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Elucidating the Reaction Pathway of Decarboxylation-Assisted Olefination Catalyzed by a Mononuclear Non-Heme Iron Enzyme

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Abstract

Installation of olefins into molecules is a key transformation in organic synthesis. The recently discovered decarboxylation-assisted olefination in the biosynthesis of rhabduscin by a mononuclear non-heme iron enzyme (*P*IsnB) represents a novel approach in olefin construction. This method is commonly employed in natural product biosynthesis. Herein, we demonstrate that a ferryl intermediate is used for C–H activation at the benzylic position of the substrate. We further establish that *P*IsnB reactivity can be switched from olefination to hydroxylation using electron-withdrawing groups appended on the phenyl moiety of the analogues. These experimental observations imply that a pathway involving an initial C–H activation followed by a benzylic carbocation species or by electron transfer coupled β -scission is likely utilized to complete C=C bond formation.

Members of the non-heme iron enzyme family are known to catalyze oxidative transformations responsible for both primary and secondary metabolite production.¹ Examples include hydroxylation, halogenation, epoxidation, and desaturation.^{2–4} Iron(II)- and 2-oxoglutarate-dependent (Fe/2OG) enzymes, a subclass of this enzyme family, carry out reactions by using a reactive ferryl (Fe(IV)=O) species as the key intermediate.^{3,5} In the past decade, the Fe(IV)=O species along with other intermediates involved in hydroxylation and halogenation have been extensively studied (Figure 1a).^{6–8} In contrast, reaction mechanisms for other biologically and chemically intriguing transformations, such as decarboxylation-assisted olefination, have not been thoroughly investigated.

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/jacs.8b10077.

Experimental methods, additional comments on SF-Abs and Mössbauer analyses, Figures S1–S31, Tables S1 and S2, and references (PDF)

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A majority of the identified biosynthetic pathways leading to vinyl isonitriles, such as found in hapalindoles, ambiguine, and rhabduscin, utilize a conserved approach to produce the vinyl isonitrile group.^{9,10} Among these pathways, Fe/2OG enzymes install an olefin group via loss of CO₂ and hydride removal.¹¹ Compared to other reactions types, this reaction represents a unique approach for constructing olefins. Recently, *P.IsnB*, an Fe/2OG enzyme, was reported to catalyze the installation of an olefin group in the isonitrile-containing tyrosine **1**. Subsequently, the vinyl isonitrile intermediate **2** would then be subjected to further modifications to generate rhabduscin (Figure 1b).¹⁰ Elucidating the mechanism will provide a fundamental understanding of this transformation and will shed light on the factors that direct and govern the reaction outcomes. Herein, we employ a complementary approach including mechanistic probe design, transient kinetics, CW EPR, 2D pulsed EPR hyperfine sublevel correlation spectroscopy (HYSCORE), Mössbauer, and LC-UV/MS to elucidate the plausible pathways of this novel reaction. Different from the originally proposed hydroxylation and CO₂ elimination pathway,^{12,13} our results suggest that alternate pathways involving a benzylic carbocation or an electron transfer coupled decarboxylation is likely to be utilized to trigger the olefination (Scheme 1).

To study *P.IsnB*-catalyzed reaction, the substrate (**1**), its deuterium-labeled analogue at the benzylic position (**D-1**), and the product standard (**2**) were synthesized (see SI for procedures). *P.IsnB* was heterologously expressed in *E. coli* and purified (Figure S23). The activity of *P.IsnB* was tested by stopped-flow absorption spectroscopy (SF-Abs) to monitor the reaction after a rapid mixing of the anaerobic *P.IsnB*-Fe(II)·2OG·substrate (**1** or **D-1**) complex with oxygenated buffer at 5°C. Similar to other characterized Fe/2OG enzymes, an Fe(II)-2OG metal-to-ligand charge transfer (MLCT) band centered at 512 nm was observed in the *P.IsnB* quaternary complex (Figure S25), which also exhibited distinct absorption shoulders at 465 and 570 nm.¹⁴ When **1** was used, the MLCT band depleted within 0.07 s after O₂ mixing and a rapid increase of absorption features at ~330 nm was detected. In contrast, the use of **D-1** resulted in an additional rise of an optical feature at ~440 nm that reached a maximum at ~0.05 s and then decayed (Figure 2a,b). In both cases, the absorption change below 350 nm cannot be unambiguously assigned to the kinetics of a single species and may partially result from product absorption (Figure S26, $\epsilon_{330\text{ nm}} \approx 1600\text{ M}^{-1}\text{ cm}^{-1}$ for **2**). Thus, the absorption feature at 440 nm was used to follow the Fe(IV)=O kinetics, while the identification of the Fe(IV)=O species was verified by Mössbauer spectroscopy (see below). A three-step kinetic model (*P.IsnB*-Fe(II)·2OG·substrate + O₂ → Fe(IV)=O → *P.IsnB*-Fe(II)·product → *P.IsnB*-Fe(II)·2OG) reproduced the time-dependent changes of the 440 and 512 nm absorption generated in the course of reactions with **1** and **D-1** (see SI for discussions, Figures S27, S28). The decay rate constant for the *P.IsnB* quaternary complex, which also represents the rate of Fe(IV)=O formation, is ~65 mM⁻¹ s⁻¹ for both reactions (**1** and **D-1**). However, the Fe(IV)=O decay rate constants, k_2 , differed significantly. For **D-1**, k_2 is ~3 s⁻¹, but for **1**, since the Fe(IV)=O species could not be observed in SF-Abs (the corresponding 440 nm kinetic trace mainly reflects the decay of the Fe(II)-2OG MLCT band, Figure 2b) or Mössbauer experiments (see below), we estimated k_2 is larger than 300 s⁻¹. Thus, the H/D kinetic isotope effect (KIE) of the Fe(IV)=O decay reaches >100, which implies the initial C–H activation site is at the benzylic position of the substrate.

Additional evidence for the substrate position relative to the iron center was provided by EPR measurement where nitric oxide (NO) was used as the O₂ surrogate. Addition of NO to Fe(II) centers in non-heme enzymes typically yields {FeNO}⁷ species, of which the EPR signal could be used to elucidate the interactions between the substrate and the non-heme iron centers.^{15–18} The HYSCORE Q-band (34 GHz) spectrum of the NO-treated *P*.IsnB-Fe(II)-2OGD-**1** complex was obtained at a magnetic field corresponding to the maximum peak intensity of the echo-detected field-swept EPR spectrum (effective $g = 3.97$, magnetic field of 6094 G, Figures 3 and S30) at $T = 3.1$ K. This field position corresponds to the $g \approx 4$ resonance in the X-band (9 GHz) CW EPR spectrum (Figure S29) attributed to the substrate-bound *P*.IsnB complex. The most pronounced features observed in the (+, +) quadrant of the HYSCORE spectrum are due to deuterons at the benzylic position located in relative proximity to the Fe center (Figure 3A). While the splitting along the main diagonal is caused by the quadrupolar interaction, an extension along the anti-diagonal is due to an electron–nuclear hyperfine interaction of <1 MHz in amplitude. Such a small coupling is indicative of Fe–²H interspin distances longer than the typical bond length, thus, justifying the point–dipole approximation for the analysis. Figure 3B shows the optimal simulation with the following hyperfine interaction parameters: electron–nuclear hyperfine tensor $\mathbf{A} = [0.9 \pm 0.3, -0.45 \pm 0.15, -0.45 \pm 0.15]$ MHz, quadrupolar parameter $\mathbf{K} = 0.05 \pm 0.05$ MHz, and quadrupolar asymmetry parameter $\eta = 0.3 \pm 0.3$. By using point-dipole approximation, $\mathbf{A}$ can be converted to the distance between the spin center of the Fe–NO unit and the deuterons of D-**1**, yielding $r = 3.78 \pm 0.5$ Å. This value is comparable to the distances revealed in other Fe/2OG enzymes by HYSCORE and ESEEM techniques^{16–18} and provides an additional support for the benzylic C–H activation during *P*.IsnB catalysis.

Freeze-quench (FQ)-coupled Mossbauer spectroscopy experiments were also performed (Figure S31 and Supporting Information for discussion). In short, three time-dependent iron species were observed upon rapid mixing of *P*.IsnB-Fe(II)-2OG-**1** (or D-**1**) with an oxygenated buffer at various time intervals: (1) a quadrupole doublet representing the *P*.IsnB quaternary complex ($\delta = 1.18$ mm/s, $|E_Q| = 2.76$ mm/s), (2) another doublet representing the Fe(IV)=O species ($\delta = 0.29$ mm/s, $|E_Q| = 1.06$ mm/s), and (3) a third doublet representing the *P*.IsnB-Fe(II)-product and/or the *P*.IsnB-Fe(II)-2OG complex ($\delta = 1.28$ mm/s, $|E_Q| = 2.84$ mm/s). The Fe(IV)=O doublet was only observed in the reaction when D-**1** was used, but not with **1** even at the quench time of 0.02 s. The failure to detect Fe(IV)=O species in the reaction with **1** is likely due to the fast decay of such an intermediate. In addition, Mössbauer data suggest that only ~50% of the total *P*.IsnB quaternary complex is active. Furthermore, ~20–25% of the isonitrile–Fe(II) complex was observed, which is likely generated through the direct binding of the isonitrile group to the iron center (Table S2).^{13b}

LC-UV/MS was used to monitor the *P*.IsnB reaction. The product, which has an identical retention time to that of the synthetic standard **2**, was observed in the reaction mixture containing *P*.IsnB, O₂, 2OG, and **1** (Figure 4a). Combined with SF-Abs and Mossbauer observations, these results suggest that *P*.IsnB utilizes an Fe(IV)-oxo species as the key intermediate to trigger benzylic C–H bond cleavage and installation of a C=C bond. However, the governing factors that direct reaction outcome to olefination remain to be

elucidated. Subsequent to C–H activation and substrate radical formation, the reaction may proceed through a pathway involving a hydroxylated intermediate or a substrate radical/cation to trigger β -scission and complete the olefin installation (Scheme 1). To distinguish these pathways, analogues with a H, F, or CF₃ (**3**, **4**, or **5**, Scheme 1) at the *para* position were synthesized and subjected to the *P*.IsnB-catalyzed reaction. SF-Abs results revealed that all the analogues showed a similar Fe(II)-2OG MLCT band decay kinetics when reacting with O₂ (Figure S27, ranging between 40 and 50 mM⁻¹ s⁻¹), resembling that of the native substrate (**1**). The decay rate constants of the Fe(IV)=O species were estimated to be 19, 10, and 3 s⁻¹ for **3**, **4**, and **5**, respectively (Figures 2, S27). Although these analogues show a decreased Fe(IV)=O decay rate comparable with that of the native substrate, the similar Fe(IV)=O formation rate indicates these analogues are substrates for *P*.IsnB. Furthermore, CW EPR measurement of NO-*P*.IsnB-Fe(II)-2OG-**5** (or **1**) showed no obvious perturbation (Figure S29), thus implying a similar binding configuration of **5** and **1**.

LC-UV/MS analysis revealed that a substantial decrease of the olefin products (**6** and **7**) when **3** and **4** were used (Figure S24), while **5** resulted in no detectable product (**8**) formation. Meanwhile, a plausible hydroxylated product with an *m/z* value of +16 from the substrates (**3**, **4**, and **5**) was detected, but the corresponding species was not observed when **1** was used (Figure 4b). Next, the hydroxylated product standard (**9**) of **3** was synthesized. LC-MS revealed that **9** has an identical elution time to that of the reaction product when **3** was used. Additionally, the amount of the hydroxylated product correlates with the electron-withdrawing property of the analogues tested (**5** > **4** > **3** (CF₃ > F > H)) (Figure 4b). Two possible scenarios can account for this observation: (1) due to nonoptimum substrate positioning, the hydroxylated intermediate dissociates from the active site prior to decarboxylation, or (2) the electron-withdrawing property of the *para*-substituent redirects the reaction pathway from olefination to hydroxylation. To distinguish these possibilities, **9** was incubated with *P*.IsnB. If the hydroxylation is the on-pathway intermediate, by incubating *P*.IsnB with **9**, one should be able to observe formation of the olefin product **6**. However, such a product was not detected during 20 min incubation of **9** with *P*.IsnB under anaerobic conditions (Figure 4c). Thus, hydroxylation is unlikely to serve as an intermediate. Furthermore, a competition experiment revealed that **9** can inhibit the formation of **2** (Figure S24), implying that **9** can bind to *P*.IsnB. Taken together, these results suggest that the pathway utilizing a hydroxylated intermediate is unlikely to operate. Alternatively, a pathway involving a carbocation or electron transfer coupled decarboxylation may be utilized. When the analogues bearing an electron-withdrawing group are used, the instability of the benzylic cation or the transient species involved in the latter pathway alter the reaction pathway and the incipient substrate radical is quenched by the Fe(III)-OH species to produce the hydroxylated product. The ¹⁸O labeling experiment using ¹⁸OH₂ revealed no ¹⁸O incorporation into **9** (Figure S24), thus suggesting a OH rebound mechanism for hydroxylation.

In addition to Fe/2OG enzymes, a decarboxylation-assisted olefination approach has been reported in other enzymatic transformations where various cofactors, such as thiolate-heme and [4Fe-4S]-SAM, are involved.^{19–22} Herein, our results provide experimental evidence to

support a pathway utilizing C–H activation followed by a carbocation or by an electron transfer coupled β -scission to trigger olefin installation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Proposed pathway of Fe/2OG enzyme catalyzed reactions. (b) *P.IsnB* catalyzed decarboxylation-assisted olefination found in rhabduscin biosynthesis.

Figure 2.

SF-Abs results of *P.IsnB* reactions. (a) Change in absorbance at the indicated reaction times after mixing the *P.IsnB*·Fe(II)·2OGD·**1** complex with O₂. The spectra were obtained by subtracting the spectrum at 0.002 s. (b) Kinetic traces at 440 nm used to follow the kinetics of the Fe(IV)=O intermediate in the reaction using **1** (blue), D-**1** (red), and **5** (green). Simulations are shown in black.

Figure 3.

Q-band (34 GHz) HYSCORE spectrum of the NO.*P.IsnB*-Fe(II)-2OGD-**1** obtained at a magnetic field corresponding to $g \approx 3.97$ resonance in the echo-detected field-swept EPR spectrum and $T = 3.1$ K. (A) Spectral region showing the features resulting from ^2H and FeNO interactions and (B) the corresponding spectral simulations.

Figure 4.

(a) LC-UV chromatograms of *P.IsnB*-catalyzed conversion of **1** to **2** in the presence of O₂ and 2OG detected at λ_{266} . (b) LC-MS chromatograms of *P.IsnB*-catalyzed hydroxylation when substrate analogues were used. The bottom trace represents the product standard **9**. (c) LC-UV chromatograms of reacting **9** with *P.IsnB* anaerobically and the product standard **6** detected at λ_{266} .

Scheme 1.

Possible Pathways Account for *P.IsnB*-Catalyzed Olefination; Products and Probes Employed in This Study (1–9)