Communication

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

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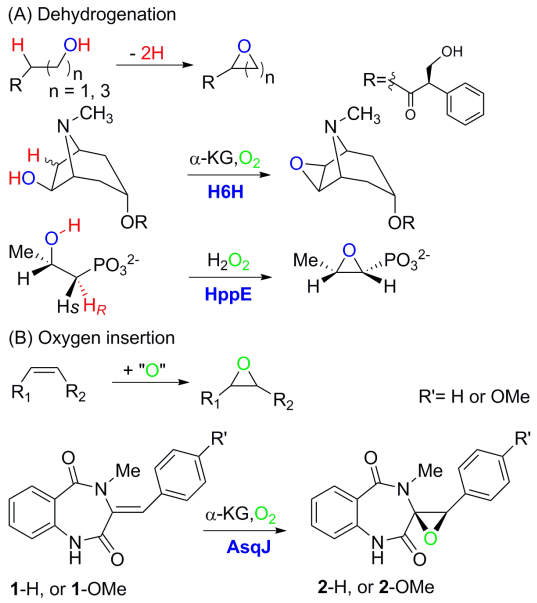
Supporting Information Placeholder

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 not examined in detail. Using a combination of methods including transient kinetics, Mössbauer spectroscopy and mass spectrometry, we demonstrate that AsqJ catalyzed (-)-4'methoxy-cyclopenin 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'-methoxy-dehydrocyclopeptin, 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, a.k.a. oxirane, is typically installed via oxidative approaches by highly reactive intermediates that are derived from different cofactors such as flavin,[1] thiolate-heme,[2e, 2f] or non-heme iron.[1, 2a-2d] The majority of epoxide formations require molecular oxygen (O_2), which is used as an oxygen source of the epoxide, an oxidant, or both.[1-2] In some cases, instead of O_2, H_2O_2 can also 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_ε-epoxysuccinamoyl-DAP-Val,[6] PenD (PntD) in pentalenolactone,[7] and the recently discovered AsqJ in quinolone alkaloid biosyntheses.[8]

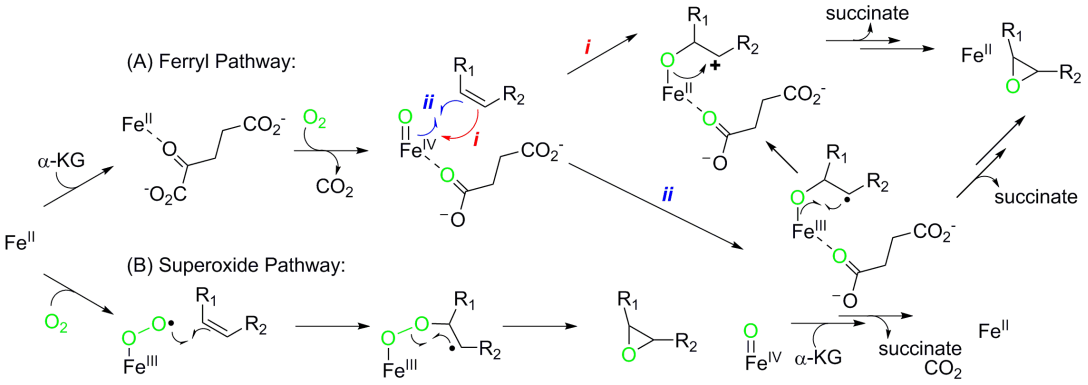
Two fundamentally different approaches in constructing epoxide group are utilized by non-heme iron enzymes. First, in HppE and H6H,[3d, 9] despite using different oxidants (O_2 in H6H, or H_2O_2 in HppE), the epoxide formation is a formal dehydrogenation process (-2H) where consecutive cleavages of C-H 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'-methoxy-cyclopenin (**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]



Scheme 1 Examples of epoxidation catalyzed by non-heme iron enzymes via (A) dehydrogenation; and (B) oxygen insertion (+O).

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

iron enzymes that uses α-KG and O_2 as co-substrates to catalyze a broad array of transformations, including hydroxylation, halogenation, desaturation, stereoinversion, endoperoxide formation and expoxidation.[13,14] For all 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). Firstly, in a ferryl pathway, an Fe(IV)-oxo species is proposed to trigger the C_{sp2}-C_{sp2} 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. Secondly, owing to the highly reactive nature of C=C bonds, a 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 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 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, e.g. hydroxyl-ethylphosphonate dioxygenase[17] and ethylphosphonate synthase.[18, 19]



Scheme 2. Mechanistic consideration of AsqJ catalyzed epoxidation: (A) ferryl pathway where an Fe(IV)-oxo species is used to react with a double bond followed by (i) carbocation intermediate or (ii) substrate radical formation; (B) superoxide pathway where an Fe(III)-superoxide is used as the reactive intermediate to trigger the oxygen insertion. Subsequently, the resulting Fe(IV)-oxo species is then reduced to Fe(II) by an α-KG.

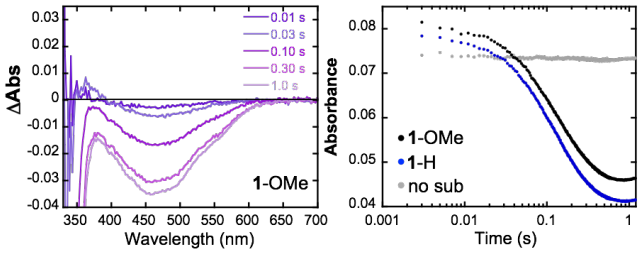


Figure 1. SF-Abs kinetics of the decay of Fe(II)-α-KG MLCT band. Left Panel: Change centered at 470 nm at the indicated reaction times after mixing the AsqJ•Fe(II)•α-KG•**1**-OMe complex with O_2; Right Panel: Kinetics of the absorbance at 470 nm in the reactions of the AsqJ•Fe(II)•α-KG• complex and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

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) is obtained by heterologous overexpression in *E.coli* and purified as N-His_6-tagged fusion (SI). We first carried out a stopped-flow optical absorption (SF-Abs) experiment by rapid mixing anaerobic AsqJ•Fe(II)•α-KG solution with O_2-saturated buffer in the presence or the 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 caused by substrate ("substrate triggering") is a common theme in Fe/α-KG enzymes and other Fe/oxygenases.[14,15] Furthermore, the similar "substrate triggering" effect observed in the presence of **1**-H and **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 originated from a Fe(IV)-oxo intermediate.[16] However, such a feature cannot be de-convoluted here due to the strong absorptions of **1**-OMe and **1**-H in the similar near UV region.

To test whether a Fe(IV)-oxo species is also involved in the AsqJ catalyzed epoxidation, we carried out a freeze quench (FQ) experiment by rapid mixing anaerobic AsqJ•Fe(II)•α-KG•**1**-OMe (or **1**-H) with O_2-saturated buff-

er, 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 (Figure 2 and S6). This doublet has parameters 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%, 8% at 0.03 s, 0.07 s 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 s, 0.2 s 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 be originated 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 (Figure 2 and S6).

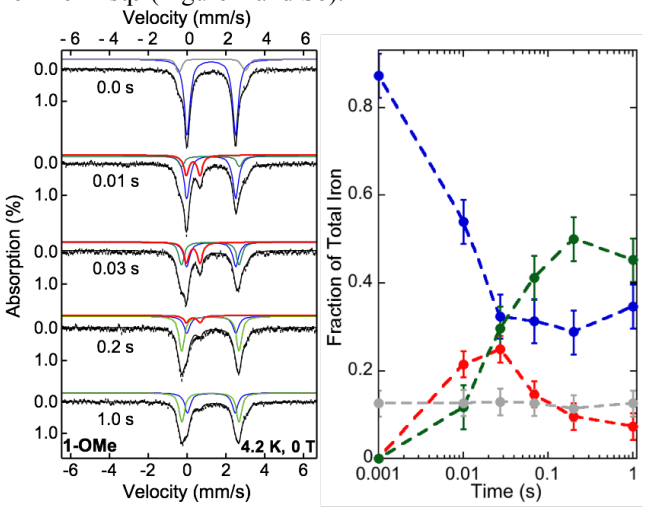


Figure 2. 4.2 K zero field Mössbauer spectra of the reaction of AsqJ•^{57}Fe(II)•α-KG•**1**-OMe complex with O_2 at different times (left panel) and the iron speciation determined by the spectral simulations (right panel). Black vertical bars: experimental spectra; Black line: overall spectral simulations (parameters are listed in Table S1); Blue line: AsqJ•^{57}Fe(II)•α-KG• **1**-OMe complex; Grey line: inactive enzyme; Red line: Fe(IV)-oxo intermediate; Green line: Fe(II) product complex.

The observation of a 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 condition,[12] the observed earlier onset and higher accumulation of such a species in the reaction of **1**-H clearly suggests the *para* substitution of the substrate (OMe vs. H) results in the perturbation of the overall kinetics of the Fe(IV)-oxo species.

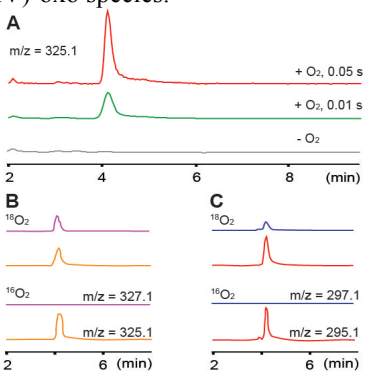


Figure 3. LC-MS chromatogram of the AsqJ catalyzed reactions. (A) Chemical quench of AsqJ•Fe(II)•α-KG•**1**-OMe reaction with O_2-saturated buffer at various time points. (B) and (C) $^{16}O_2$ and $^{18}O_2$ treatment of the AsqJ reactions with **1**-OMe and **1**-H, respectively.

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 AsqJ•Fe(II)•α-KG•**1**-OMe complex with O_2-saturated buffer reveals the formation of a peak at 4.2 min elution time corresponding to the product peak (**2**-OMe) with mass/charge (*m/z*) = 325.1. The same peak with less intensity (of ~30% peak area) was detected at an earlier quench time, 0.01 s, when the Fe(IV)-oxo is 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 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 the identical retention time as 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, a $^{18}O/^{16}O$ ratio of ~ 0.79±0.03, could be attributed to $^{16}O_2$ contamination during the experiment. On the other hand, 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 $^{18}O_2$ environment reveals an $^{18}O/^{16}O$ (297.1/295.1) 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 an equal amount of **1**-H and **1**-OMe were incubated with AsqJ and then exposed to $^{18}O_2$. 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 the *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 $^{16}O_2/^{18}O_2$ 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/non-heme model complexes and enzyme systems.[23,24]

ASSOCIATED CONTENT

Supporting Information.

This material is available free of charge on the ACS Publications website at http:// pubs.acs.org.

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Notes

The authors declare no competing financial interests.

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