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Mechanistic Investigation of a Non-Heme Iron Enzyme Catalyzed Epoxidation in (−)-4′-Methoxycyclopenin Biosynthesis

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Supporting Information

ABSTRACT: Mechanisms have been proposed for α-KG-dependent non-heme iron enzyme catalyzed oxygen atom insertion into an olefinic moiety in various natural products, but they have not been examined in detail. Using a combination of methods including transient kinetics, Mössbauer spectroscopy, and mass spectrometry, we demonstrate that AsqJ-catalyzed (−)-4′-methoxycyclopenin formation uses a high-spin Fe(IV)-oxo intermediate to carry out epoxidation. Furthermore, product analysis on $^{16}O/^{18}O$ isotope incorporation from the reactions using the native substrate, 4′-methoxydehydrocyclopeptin, and a mechanistic probe, dehydrocyclopeptin, reveals evidence supporting oxo↔hydroxo tautomerism of the Fe(IV)-oxo species in the non-heme iron enzyme catalysis.

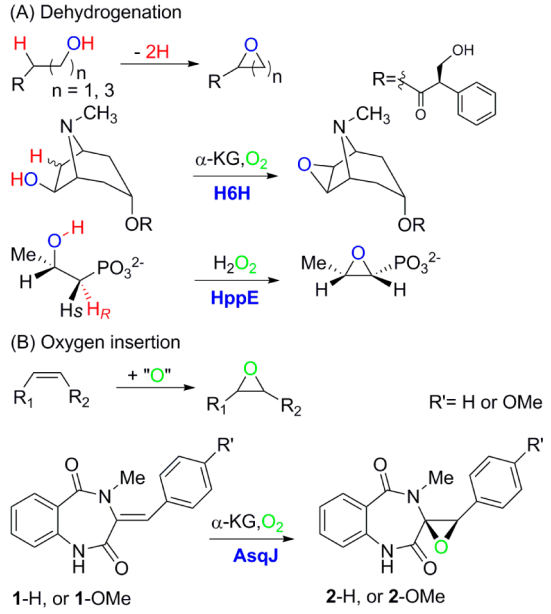
The biological activities of natural products are often conveyed by structural modifications involving heteroatoms. Among various modifications, the epoxide moiety, with a strained C−O−C three-membered ring structure, is widely distributed.[1] In nature, an epoxide, also called an oxirane, is typically installed via oxidative approaches by highly reactive intermediates that are derived from different cofactors such as flavin,[1] thiolate-heme,[2e,f] or non-heme iron.[1,2a−d] The majority of epoxide formations require molecular oxygen (O_2), which is used as an oxygen source for the epoxide, an oxidant, or both.[1,2] In some cases, instead of O_2, H_2O_2 can be utilized.[3]

Non-heme iron-dependent epoxidases have been reported in various biosynthetic pathways, e.g., H6H (hyoscyamine 6β-hydroxylase) in scopolamine,[4] HppE (2-hydroxyl propyl phosphonate epoxidase) in fosfomycin,[5] DdaC in $N_β$-epoxysuccinamoyl-DAP-Val,[6] PenD (PntD) in pentalenolactone,[7] and the recently discovered AsqJ in quinolone alkaloid biosyntheses.[8]

Two fundamentally different approaches to construct an epoxide group are utilized by non-heme iron enzymes. First, in HppE and H6H,[3d,9] despite using different oxidants (O_2 in H6H and H_2O_2 in HppE), the epoxide formation is a formal dehydrogenation process (−2H) where consecutive cleavages of C−H and O−H bonds occur (Scheme 1A). Second, in DdaC, PenD, and AsqJ,[6−8] the reactions proceed through an oxygen atom insertion into a double bond moiety of the substrate (Scheme 1B). In the first case, the dehydrogenation mechanism has been characterized in HppE, where a ferryl (Fe(IV)-oxo) species is proposed to trigger unactivated C−H bond cleavage to initiate the reaction.[3d,10] In the second case, although an epoxide intermediate has been suggested and observed when a mechanistic probe was used in phenylalanine hydroxylase, and an Fe(IV)-oxo species has been established in its native hydroxylation reaction,[11] no direct mechanistic investigation on epoxidation has been reported. Herein, we provide experimental evidence for a plausible oxygen insertion reaction mechanism in the formation of (−)-4′-methoxycyclopenin (**2**-OMe) and (−)-cyclopenin (**2**-H) catalyzed by AsqJ, using 4′-methoxydehydrocyclopeptin (**1**-OMe) and dehydrocyclopeptin (**1**-H) as substrates (Scheme 1B). In addition to the epoxidase activity, AsqJ also catalyzes a desaturation reaction.[8] AsqJ was discovered by Ishikawa et al.[8] and structurally characterized by Bräuer et al.[12]

AsqJ belongs to the α-keto-glutarate (α-KG)-dependent iron (Fe/α-KG) enzyme family, a subclass of non-heme iron

Scheme 1. Examples of Epoxidation Catalyzed by Non-Heme Iron Enzymes via (A) Dehydrogenation and (B) Oxygen Insertion



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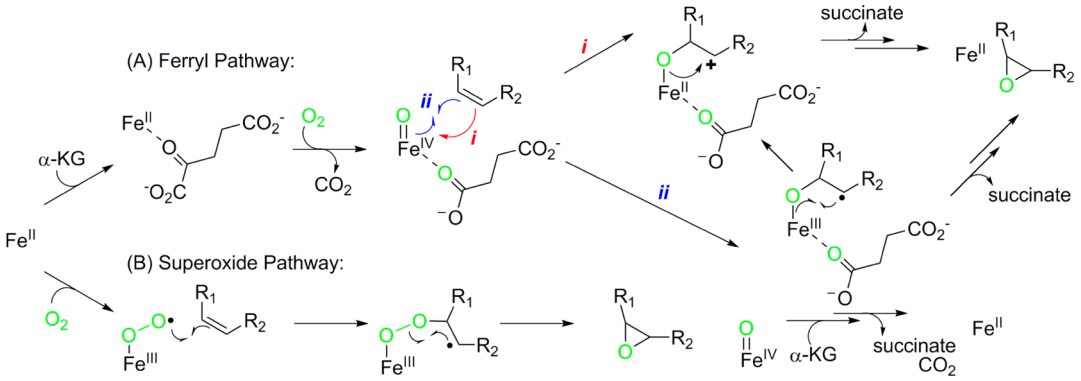
Scheme 2. Mechanistic Consideration of AsqJ-Catalyzed Epoxidation[a]



[a](A) In the ferryl pathway, an Fe(IV)-oxo species is used to react with a double bond followed by (i) carbocation intermediate or (ii) substrate radical formation. (B) In the superoxide pathway, an Fe(III)-superoxide is used as the reactive intermediate to trigger the oxygen insertion; subsequently, the resulting Fe(IV)-oxo species is reduced to Fe(II) by an α-KG.

enzymes that use α-KG and O_2 as co-substrates to catalyze a broad array of transformations, including hydroxylation, halogenation, desaturation, stereoinversion, endoperoxide formation, and epoxidation.[13,14] For all the reactions involving a C−H bond activation step, a reactive Fe(IV)-oxo species has been demonstrated as the key intermediate.[15] However, oxygen addition to a C═C double bond does not involve C−H bond activation. Thus, reaction mechanism(s) may differ from the canonical pathway, and different reactive species may be involved. Herein, two mechanistic possibilities are considered (Scheme 2). First, in a ferryl pathway, an Fe(IV)-oxo species is proposed to trigger the C_{sp^2}−C_{sp^2} bond cleavage. Based on the nature of the resulting species, a mechanistic branching point can be envisioned. On the one hand, a substrate carbocation intermediate can be generated through a polar mechanism, which then undergoes epoxide formation (Scheme 2A, pathway *i*). On the other hand, a radical mechanism can be utilized to generate a carbon-centered radical species, followed by the C−O bond formation to form an epoxide (Scheme 2A, pathway *ii*). Alternatively, a carbocation species can also be produced via an electron-transfer step from the substrate radical to the Fe(III) species. Second, owing to the highly reactive nature of C═C bonds, an Fe(III)-superoxide pathway also needs to be considered. Due to the relatively weak electrophilicity of the Fe(III)-superoxo species, the reaction is likely to proceed through a radical mechanism (Scheme 2B). In this pathway, α-KG plays the role of a scavenger for "quenching" the Fe(IV)-oxo species formed after the epoxidation step, rather than a co-substrate used to generate Fe(IV)-oxo species.[16] Although both the superoxo pathway and the Fe(IV)-oxo species reduction by an α-KG have no literature precedent in the Fe/α-KG enzymes, in other non-heme iron enzymes, depending on the nature of the reactions, utilization of Fe(III)-superoxide has been suggested in, e.g., hydroxyl-ethylphosphonate dioxygenase[17] and ethylphosphonate synthase.[18,19]

To distinguish among the mechanistic possibilities, we synthesized the substrate **1**-OMe and a mechanistic probe **1**-H according to known methods[20] with minor modification as described in the Supporting Information (SI). *Aspergillus nidulans* AsqJ (AsqJ) was obtained by heterologous overexpression in *Escherichia coli* and purified as N-His_6-tagged fusion (SI). We first carried out a stopped-flow optical absorption (SF-Abs) experiment by rapidly mixing an anaerobic solution containing the AsqJ·Fe(II)·α-KG complex with O_2-saturated buffer in the presence or absence of substrates (**1**-OMe or **1**-H). An optical absorption feature centered at ∼470 nm, assigned as a metal-to-ligand charge-transfer (MLCT) band between Fe(II) and α-KG,[21,22] decays rapidly to a minimum at a reaction time of ∼1 s in the presence of either **1**-OMe or **1**-H. In the absence of substrate, no obvious decay of this feature was detected (Figure 1). This activation effect

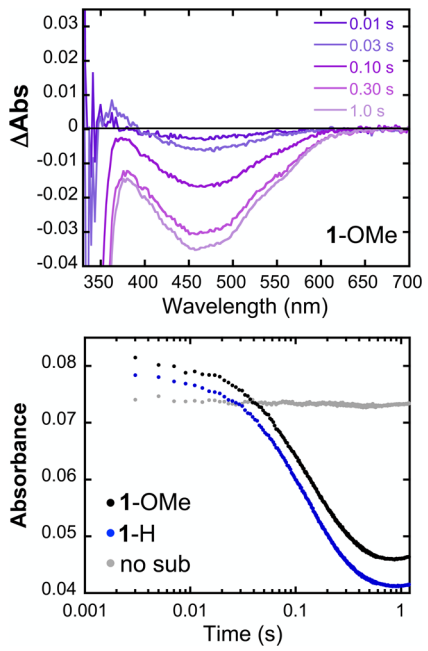


Figure 1. SF-Abs kinetics of the decay of Fe(II)-α-KG MLCT band. Top: Change centered at 470 nm at the indicated reaction times after mixing the AsqJ·Fe(II)·α-KG·**1**-OMe complex with O_2. Bottom: Kinetics of the absorbance at 470 nm in the reactions of the AsqJ·Fe(II)·α-KG complex and O_2 with substrate (**1**-OMe, black; or **1**-H, blue) or without substrate (no sub, gray).

caused by substrate ("substrate triggering") is a common theme in the Fe/α-KG enzymes and other Fe/oxygenases.[14,15] Furthermore, the similar "substrate triggering" effect observed in the presence of **1**-H or **1**-OMe suggests that **1**-H and **1**-OMe have similar efficacy in triggering α-KG consumption. In other characterized Fe/α-KG enzymes, a transient 318 nm absorption feature has been observed and assigned as originating from the

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Fe(IV)-oxo intermediate.[16] However, such a feature could not be deconvoluted here due to the strong absorptions of **1**-OMe and **1**-H in the similar near-UV region.

To test whether an Fe(IV)-oxo species is also involved in the AsqJ-catalyzed epoxidation, we carried out a freeze-quench (FQ) experiment by rapidly mixing the anaerobic AsqJ·Fe(II)·α-KG·**1**-OMe (or **1**-H) complex with O_2-saturated buffer and quenching the reaction at various time points. At 0.01 s, the shortest time that can be achieved on our apparatus, a new quadrupole doublet is developed to ∼22% and ∼31% of the total iron in the sample for substrates **1**-OMe and **1**-H, respectively (Figures 2 and S6). This doublet has parameters

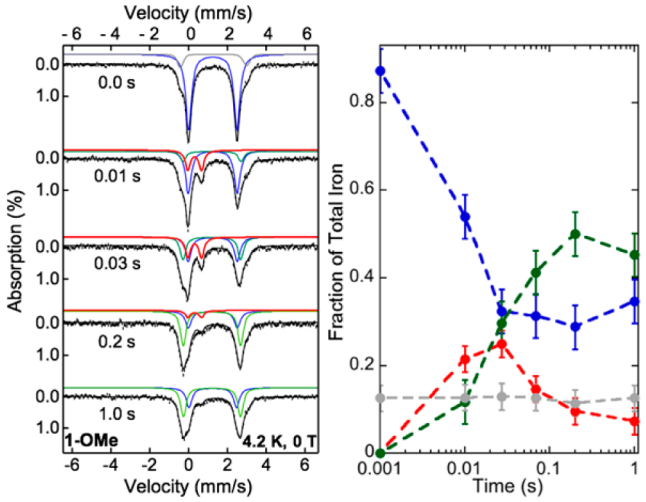

Figure 2. Left: 4.2 K zero-field Mössbauer spectra of the reaction of the AsqJ·^{57}Fe(II)·α-KG·**1**-OMe complex with O_2 at different times. Right: Iron speciation determined by the spectral simulations. Black vertical bars mark experimental spectra; black line, overall spectral simulations (parameters are listed in Table S1); blue line, AsqJ·^{57}Fe(II)·α-KG·**1**-OMe complex; gray line, inactive enzyme; red line, Fe(IV)-oxo intermediate; green line, Fe(II) product complex.

typical of a high-spin Fe(IV)-oxo species (δ = 0.31 mm/s, $|\Delta E_Q|$ = 0.68 mm/s) (Table S2).[15] In the case of **1**-H, this Fe(IV)-oxo species decays to 26%, 19%, and 8% at 0.03, 0.07, and 0.2 s, and further decays to ≤5% at 1 s (Figure S6). In contrast, in the case of **1**-OMe, the same Fe(IV)-oxo species increases slightly to ∼25% at 0.03 s and then decays to 15%, 10%, and ≤6% at 0.07, 0.2, and 1 s, respectively (Figure 2). Nonetheless, for both substrates, the decay of the Fe(IV)-oxo intermediate is followed by the formation of a new Fe(II) species (δ = 1.20 mm/s, $|\Delta E_Q|$ = 2.95 mm/s), which may originate from the enzyme−product complex and is clearly distinguishable from the AsqJ·Fe(II)·α-KG·substrate complex (δ = 1.24 mm/s, $|\Delta E_Q|$ = 2.52 mm/s). There is another minority Fe(II) species shown in the anaerobic control sample (δ = 1.29 mm/s, $|\Delta E_Q|$ = 3.46 mm/s), representing ∼12% of the total iron. Based on its steady presence throughout the reaction, this Fe(II) species can be attributed to the "inactive" form of AsqJ (Figures 2 and S6).

The observation of an Fe(IV)-oxo species in the reactions of AsqJ with both **1**-OMe and **1**-H indicates that such a reactive intermediate is also operative in the AsqJ epoxidation. Although literature suggests no obvious difference between the reactions using either **1**-OMe or **1**-H under multiple turnover conditions,[12] the observed earlier onset and higher accumulation of such a species in the reaction of **1**-H clearly suggests that *para* substitution of the substrate (OMe vs -H) results in the perturbation of the overall kinetics of the Fe(IV)-oxo species.

To demonstrate that the observed Fe(IV)-oxo species is the committed intermediate for the AsqJ epoxidation, we carried out a chemical-quench experiment. Liquid chromatography−mass spectrometry (LC-MS) analysis of the reaction sample quenched at 0.05 s after the mixing of the AsqJ·Fe(II)·α-KG·**1**-OMe complex with O_2-saturated buffer revealed the formation of a peak at 4.2 min elution time corresponding to the product peak (**2**-OMe) with a mass/charge ratio (m/z) of 325.1. The same peak with lower intensity (∼30% peak area) was detected at an earlier quench time, 0.01 s, when the Fe(IV)-oxo was still in its formation phase, as shown in the Mössbauer results. As expected, the chromatogram of a reaction mixture sample without exposure to O_2 contains no desired product (Figure 3A, bottom trace). This result implies that the Fe(IV)-oxo

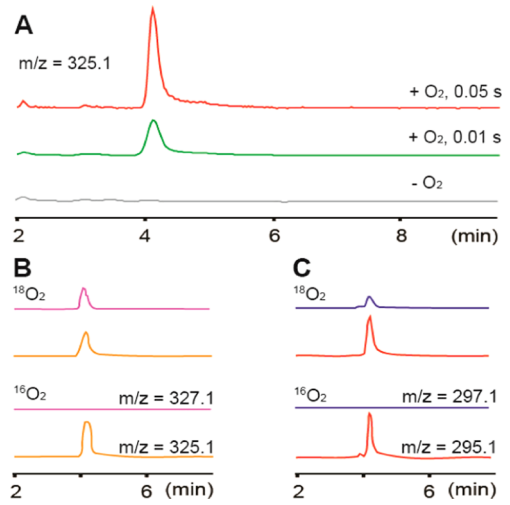

Figure 3. LC-MS chromatogram of the AsqJ-catalyzed reactions. (A) Chemical quench of the reaction of AsqJ·Fe(II)·α-KG·**1**-OMe complex with O_2-saturated buffer at various time points. (B,C) $^{16}O_2$ and $^{18}O_2$ treatment of the AsqJ reactions with **1**-OMe and **1**-H, respectively.

species is generated prior to the epoxide formation and used to react with the C=C bond, which is consistent with the ferryl pathway in Scheme 2A. Next, parallel experiments were carried out by exposing the reaction mixture to $^{16}O_2$ and $^{18}O_2$, separately. Under $^{18}O_2$ environment, formation of a product peak is observed, which has a retention time identical to that of the product peak in the reaction with $^{16}O_2$, but with m/z shifted from 325.1 to 327.1 (Figure 3B, purple trace). This observation suggests that the epoxide oxygen is derived from molecular oxygen. In addition, a peak with m/z 325.1 was also detected. A significant amount of ^{16}O incorporation under $^{18}O_2$ environment—an $^{18}O/^{16}O$ ratio of ∼0.79 ± 0.03—could be attributed to $^{16}O_2$ contamination during the experiment. However, it may also result from an "oxygen exchange" event at or prior to the Fe(IV)-oxo formation. If the latter scenario operates, we anticipate a greater level of $^{18}O/^{16}O$ exchange in the reaction using **1**-H in place of **1**-OMe, as suggested by Mössbauer analysis, which shows that the overall accumulation of the Fe(IV)-oxo species is higher when **1**-H is used instead of **1**-OMe. Indeed, the chromatogram of the reaction using **1**-H under an $^{18}O_2$ environment reveals an $^{18}O/^{16}O$ (297.1/295.1)

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ratio of ~0.21 ± 0.01, indicating ~3−4-fold less ^{18}O incorporation than in the reaction using **1**-OMe. Furthermore, a competition experiment was conducted, where equal amounts of **1**-H and **1**-OMe were incubated with AsqJ and then exposed to $^{18}O_2$. A similar result was detected, in which **2**-H has ~3−4-fold less ^{18}O incorporation than **2**-OMe. Thus, FQ-Mössbauer and LC-MS results suggest that the oxygen atom exchange most likely happens on the Fe(IV)-oxo species.

In summary, this study reveals that an Fe(IV)-oxo species is the key intermediate responsible for the epoxide formation in the AsqJ-catalyzed epoxidation. Our results also suggest that *para* substitution on the substrate (-OMe vs -H) affects the kinetics of the Fe(IV)-oxo intermediate. By using pre-steady-state kinetics coupled with spectroscopic analysis and $^{16}O_2/^{18}O_2$ isotopic substitution experiment, our study provides direct experimental evidence to support a fast oxo↔hydroxo tautomerism of the Fe(IV)-oxo species, which has been indicated in heme and non-heme model complexes and enzyme systems.[23,24]

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b05400.

Experimental details and data, including Figures S1−S6 and Tables S1 and S2 (PDF)

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Notes

The authors declare no competing financial interest.

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