

Nitrene Transfer Catalyzed by a Non-Heme Iron Enzyme and Enhanced by Non-Native Small-Molecule Cofactors

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Supporting Information Placeholder

ABSTRACT: Transition-metal catalysis is a powerful tool for the construction of chemical bonds. Here we show that a non-heme iron enzyme can catalyze olefin aziridination and nitrene C–H insertion, and that these activities can be improved by directed evolution. The non-heme iron center allows for facile modification of the primary coordination sphere by addition of metal-coordinating molecules, enabling control over enzyme activity and selectivity using small molecules.

Over the last century, chemists have developed myriad synthetic transition-metal catalysts to access new chemical transformations and modes of reactivity. Nature has been developing catalysts for far longer: over billions of years, she has evolved a rich repertoire of proteins that perform most of the chemical reactions of life. But nature's inventions do not include many of the best inventions of human chemists. Our efforts to merge abiological transition-metal chemistry with nature's vast toolbox of metalloproteins have focused on heme-binding proteins¹, as the heme cofactor and its analogues are well-studied in synthetic transition-metal chemistry. However, heme-binding proteins represent only a small fraction of the chemical diversity present in natural metalloproteins. Metalloproteins comprise greater than 30% of all proteins² and are responsible for some of the most fundamental chemical reactions in biology, including nitrogen fixation, photosynthesis, and DNA synthesis. Natural metalloproteins bind a variety of metals in a wide range of metal-binding sites, either coordinating the metal ion itself or a more complex metal-containing cofactor. Nearly any heteroatom-containing side chain can coordinate to a metal, in addition to the peptide backbone, allowing myriad possible coordination environments³. Many coordination environments in non-heme metalloenzymes have multiple open coordination sites at the metal center, a key feature of numerous synthetic transition-metal catalysts. Expanding new-to-nature catalysis to non-heme metalloenzymes would open a new world of transition-metal biocatalysis.

For decades, humans have used small-molecule ligands to understand and control Nature's catalysts. Nature employs allostery to regulate enzyme activity with small molecules (Figure 1A). Allosteric regulation of enzymes has been studied at length⁴ and there have been successful examples of engineered⁵ or designed⁶ allosteric enzyme regulation, but allostery is still challenging to understand or adapt to engineered systems. Cofactor-dependent enzymes, in which a small molecule is directly involved in catalysis, are often more readily understood and more easily manipulated by small molecules. Biochemists have knocked out catalytic activity *via* mutagenesis and restored it with exogenous effectors⁷ and sub-

stituted native cofactors with synthetic analogues with altered properties⁸. In the case of heme-binding proteins, scientists have substituted the metal ion⁹ or modified the porphyrin prosthetic group¹⁰ to modulate or expand enzymatic activity. A non-heme metalloprotein, in which a metal ion is coordinated directly by the protein and by exogenous small molecules, would allow for direct manipulation of the primary metal coordination environment.

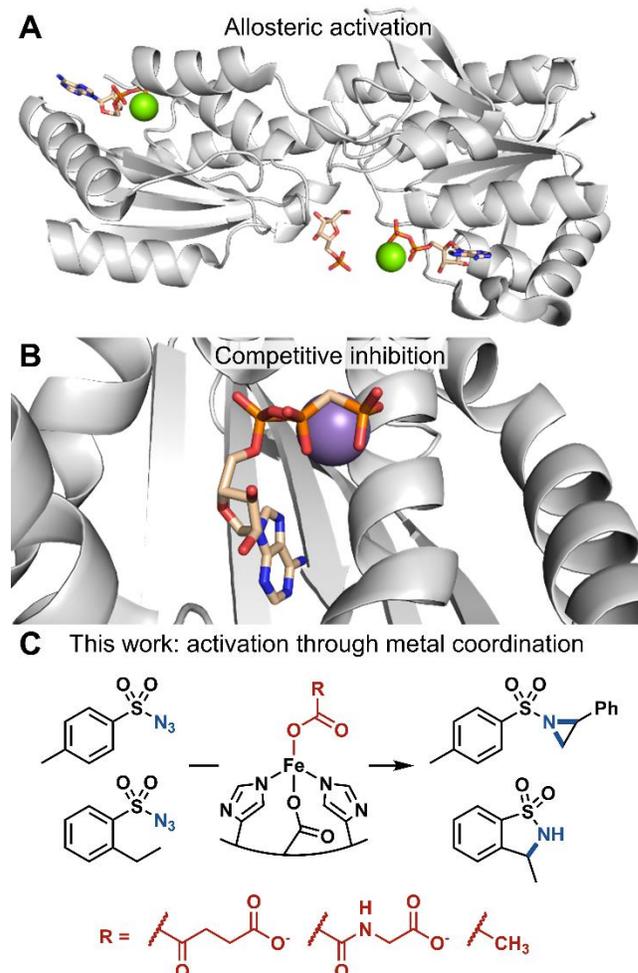


Figure 1. Examples of enzyme activity control using small molecules. (A) ADP acts as a natural allosteric regulator of phosphofructokinase (PDB ID: 4PFK). (B) Non-natural analogues of ATP act as competitive inhibitors of ATP-dependent enzymes (PDB ID: 115A). (C) This work: small-molecule activation of a non-heme iron center for nitrene transfer.

To search for abiological catalytic promiscuity among natural metalloproteins, we looked at α -ketoglutarate (α KG)-dependent iron enzymes, a family of enzymes which features a conserved metal-binding active site with iron coordinated by two histidines and one aspartate or glutamate¹¹. In nature, these enzymes perform similar chemistry to the heme-binding cytochrome P450 family, in which a high-valent iron-oxo intermediate performs C–H hydroxylation, olefin epoxidation, or other oxidative transformations¹². Though members of this enzyme family have been reported to catalyze reactions beyond their native functions, all the reactions reported proceed through the native iron-oxo mechanism¹³. We hypothesized that non-heme iron enzymes might also be able to catalyze abiological transformations similar to heme-binding proteins through a non-natural mechanistic pathway.

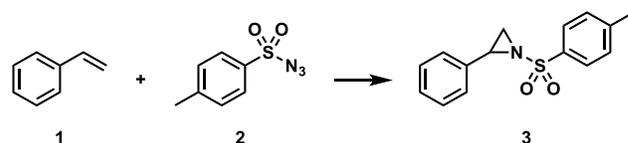
We screened a set of seven purified α -ketoglutarate (α KG)-dependent iron dioxygenases against the intermolecular aziridination reaction of styrene **1** and *p*-toluenesulfonyl azide **2**. Aziridination¹⁴ and carbon–hydrogen (C–H) bond insertions of nitrenes¹⁵ have been reported using engineered heme-binding proteins and were subsequently proposed in a natural product biosynthetic pathway¹⁶. Chang *et al.* speculated the existence of a transient iron-nitrene intermediate in their report of the transformation of alkyl azides to nitriles by an α KG-dependent iron dioxygenase, but this reaction still proceeds through the canonical iron-oxo catalytic cycle^{13b}. To date, no reported non-heme iron enzyme, natural or engineered, has been reported to catalyze productive nitrene transfer.

From the set of enzymes we tested, only *Pseudomonas savastanoi* ethylene-forming enzyme (*PsE*FE, UniProt ID P32021), formed aziridine **3** significantly above background (Supplementary Table S2). Compared to other members of the α KG-dependent iron dioxygenase family *PsE*FE is mechanistically and structurally distinct. While most enzymes of this family catalyze the oxidation of a substrate, often C–H hydroxylation, *PsE*FE natively catalyzes the fragmentation of the usual co-substrate α -ketoglutarate to ethylene, as well as the hydroxylation of L-arginine¹⁷. Structurally, *PsE*FE possesses a hybrid fold, combining elements of both type I and type II α KG-dependent iron enzymes. It binds α -ketoglutarate in a strained conformation in an unusually hydrophobic pocket, which is likely responsible for the atypical catalytic activity¹⁸.

As the iron-binding site in *PsE*FE is quite unlike that of the heme-binding proteins that perform nitrene-transfer chemistry, we sought to characterize the necessary components of the reaction. Iron is required, with a single added equivalent of iron(II) sufficient to fully restore catalytic activity of the wild-type apoenzyme. *PsE*FE has three coordination sites filled by amino-acid side chains (two histidines and one aspartate), leaving up to three additional sites open for binding. In the native catalytic mechanism of *PsE*FE and other members of its family, α -ketoglutarate occupies two of these sites and is required for activity, as it is oxidatively decarboxylated to succinate to generate the reactive iron-oxo intermediate. *PsE*FE has been shown to catalyze arginine hydroxylation with α -ketoadipate instead of α -ketoglutarate, but with 500-fold lower activity. Other α -ketoacids were reported to give no activity¹⁹. Nitrene transfer, however, does not proceed through the native catalytic cycle and therefore does not require α KG as a co-substrate; the α KG is now more a cofactor and as such could potentially be replaced by different small-molecule ligands. Intrigued by the possibility of modulating enzyme activity by changing the primary co-

ordination sphere of the catalytic iron, we tested *PsE*FE for aziridination with a set of α -ketoglutarate mimics and related molecules as additives. We found that whereas addition of a carboxylate is beneficial for activity (though not required), the wild-type enzyme is significantly more active for aziridination with added acetate or *N*-oxalylglycine (NOG, a general α KG-dependent enzyme competitive inhibitor¹¹) compared to α -ketoglutarate (Table 1).

Table 1. Aziridination catalyzed by wild-type *PsE*FE



Deviation from standard conditions ¹	Relative activity
None	1.00
No iron	0.08
No α KG	0.52
Acetate ² instead of α KG	6.72
<i>N</i> -oxalylglycine ³ instead of α KG	7.75
Acetate instead of α KG, no ascorbic acid	6.01

¹Standard conditions: Reactions were performed in MOPS buffer (20 mM pH 7.0) with 5% ethanol co-solvent, with 20 μ M apoenzyme, 1 mM Fe(NH₄)₂(SO₄)₂, 1 mM α KG (as disodium salt), 1 mM L-ascorbic acid, and 10 mM **1** and **2**. ²Sodium salt. ³Free acid.

We then sought to improve *PsE*FE for aziridination *via* directed evolution, targeting active-site residues with site-saturation mutagenesis and screening for enhanced activity. During directed evolution, we screened with added acetate, as it enhanced the activity of the wild-type enzyme significantly more than the native α -ketoglutarate, it is biologically ubiquitous, and it is inexpensive. Although α -ketoglutarate is the native cofactor and is naturally present at near-millimolar intracellular concentration in *Escherichia coli*²⁰, we reasoned that by supplementing the reaction medium with acetate we could evolve *PsE*FE to be dependent on acetate instead.

After two rounds of site-saturation mutagenesis and one round of recombination, we found a variant with five mutations from the wild type (T97M R171L R277H F314M C317M, *PsE*FE MLHMM) which catalyzed the formation of **3** with 120 total turnover number (TTN) and 88% enantiomeric excess (ee) favoring the (*R*)-enantiomer (Figure 2A). Four of the five introduced mutations are in the binding pocket of the native substrate arginine and presumably are involved in substrate binding. The fifth beneficial mutation is at Arg-277, the residue whose guanidino group natively binds the distal carboxylate of α -ketoglutarate (Figure 2B). The R277H mutation presumably abolishes binding of the native cofactor α -ketoglutarate; as a result, *PsE*FE MLHMM shows no significant increase in aziridination activity when α -ketoglutarate is added, but an 11-fold increase when acetate is added (Supplementary Table S4). Thus the evolved MLHMM variant is more activated by acetate than the wild type and is no longer activated by α -ketoglutarate at all, demonstrating the tunability of the cofactor dependence of *PsE*FE.

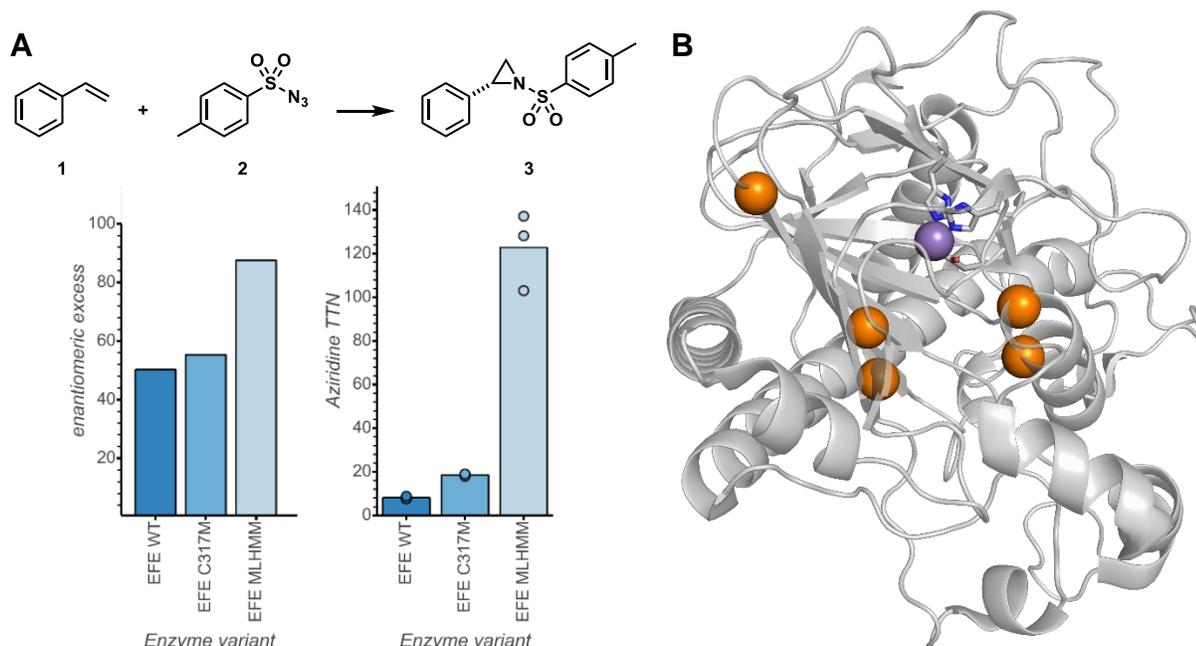


Figure 2. Directed evolution of *PsEFE* for aziridination. (A) Evolutionary lineage. Reactions were performed in triplicate anaerobically with acetate and quantified by analytical HPLC-UV. Full experimental details are given in the Supporting Information. (B) Structural representation of *PsEFE* with mutated sites highlighted in orange; metal-coordinating residues H189, D191, and H268 are represented in sticks and Mn (the metal with which the protein was crystallized) is represented as a purple sphere (PDB ID: 6CBA).

We reasoned that variants of *PsEFE* generated by directed evolution for aziridination could exhibit promiscuous activity for additional nitrene-transfer reactions beyond aziridination. Screening a panel of variants for the intramolecular C–H bond insertion reaction of 2-ethylbenzenesulfonyl azide **4** to form sultam **5**, we identified multiple variants that perform this reaction with good TTN, excellent chemoselectivity, and moderate enantioselectivity. Remarkably, *PsEFE* R171V F314M C317M (*PsEFE* VMM) is significantly more active and more chemo- and enantioselective with *N*-oxalylglycine added than with either acetate or α -ketoglutarate, forming **5** with up to 730 TTN and greater than 100:1 selectivity for insertion over reduction (Table 2). This chemoselectivity is much higher than that of the heme proteins previously reported to catalyze similar reactions^{15a,b}. To probe the specific effect of cofactor binding on activity, we tested *PsEFE* R171V R227H F314M C317M (*PsEFE* VHMM), a variant in which α KG and NOG binding are disrupted by the R277H mutation. Whereas *PsEFE* VHMM's activity is still enhanced nearly ten-fold with acetate, there is no significant difference between its activity with no additive, α KG, or NOG added to the reaction (Table 2). α KG or its analogues therefore appear to bind within the native α KG binding site, activating the protein through the primary metal coordination sphere.

Aziridination with *PsEFE* is reasonably oxygen tolerant, with the MLHMM variant maintaining 20% activity for aziridination when performed in air. C–H insertion, however, does not proceed detectably in aerobic conditions with any variant tested. These observations suggest that formation of the putative iron-nitrene intermediate is not significantly inhibited by oxygen, but that a subsequent mechanistic step in the C–H insertion reaction is inhibited aerobically. Unlike nitrene transfer with heme proteins^{15a}, an additional reductant is not required when reactions are performed anaerobically (Supplementary Table S4).

Table 2. Nitrene C–H insertion catalyzed by *PsEFE*

<i>PsEFE</i> variant	Additive	TTN (5)	ee (%)	5/6
Wild type	Acetate	12	n.d. ¹	1.6
VHMM	None	27	n.d. ¹	3.4
VHMM	α KG	31	n.d. ¹	3.8
VHMM	Acetate	240	7.3	32
VHMM	NOG	33	n.d. ¹	4.1
VMM	None	25	n.d. ¹	0.9
VMM	α KG	130	61	9.0
VMM	Acetate	310	9.4	24
VMM	NOG	450	48	105
VMM ²	NOG	730	47	100

Reactions were performed anaerobically in MOPS buffer (20 mM pH 7.0) with 2.5% ethanol co-solvent, 20 μ M apoenzyme, 1 mM $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$, 1 mM additive, 1 mM *L*-ascorbic acid, and 10 mM **4** (maximum 500 TTN). Reactions were quantified by analytical HPLC-UV. TTNs are shown for **5** only. ¹Not determined due to low conversion. ²10 μ M enzyme concentration (max. 1000 TTN).

PsEFE is highly expressed (>200 mg/L *E. coli* culture) and is catalytically active in whole *E. coli* cells and in cell lysate. We observe that in cell lysate the enzyme maintains high activity with no external additive; the enzyme presumably retains a cofactor from the intracellular environment during lysis. Addition of *N*-oxalylglycine nevertheless enhances C–H insertion yield and chemoselectivity when *PsEFE* VMM is in cell lysate. In whole cells, however,

there is no significant change upon addition of *N*-oxalylglycine (Supplementary Table S6). This is unsurprising as *N*-oxalylglycine is not reported to be cell-permeable. Future biochemical studies and further mutagenesis will likely enhance the selectivity for cofactor analogues and impart *in vivo* activation to PsEFE variants.

In conclusion, we have discovered a non-heme iron enzyme capable of performing nitrene-transfer chemistry and enhanced that activity *via* directed evolution. This is the first example of enzymatic nitrene transfer catalyzed by a non-heme metalloprotein. PsEFE features a metal center whose primary coordination sphere can be altered by simple reaction additives, allowing for modulation of catalytic activity and selectivity. We anticipate that this biocatalytic system will lead to discovery of new metalloenzymatic transformations not possible with previously reported enzymes.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Materials and experimental methods, compound characterization data (PDF)

Full nucleotide and amino-acid sequences for all reported enzyme variants (XLSX)

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Notes

A provisional patent has been filed through the California Institute of Technology based on the results presented here.

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REFERENCES

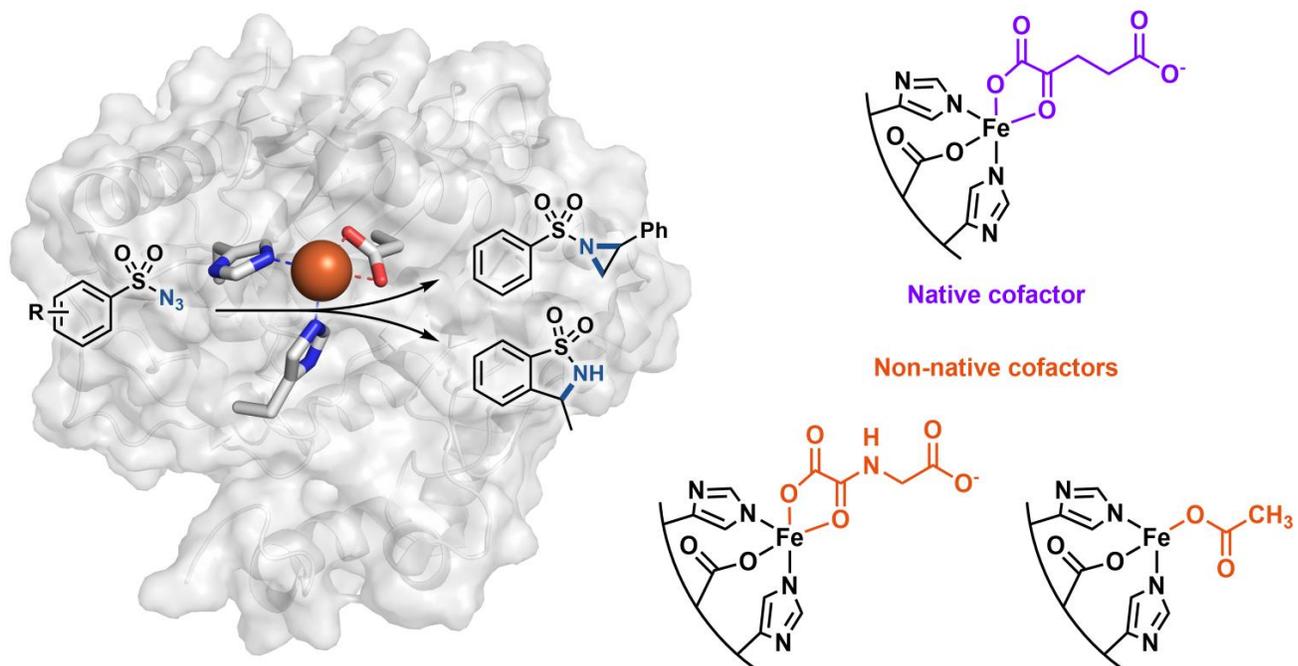
- (1) Brandenberg, O. F.; Fasan, R.; Arnold, F. H. Exploiting and engineering hemoproteins for abiological carbene and nitrene transfer reactions. *Curr. Opin. Biotechnol.* **2017**, *47*, 102–111.
- (2) Degtyarenko, K. Metalloproteins. In *Encyclopedia of Genetics, Genomics, Proteomics and Bioinformatics*; Jorde, L.; Little, P.; Dunn, M.; Subramaniam, S., Eds.; John Wiley & Sons, Ltd., **2005**.
- (3) Holm, R. H.; Kennepohl, P.; Solomon, E. I. Structural and Functional Aspects of Metal Sites in Biology. *Chem. Rev.* **1996**, *96*, 2239–2314.
- (4) Motlagh, H. N.; Wrabl, J. O.; Li, J.; Hilsner, V. J. The ensemble nature of allostery. *Nature* **2014**, *508*, 331–339.
- (5) Guntas, G.; Mansell, T. J.; Kim, J. R.; Ostermeier, M. Directed evolution of protein switches and their application to the creation of ligand-binding proteins. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11224–11229.

- (6) Langan, R. A.; Boyken, S. E.; Ng, A. H.; Samson, J. A.; Dods, G.; Westbrook, A. M.; Nguyen, T. H.; Lajoie, M. J.; Chen, Z.; Berger, S.; Mulligan, V. K.; Dueber, J. E.; Novak, W. R. P.; El-Samad, H.; Baker, D. De novo design of bioactive protein switches. *Nature* **2019**, *572*, 205–210.
- (7) Qiao, Y.; Molina, H.; Pandey, A.; Zhang, J.; Cole, P. A. Chemical Rescue of a Mutant Enzyme in Living Cells. *Science* **2006**, *311*, 1293–1297.
- (8) (a) Khanna, N.; Esmieu, C.; Mészáros, L. S.; Lindblad, P.; Berggren, G. *In vivo* activation of an [FeFe] hydrogenase using synthetic cofactors. *Energy Environ. Sci.* **2017**, *7*, 1563–1567. (b) Ji, X.; Mandalapu, D.; Cheng, J.; Ding, W.; Zhang, Q. Expanding the Chemistry of the Class C Radical SAM Methyltransferase NosN by Using an Allyl Analogue of SAM. *Angew. Chem. Int. Ed.* **2018**, *57*, 6601–6604. (c) Drenth, J.; Trajkovic, M.; Fraaije, M. W. Chemoenzymatic Synthesis of an Unnatural Deazaflavin Cofactor That Can Fuel F₄₂₀-Dependent Enzymes. *ACS Catal.* **2019**, *9*, 6435–6443.
- (9) (a) Sreenilayam, G.; Moore, E. J.; Steck, V.; Fasan, R. Metal Substitution Modulates the Reactivity and Extends the Reaction Scope of Myoglobin Carbene Transfer Catalysts. *Adv. Synth. Catal.* **2017**, *359*, 2076–2089. (b) Wolf, M. W.; Vargas, D. A.; Lehnert, N. Engineering of RuMb: Toward a Green Catalyst for Carbene Insertion Reactions. *Inorg. Chem.* **2017**, *56*, 5623–5635. (c) Natoli, S. N.; Hartwig, J. F. Noble-Metal Substitution in Hemoproteins: An Emerging Strategy for Abiological Catalysis. *Acc. Chem. Res.* **2019**, *52*, 326–335.
- (10) (a) Reynolds, E. W.; McHenry, M. W.; Cannac, F.; Gober, J. G.; Snow, C. D.; Brustad, E. M. An Evolved Orthogonal Enzyme/Cofactor Pair. *J. Am. Chem. Soc.* **2016**, *138*, 12451–12458. (b) Sreenilayam, G.; Moore, E. J.; Steck, V.; Fasan, R. Stereoselective Olefin Cyclopropanation under Aerobic Conditions with an Artificial Enzyme Incorporating an Iron-Chlorin e6 Cofactor. *ACS Catal.* **2017**, *7*, 7629–7633.
- (11) Hausinger, R. P. Fe(II)/ α -Ketoglutarate-Dependent Hydroxylases and Related Enzymes. *Crit. Rev. Biochem. Mol. Biol.* **2004**, *39*, 21–68.
- (12) Islam, M. D.; Leissing, T. M.; Chowdhury, R.; Hopkinson, R. J.; Schofield, C. J. 2-Oxoglutarate-Dependent Oxygenases. *Annu. Rev. Biochem.* **2018**, *87*, 585–620.
- (13) (a) Matthews, M. L.; Chang, W.-c.; Layne, A. P.; Miles, L. A.; Krebs, C.; Bollinger, J. M. Jr. Direct nitration and azidation of aliphatic carbons by an iron-dependent halogenase. *Nat. Chem. Biol.* **2014**, *10*, 209–215. (b) Davidson, M.; McNamee, M.; Fan, R.; Guo, Y.; Chang, W.-c. Repurposing Nonheme Iron Hydroxylases To Enable Catalytic Nitrile Installation through an Azido Group Assistance. *J. Am. Chem. Soc.* **2019**, *141*, 3419–3423. (c) Neugebauer, M. E.; Sumida, K. H.; Pelton, J. G.; McMurry, J. L.; Marchand, J. A.; Chang, M. C. Y. A family of radical halogenases for the engineering of amino-acid-based products. *Nat. Chem. Biol.* **2019**, *15*, 1009–1016.
- (14) Farwell, C. C.; Zhang, R. K.; McIntosh, J. A.; Hyster, T. K.; Arnold, F. H. Enantioselective Enzyme-Catalyzed Aziridination Enabled by Active-Site Evolution of a Cytochrome P450. *ACS Cent. Sci.* **2015**, *1*, 89–93.
- (15) (a) McIntosh, J. A.; Coelho, P. S.; Farwell, C. C.; Wang, Z. J.; Lewis, J. C.; Brown, T. R.; Arnold, F. H. Enantioselective Intramolecular C–H Amination Catalyzed by Engineered Cytochrome P450 Enzymes *In Vitro* and *In Vivo*. *Angew. Chem. Int. Ed.* **2013**, *52*, 9309–9312. (b) Singh, R.; Bordeaux, M.; Fasan, R. P450-Catalyzed Intramolecular sp³ C–H Amination with Arylsulfonyl Azide Substrates. *ACS Catal.* **2014**, *4*, 546–552. (c) Prier, C. K.; Zhang, R. K.; Buller, A. R.; Brinkmann-Chen, S.; Arnold, F. H. Enantioselective, intermolecular benzylic C–H amination catalysed by an engineered iron-haem enzyme. *Nat. Chem.* **2017**, *9*, 629–634.
- (16) Tsutsumi, H.; Katsuyama, Y.; Izumikawa, M.; Takagi, M.; Fujie, M.; Satoh, N.; Shin-ya, K.; Ohnishi, Y. Unprecedented Cyclization Catalyzed by a Cytochrome P450 in Benzastatin Biosynthesis. *J. Am. Chem. Soc.* **2018**, *140*, 6631–6639.
- (17) Fukuda, H.; Ogawa, T.; Tazaki, M.; Nagahama, K.; Fujii, T.; Tanase, S.; Morino, Y. Molecular cloning in *Escherichia coli*, expression, and nucleotide sequence of the gene for the ethylene-forming enzyme of *Pseudomonas syringae* pv. *phaseolicola* PK2. *Biochem. Biophys. Res. Commun.* **1992**, *188*, 483–489.
- (18) (a) Zhang Z., Smart T. J.; Choi, H.; Hardy, F.; Lohans, C. T.; Abboud, M. I.; Richardson, M. S. W.; Paton, R. S.; McDonough, M. A.; Schofield, C. J. Structural and stereoelectronic insights into oxygenase-catalyzed formation of ethylene from 2-oxoglutarate. *Proc. Natl. Acad. Sci. U.S.A.* **2017**, *114*, 4467–4472. (b) Martinez, S.; Fellner, M.; Ferr, C. Q.; Ritchie, A.; Hu, J.; Hausinger, R. P. Structures and Mechanisms of the Non-Heme Fe(II)- and 2-Oxoglutarate-Dependent Ethylene-Forming Enzyme: Substrate Binding Creates a Twist. *J. Am. Chem. Soc.* **2017**, *139*, 11980–11988.

(19) Martinez, S.; Hausinger, R. P. Biochemical and Spectroscopic Characterization of the Non-Heme Fe(II)- and 2-Oxoglutarate-dependent Ethylene-Forming Enzyme from *Pseudomonas syringae* pv. *phaseolicola* PK2. *Biochemistry* **2016**, *55*, 5989–5999.

(20) Bennett, B. D.; Kimball, E. H.; Gao, M.; Osterhout, R.; Van Dien, S. J.; Rabinowitz, J. D. Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*. *Nat. Chem. Biol.* **2009**, *5*, 593–599.

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Non-natural nitrene transfer is enhanced by substitution of native iron-binding cofactor
