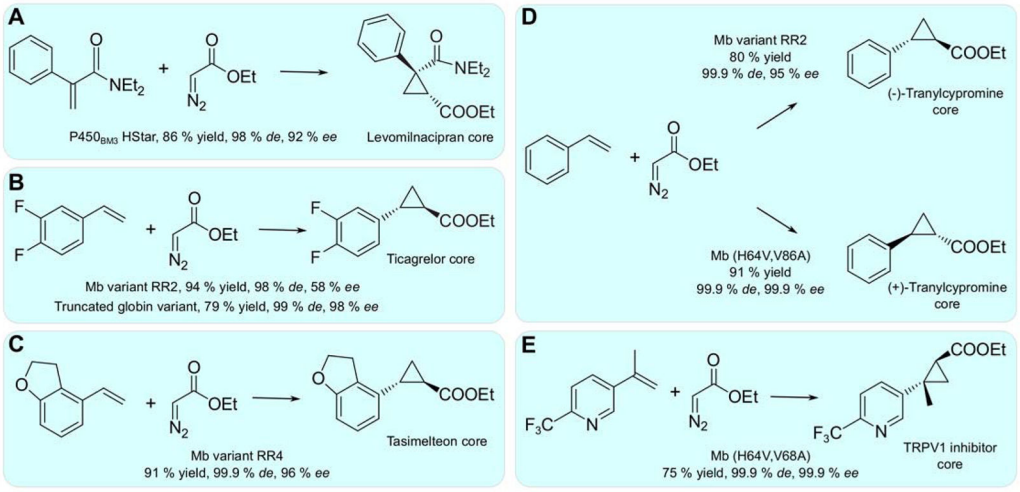

Figure 3. Preparative-scale synthesis of cyclopropane-containing drug precursors

Preparative-scale cyclopropanation reactions reported to date using engineered hemoproteins, providing access to various pharmaceutical intermediates with excellent yields, diastereo- and enantioselectivities.

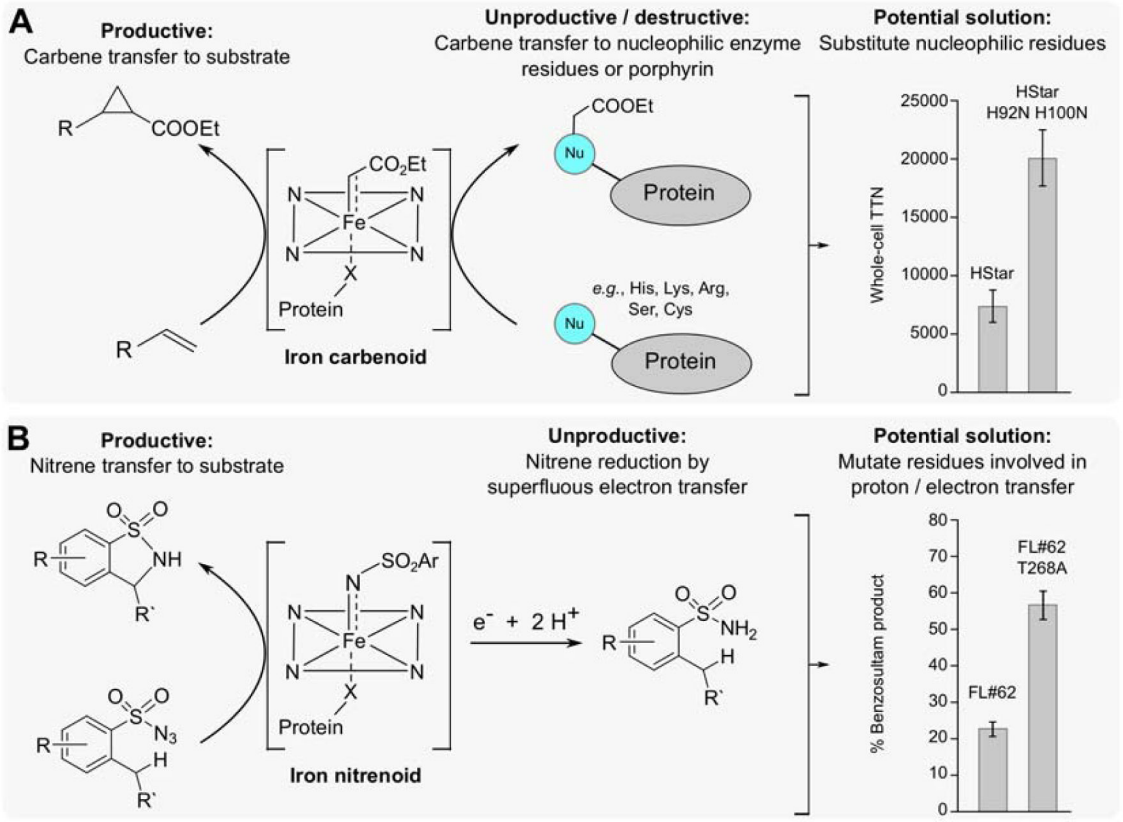

Figure 4. Limitations to carbene and nitrene transfer efficiency

(A) Carbene transfer biocatalysts suffer from alkylation of the porphyrin ring or nucleophilic protein residues by the reactive carbenoid intermediate, ultimately resulting in enzyme inactivation. Replacing nucleophilic residues, as shown by Renata *et al.* [44•] or otherwise promoting productive transfer to the desired substrate could ameliorate the problem and deliver improved cyclopropanation enzymes. (B) Nitrene transfer catalysts are affected by overreduction/protonation of the heme nitrene intermediate, resulting in unproductive consumption of azide substrate. For intramolecular C–H amination, mutation of a residue involved in proton transfer within the native P450 catalytic cycle (T268) results in higher product formation [11].