Communication

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J. Am. Chem. Soc., **Just Accepted Manuscript** • Publication Date (Web): 02 Dec 2019

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

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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 *Pseudomonas savastanoi* ethylene-forming enzyme (*Ps*EFE), 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 proteins[1], 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 proteins[2] 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 environments[3]. 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. In this work we show that a non-heme iron enzyme can catalyze nitrene-transfer chemistry (Figure 1). This non-native activity is enhanced by binding of non-native small-molecule ligands and has been improved by directed evolution.

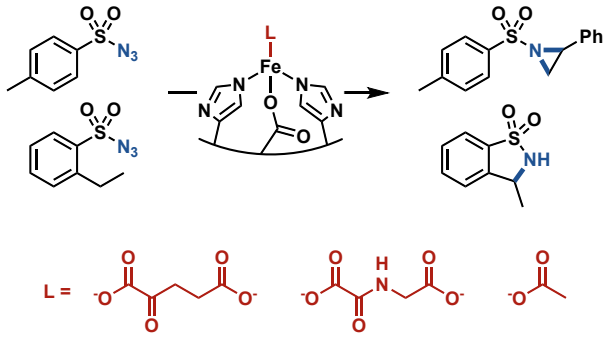


Figure 1. Small-molecule activation of a non-heme iron center for nitrene transfer. Carboxylate-containing ligands α-ketoglutarate, *N*-oxalylglycine, and acetate modulate the nitrene-transfer activity of variants of *P. savastanoi* ethylene-forming enzyme.

To search for abiological catalytic promiscuity among natural metalloproteins, we looked at α-ketoglutarate (αKG)-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 glutamate[4]. 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 transformations[5]. 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 mechanism[6]. 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.

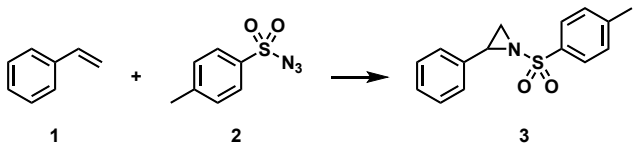
We screened a set of seven purified α-ketoglutarate (αKG)-dependent iron dioxygenases against the intermolecular aziridination reaction of styrene **1** and *p*-toluenesulfonyl azide **2**. Aziridination[7] and carbon–hydrogen (C–H) bond insertions of nitrenes[8] have been reported using engineered heme-binding proteins and were subsequently proposed in a natural product biosynthetic pathway[9]. Chang and coworkers speculated the existence of a transient iron-nitrene intermediate in their report of the transformation of alkyl azides to nitriles by an αKG-dependent iron dioxygenase, but this reaction still proceeds through the canonical iron-oxo catalytic cycle[6b]. 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 (*Ps*EFE, UniProt ID P32021), formed aziridine **3** significantly above background

(Supplementary Table S2). Compared to other members of the αKG-dependent iron dioxygenase family *Ps*EFE is mechanistically and structurally distinct. While most enzymes of this family catalyze the oxidation of a substrate, often C–H hydroxylation, *Ps*EFE natively catalyzes the fragmentation of the usual co-substrate α-ketoglutarate to ethylene, as well as the hydroxylation of L-arginine[10]. Structurally, *Ps*EFE possesses a hybrid fold, combining elements of both type I and type II αKG-dependent iron enzymes. It binds α-ketoglutarate in a strained conformation in an unusually hydrophobic pocket, which is likely responsible for the atypical catalytic activity[11].

As the iron-binding site in *Ps*EFE 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. *Ps*EFE 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 *Ps*EFE 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. *Ps*EFE 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 activity[12]. Nitrene transfer, however, does not proceed through the native catalytic cycle and therefore does not require αKG as a co-substrate; the αKG is now more a ligand and as such could potentially be replaced by different small-molecule ligands. Intrigued by the possibility of modulating enzyme activity by changing the primary coordination sphere of the catalytic iron, we tested *Ps*EFE 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 αKG-dependent enzyme competitive inhibitor[4]) compared to α-ketoglutarate (Table 1).

Table 1. Aziridination catalyzed by wild-type *Ps*EFE



1 2 3

Deviation from standard conditions[1]	Relative activity
None	1.00
No iron	0.08
No αKG	0.52
Acetate[2] instead of αKG	6.72
N-oxalylglycine[3] instead of αKG	7.75
Acetate instead of αKG, no ascorbic acid	6.01

[1]Standard conditions: Reactions were performed in MOPS buffer (20 mM pH 7.0) with 5% ethanol co-solvent, with 20 μM apoenzyme, 1 mM $Fe(NH_4)_2(SO_4)_2$, 1 mM αKG (as disodium salt), 1 mM L-ascorbic acid, and 10 mM **1** and **2**. [2]Sodium salt. [3]Free acid.

We then sought to improve *Ps*EFE for aziridination *via* directed evolution, targeting active-site residues with site-saturation mutagenesis and screening for enhanced activity. Details of our engineering strategy are in the Supporting Information. Initial screening for the first round was performed in the presence of exogenous α-ketoglutarate, but for validation of this round and for all subsequent evolution we screened with exogenous acetate. Acetate was chosen as the preferred ligand because 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 ligand and is naturally present at near-millimolar intracellular concentration in *Escherichia coli*[13], we reasoned that by supplementing the reaction medium with acetate we could evolve *Ps*EFE to be dependent on acetate instead, despite screening under whole cell or lysate conditions.

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, *Ps*EFE 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 likely interferes with binding of the native ligand α-ketoglutarate; as a result, *Ps*EFE 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 highly activated by acetate but is no longer activated by α-ketoglutarate at all, demonstrating the tunability of the ligand dependence of *Ps*EFE.

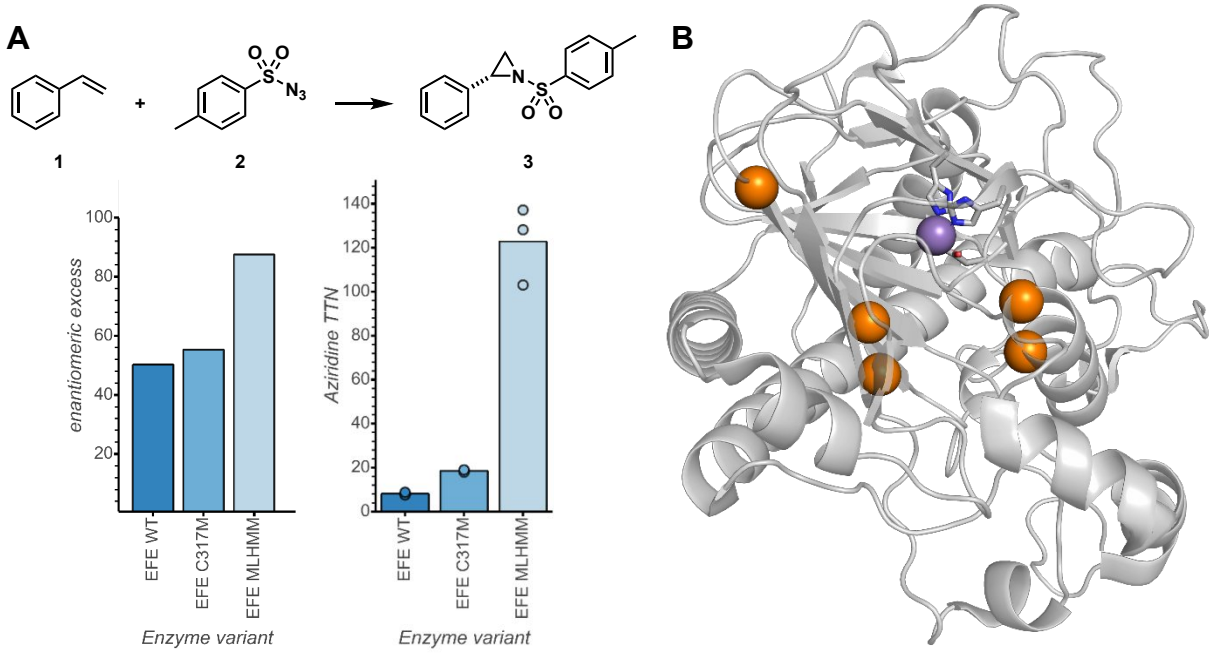
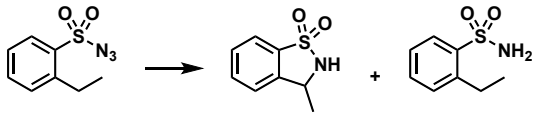


Figure 2. Directed evolution of *Ps*EFE for aziridination. (A) Evolutionary lineage. Reactions were performed in triplicate anaerobically with acetate and quantified by analytical HPLC-UV. Full experimental details are given in the Supporting Information. (B) Structural representation of *Ps*EFE with 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 *Ps*EFE 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, *Ps*EFE R171V F314M C317M (*Ps*EFE 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[8a,b]. To probe the specific effect of ligand binding on activity, we tested *Ps*EFE R171V R227H F314M C317M (*Ps*EFE VHMM), a variant in which αKG and NOG binding are disrupted by the R277H mutation. Whereas *Ps*EFE VHMM's activity is still enhanced nearly ten-fold with acetate, there is no significant difference between its activity with no additive, αKG, or NOG added to the reaction (Table 2). αKG or its analogues therefore appear to bind within the native αKG binding site, activating the protein through the primary metal coordination sphere.

Aziridination with *Ps*EFE 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[8a], an additional reductant is not required when reactions are performed anaerobically (Supplementary Table S4).

Table 2. Nitrene C–H insertion catalyzed by *Ps*EFE



4 5 6

*Ps*EFE 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	αKG	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	αKG	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 μM apoenzyme, 1 mM $Fe(NH_4)_2(SO_4)_2$, 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. [1]Not determined due to low conversion. [2]10 μM enzyme concentration (max. 1000 TTN).

*Ps*EFE is highly expressed (>200 mg/L *E. coli* culture) and is catalytically active for nitrene transfer 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 ligand from the intracellular environment during lysis. Addition of *N*-oxalylglycine nevertheless enhances C–H insertion yield and chemoselectivity when *Ps*EFE 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 ligand analogues and impart *in vivo* activation to *Ps*EFE 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. *Ps*EFE 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 and sequences of all oligonucleotides used for mutagenesis (XLSX)

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Notes

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

ACKNOWLEDGMENTS

This work was supported by the National Science Foundation (NSF) Division of Molecular and Cellular Biosciences (grant MCB-1513007). N. W. G., A. M. K., and R. K. Z. acknowledge support from the NIH training grants NIH T32 GM07616 (N. W. G.) and NIH T32 GM112592 (A. M. K., R. K. Z.) and NSF Graduate Research Fellowship DGE-1144469 (A. M. K, R. K. Z.). We thank Sabine Brinkmann-Chen for critical reading of the manuscript and Noah P. Dunham, S. B. Jennifer Kan, and Benjamin J. Levin for helpful discussions. We thank Professor Hans Renata and Professor Harry Gray for generously sharing plasmids.

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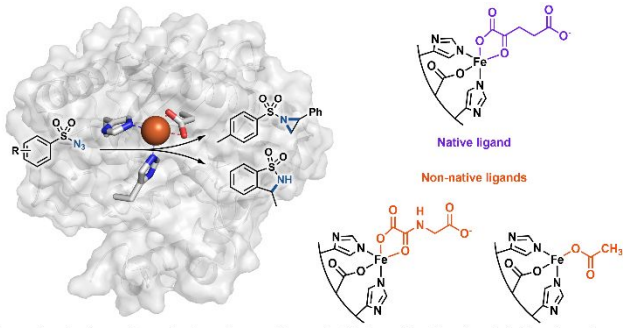

Non-natural nitrene transfer is enhanced by substitution of native iron-binding ligand