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α -Amine Desaturation of D-Arginine by the Iron(II)- and 2-(Oxo)glutarate-Dependent L-Arginine 3-Hydroxylase, VioC

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Abstract

When challenged with substrate analogues, iron(II)- and 2-(oxo)glutarate-dependent (Fe/2OG) oxygenases can promote transformations different from those they enact upon their native substrates. We show here that the Fe/2OG enzyme, VioC, which is natively an L-arginine 3-hydroxylase, catalyzes an efficient oxidative deamination of its substrate enantiomer, D-Arg. The reactant complex with D-Arg retains all interactions between enzyme and substrate functional groups, but the required structural adjustments and opposite configuration of C2 position this carbon more optimally than C3 to donate hydrogen (H[•]) to the ferryl intermediate. The simplest possible mechanism, C2 hydroxylation followed by elimination of ammonia, is inconsistent with the demonstrated solvent origin of the ketone oxygen in the product. Rather, the reaction proceeds via a hydrolytically labile C2-iminium intermediate, demonstrated by its reductive trapping in solution with NaB²H₄ to produce racemic [²H]Arg. Of two alternative pathways to the iminium species, C2 hydroxylation followed by dehydration versus direct desaturation, the latter possibility appears to be more likely, because the former mechanism would be expected to result in detectable incorporation of ¹⁸O from ¹⁸O₂. The direct desaturation of a C–N bond implied by this analysis is analogous to that recently posited for the reaction of the L-Arg 4,5-desaturase, NapI, thus lending credence to the prior mechanistic proposal. Such a pathway could also potentially be operant in a subset of reactions catalyzed by Fe/2OG N-demethylases, which have instead been purported to enact C–N bond cleavage by methyl hydroxylation and elimination of formaldehyde.

Graphical Abstract

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Supporting Information

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Notes

The authors declare no competing financial interest.

The 20 standard amino acids serve as important building blocks in biology, acting as both the constituents of macromolecular protein polymers and the platforms for synthesis of small-molecule metabolites. In either role, specific modifications by enzymes provide additional biochemical diversity critical for function.¹ The iron(II)- and 2-(oxo)-glutarate-dependent (Fe/2OG) oxygenase superfamily is responsible for a large fraction of the known oxidative amino acid modifications.² In eukaryotes, amino acid-targeting Fe/2OG enzymes hydroxylate side chain functional groups and operate predominantly on protein or peptide substrates for structural or regulatory functions.^{3,4} In prokaryotes, Fe/2OG catalysts can additionally target monomeric amino acids, and many different reaction outcomes are possible.⁵ A classic example is clavaminase synthase (CAS), which performs sequential hydroxylation, cyclization, and desaturation reactions on an L-arginine (L-Arg) derivative in the biosynthesis of the β -lactamase inhibitor, clavulanic acid.⁶ Fe/2OG enzymes that utilize amino acid substrates often exhibit considerable variability in regiochemistry, as well. For example, the enzymes VioC, NapI, and OrfP all act on L-Arg but, despite sharing significant sequence similarity (~50%), target different positions of the amino acid side chain for the biosynthesis of distinct natural products: VioC is a 3-hydroxylase in the viomycin pathway,^{7,8} OrfP is a 3,4-dihydroxylase in streptothricin pathways,⁹ and NapI is a 4,5-desaturase in the naphthyridinomycin pathway.^{10,11} Examples of Fe/2OG enzymes that transform many other amino acids are known, but to date, they all target the side chain of the proteinogenic L-enantiomer. Non-native reactivity with the opposite (D) enantiomer has not, to the best of our knowledge, been investigated.

Most Fe/2OG enzymes share a HXD/EX_nH Fe(II) binding motif (known as the facial triad^{12,13}) and initial steps of catalysis, regardless of their substrate or reaction type.¹⁴ Binding of the primary substrate triggers dissociation of the remaining water ligand¹⁵ and addition of O₂ to the open coordination site of the Fe(II) cofactor.¹⁶ The noncoordinating O atom of the resulting Fe(III)–superoxo intermediate is transferred (in multiple steps) to C2 of the 2OG substrate, converting it to CO₂ and succinate, while the coordinated O atom becomes the oxo ligand of the substrate-targeting Fe(IV)–oxo (ferryl) intermediate. In transformations of aliphatic sites, the ferryl complex abstracts hydrogen (H[•]) from the substrate. Its potency typically enables activation of even very inert carbon centers. The resultant state, containing a carbon-centered substrate radical (C[•]) and Fe(III)–OH cofactor, is an important branch point: how it reacts in ensuing steps dictates the reaction outcome. In hydroxylations, the C[•] attacks the oxygen of the Fe(III)–OH complex, forming a new C–O bond and regenerating the Fe(II) cofactor for subsequent turnover. This radical-coupling step, termed oxygen rebound,¹⁷ is thought to have a low activation barrier, consistent with the failure of the C[•]/Fe(III)–OH state to have been detected in transient kinetic analyses of Fe/2OG hydroxylases.

In reaction outcomes other than hydroxylation, the C[•]/Fe(III)–OH state often has a different fate. Oxygen rebound can be almost completely suppressed, likely enabled in at least some enzymes by a different geometric structure of the ferryl complex. For example, in the Fe/2OG aliphatic halogenases, a cis-coordinated chloride or bromide ligand is transferred to the substrate radical in preference to oxygen rebound.^{18–22} For the chlorinases SyrB2 and WelO5 from the syringomycin and welwitindolinone biosynthetic pathways, respectively, it is thought that the alternative radical-coupling step is enforced by an unusual disposition of the C–H and Fe=O bonds, achieved by an ~90° relocation of the oxo ligand to an off-line position in the key ferryl complex.^{20,23–26} Such ligand reorganization could be a common strategy for suppression of rebound in Fe/2OG enzymes with other noncanonical (nonhydroxylation) reactivities.

Outcomes other than hydroxylation may also arise by pathways in which the facile rebound step does occur but the hydroxylated species then undergoes further processing. For example, a number of prokaryotic and eukaryotic Fe/2OG *N*-demethylases implicated in gene regulation and DNA repair (AlkB, ALKBH1–8, FTO, and histone demethylases) are proposed to exploit initial hydroxylation followed by fragmentation of the unstable hemiaminal intermediate to the corresponding amine and formaldehyde (Scheme 1A).^{27–32} Similarly, mechanisms involving transient C–O bond formation have been considered for a range of desaturation reactions by Fe/2OG enzymes. In these systems, the installed hydroxyl group would subsequently be jettisoned, either in an α -heteroatom-assisted dehydration (Scheme 1B) or as nucleofuge in a Grob-type fragmentation (Scheme 1C), with formation of a new C=X (X = N or O) or C=C bond, respectively.^{33–35} Although spontaneous breakdown of the hydroxylated product is certainly plausible in the demethylase reactions, enzymatic assistance after hydroxylation would be required in the desaturation reactions.

The aforementioned Fe/2OG L-Arg 3-hydroxylase, VioC, has recently been used as a platform for crystallographic characterization of reaction cycle intermediates and their stable mimics.³⁶ The enzyme can also accept alternative substrates and, in one case, was shown to promote a non-native outcome with such an analogue. Reaction of VioC with L-homoarginine (L-hArg, the L-Arg analogue with an additional methylene unit in its side chain) resulted in a 3,4-desaturation outcome in competition with hydroxylation of either site.³³ Comparative mechanistic analysis of this reaction and the native 4,5-desaturation of L-Arg by NapI showed that they proceed by different mechanisms. Whereas NapI mediates transient C5–N6 desaturation and C4–H cleavage by deprotonation (Scheme S1A), the absence of a heteroatom in the 3,4-desaturation of L-hArg by VioC necessitates a less selective mechanism involving sequential HAT steps [to the ferryl and resultant Fe(III)–OH intermediates] (Scheme S1B). Here, we report discovery and analysis of a second non-native desaturation by VioC in our ongoing efforts to map and rationalize the enzyme family's full range of catalytic capabilities. Specifically, we show that the native 3-hydroxylase readily binds and transforms the D-enantiomer of its native L-Arg substrate to the corresponding 2-ketoacid product in an oxidative deamination reaction (Scheme 2). The simplest possible adaptation of the native reaction mechanism that would rationalize the alternative outcome, C2 hydroxylation and subsequent deamination, would be reminiscent of the mechanism purported for the Fe/2OG *N*-demethylases. However, the results of in-depth analysis of the reaction by isotope-tracer and chemical-trapping experiments show that the reaction actually

proceeds through an iminium intermediate that subsequently undergoes hydrolysis. The oxidative deamination of D-Arg thus appears to proceed by α -amine desaturation, a pathway that is directly analogous to the initial steps in the proposed mechanism of olefin installation by NapI.³³

EXPERIMENTAL DETAILS

General Methods.

Liquid chromatography–mass spectrometry (LC–MS) experiments were performed on an Agilent 1260 series LC system interfaced with an Agilent 6460 triple-quadrupole mass spectrometer. All reagents were used directly as obtained from the commercial sources. The relative molecular mass and purity of enzyme samples were determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Crystallographic (Table S1) and spectroscopic methods are described in the Supporting Information.

Reactions of VioC with D-Arginine.

Assays were prepared in a Lab Master 100 anoxic chamber (MBraun) in a total volume of 200 μ L in 100 mM Tris-HCl buffer (pH 7.5) (reaction buffer). The final concentrations were 10 μ M VioC, 10 μ M (NH₄)₂Fe(SO₄)₂, 0.8 mM 2OG, and 1 mM D-arginine. Concentrated reaction mixtures were removed from the anoxic chamber, and the reaction was initiated by diluting to the final volume with cold air-saturated reaction buffer. Tubes were then opened to air, and their contents were stirred gently; the reactions were allowed to proceed for ~1 h. A small quantity of NaBH₄ (<10 mg) was added to the appropriate samples. To Fmoc-derivatize the products, 200 μ L of 7.5 mM Fmoc-OSu in CH₃CN was added to each reaction mixture, and the resultant solution was allowed to stir at room temperature for 1 h. The solutions were then filtered through 10K molecular weight cutoff centrifugal devices (Pall Corp.) prior to LC–MS analysis.

High-Performance Liquid Chromatography (HPLC) Analysis of VioC Reaction Products with D-Arginine and L-arginine.

Assay mixtures were injected onto an Agilent Zorbax Extend-C18 column (4.6 mm $\times$ 50 mm, 1.8 μ m particle size) in 100% solvent A [25 mM ammonium formate (pH 9)] and 0% solvent B (CH₃CN). The column was developed at a flow rate of 0.5 mL/min. A gradient from 100 to 50% solvent A was applied from 3 to 5 min, followed by 50% solvent A from 5 to 9 min. Another gradient from 50 to 100% solvent A was applied from 9 to 12 min, followed by a wash with 100% solvent A from 12 to 14 min. Arginine-derived products were detected using electrospray ionization in positive mode (ESI⁺).

HPLC Analysis of Succinate.

Succinate was resolved on the same reverse-phase column noted above. The flow rate was 0.5 mL/min. The column was washed with 100% solvent A (0.1% formic acid) and 0% solvent B (CH₃CN) before use. After injection of the sample, a gradient from 100 to 95% solvent A was applied from 0 to 6 min followed by a gradient from 95 to 40% solvent A from 6 to 8 min. From 8 to 10 min, the column was returned back to 100% solvent A, and

from 10 to 12 min, it was washed with solvent A. Succinate was detected by electrospray ionization in negative mode (ESI⁻).

¹⁸O Incorporation Assays.

To analyze the rate of exchange of the ketone carbonyl oxygen of 2O5GP with solvent, reaction mixtures were prepared anoxically, as described above. The final concentrations were 250 μ M VioC, 200 μ M (NH₄)₂Fe(SO₄)₂, 500 μ M 2OG, and 1 mM D-arginine. Reactions were initiated by diluting to the final volume (50 μ L) with air-saturated reaction buffer. Solutions were incubated for ~1 h before 50 μ L of 7.5 mM Fmoc-OSu in CH₃CN was added to terminate the reaction and derivatize any unreacted D-arginine. To initiate ¹⁸O exchange, 50 μ L of H₂¹⁸O was added, and the reaction was then quenched by addition of NaBH₄ after incubation for 2, 5, 10, 30, 60, and 240 min. The extents of ¹⁸O incorporation were measured by the LC-MS method described above. The theoretical maximum value of ¹⁸O incorporation is 50% (only half of the mixture is H₂¹⁸O); thus, the experimentally observed fractions of ¹⁸O in the product were multiplied by a factor of 2. The values (Table S2) plotted versus time were fit by the equation for an exponential rise (eq 1)

$$\Delta E(t) = E_0 + \Delta E_1 \left(1 - e^{-k_{\text{exc}} t}\right) \quad (1)$$

in which E_0 is the initial fraction of ¹⁸O, E_1 is the amplitude of exchange, and k_{exc} is the observed first-order rate constant for solvent exchange.

To quantify isotope incorporation in assays performed in ¹⁸O₂-charged buffer, reaction mixtures were prepared anoxically, as described above for the ¹⁸O solvent exchange control assays. The reaction-initiating ¹⁸O₂-charged reaction buffer was prepared on a vacuum line by successive 10 min purge and 10 min pump cycles with argon gas for a total of three cycles each. After the last vacuum cycle, the buffer was charged with ¹⁸O₂ gas and allowed to reach equilibrium on ice for 10 min. The reactions were initiated in the anoxic chamber by diluting to volume with the ¹⁸O₂-charged buffer and sealing the tubes. The reactions were quenched with Fmoc-OSu and NaBH₄ at the same time points used in the ¹⁸O exchange assays. The extent of ¹⁸O incorporated into the product was measured by the LC-MS analysis method described above. The oxygen isotopic composition of ¹⁸O₂ in each reaction sample was determined by measurement of the incorporation of ¹⁸O into succinate, which will not wash out in H₂¹⁶O buffer. This fraction was assumed to represent the maximum possible fraction of ¹⁸O incorporated into the product. The ¹⁸O₂-charged buffer assays using the ²H₂O solvent were performed identically but with all reagents prepared in ²H₂O reaction buffer.

For the H₂¹⁸O buffer assays, reaction mixtures were assembled anoxically as for the ¹⁸O exchange control assays. The reaction-initiating H₂¹⁸O buffer was prepared by diluting a 2 M Tris-HCl (pH 7.5) stock to 100 mM with H₂¹⁸O. Reaction mixtures were removed from the anoxic chamber, diluted to the final volume with H₂¹⁸O buffer, and quenched with Fmoc-OSu and NaBH₄ for analysis, as described above. Because the H₂¹⁸O buffer was diluted with 2 M Tris-HCl in H₂¹⁶O and the concentrated reaction mixtures were prepared

with H₂¹⁶O and 100 mM Tris-HCl, the maximum possible fraction of ¹⁸O incorporated into the product is 76%. The experimental values were corrected by dividing by 0.76.

In ¹⁸O₂ L-arginine control assays, reaction mixtures were prepared anoxically to give final concentrations of 250 μM VioC, 200 μM (NH₄)₂Fe(SO₄)₂, 500 μM 2OG, and 1 mM L-arginine or *per-d*₇-L-arginine. The reactions were initiated by diluting to the final volume with ¹⁸O₂-charged reaction buffer and quenched with Fmoc-OSu. The ¹⁸O content of the product was measured by LC-MS and corrected by dividing by the fraction ¹⁸O incorporated into the succinate co-product.

All aforementioned data sets (except the solvent exchange experiment) were fit to a linear equation (eq 2) to illustrate lack of isotope exchange.

$$\Delta E(t) = E_0 + \Delta E_1(t) \quad (2)$$

E_0 corresponds to the initial fraction of ¹⁸O incorporation, and E_1 represents the slope of change. The values for each fit are listed in Table S2.

Detection of the Iminium Intermediate by Reductive Trapping with NaB²H₄.

In the experiment that was designed to seek evidence of iminium formation in the reaction of VioC with D-arginine, the reaction mixture was prepared anoxically, as described above. The final concentrations were 250 μM VioC, 200 μM (NH₄)₂Fe(SO₄)₂, 160 μM 2OG, and 200 μM D-arginine. The mixture was removed from the anoxic chamber, and the reaction was initiated by dilution to the final volume (100 μL) with air-saturated buffer. After ~10 s, a small amount of NaB²H₄ (<10 mg) was added to the reaction mixture. The assay mixture was then derivatized with Fmoc-OSu and filtered as described above. Product detection was achieved by the LC-MS method described above.

RESULTS AND DISCUSSION

Reaction of VioC with D-Arg Yields the 2-Keto Acid Product.

Exposure of the presumptive VioC·Fe(II)·2OG·D-Arg reactant complex (formed by mixing the components in an anoxic chamber) to O₂ and analysis by (1) α-amine derivatization with fluorenylmethyloxycarbonyl (Fmoc), (2) separation by reverse-phase chromatography, and (3) detection by mass spectrometry in the positive ion mode (LC-MS) surprisingly revealed consumption of the D-Arg without formation of a new Fmoc-derivatized product. In control reactions lacking 2OG, we readily detected Fmoc-D-Arg at an elution time of 7.7 min and a mass-to-charge ratio (*m/z*) of 397 (*M* + *H*) (Figure 1, black trace), but in the complete reaction, we observed no peak at *m/z* 413 (+16) for the anticipated Fmoc-appended hydroxylation product (blue trace). Instead, we detected a single new product at *m/z* 174 eluting much earlier, at 1.3 min (green trace).

The diminished mass and elution time of the new product suggested that it lacked the Fmoc group, which increases the mass by 222 units and increases the elution time (by interaction

with the hydrophobic matrix) relative to that of the unmodified amino acid. Prevention of Fmoc attachment would most simply arise by modification or loss of the C2 primary amine. The observed (m/z) of -1 (relative to L-Arg, m/z 175) is consistent with conversion of the amine to the corresponding ketone. The ketone is expected to be susceptible to chemical reduction. Indeed, treatment of the reaction products with NaBH_4 eliminated the peak at m/z 174 and generated a new peak at a retention time of 1.5 min and m/z 176 (red trace), a (m/z) of $+2$, as expected for reduction of a ketone to an alcohol. These observations confirm that the outcome of the reaction of VioC with D-Arg is oxidative deamination to the corresponding 2-oxo-acid [2-oxo-5-guanidinopentanoate (2O5GP)].

We performed a series of assays to evaluate the efficiency of this surprising alternative activity. In a direct competition between the native and non-native Arg enantiomers (1 mM each; see the Supporting Information for a detailed procedure), VioC (0.01 mM) catalytically consumed the limiting 2OG co-substrate (0.5 mM) to generate the (3S)-hydroxy-L-Arg and 2O5GP products of the L- and D-substrates, respectively, in a ratio of 9:1 (Figure S1). A kinetic simulation of this experiment reproduced the experimental product ratio with a ratio of second-order rate constants (k_{cat}/K_M) of 12:1 in favor of the native L-enantiomer. In separate reactions of the two enantiomers, consumption of the non-native D-Arg in the presence of limiting 2OG was diminished by a factor of only ~ 2 relative to the fully coupled reaction of the native L-Arg. Despite this modest uncoupling, VioC could effect >30 turnovers of D-Arg in the presence of sufficient 2OG and ascorbate [to ensure regeneration of the active Fe(II) form of the cofactor following any adventitious oxidation associated with uncoupled events].

The Structure of the VioC/D-Arg Reactant Complex Implies HAT from C2.

To understand the structural basis for the altered outcome, the VioC·Fe(II)·2OG·D-Arg complex was crystallized under anoxic conditions to prevent turnover, and a structure was determined to 1.89 Å resolution (Figure 2A). The D-Arg substrate is evident in the active site at full occupancy, with continuous $2F_o - F_c$ composite electron density in the final refined structure. Interestingly, an omit map for the ligand exhibits diminished density for the methylene units of the side chain, suggesting local flexibility (Figure S2A). A comparison to the previously published structure of the native VioC/L-Arg reactant complex [Protein Data Bank (PDB) entry 6ALM] shows that the enantiomeric substrate maintains all of the same hydrogen bonding interactions with active site residues (Figure 2).³⁶ The native complex has C3 poised closest to the Fe(II) site, consistent with HAT from C3 to the ferryl intermediate that forms later in the reaction cycle (Scheme 2 and Figure 2D). This positioning is enforced by an array of hydrogen bonds involving all possible donors and acceptors of the substrate: the L-Arg guanidinium group donates to second-sphere carboxylates from D268 and D270, the α -amine donates to the uncoordinated carboxylate oxygen of monodentate Fe(II) ligand, E170, and the α -carboxylate accepts from S158 (Figure 2E). Strikingly, in the D-Arg reactant complex, each of these interactions is maintained. However, the inverted stereochemistry at the α -carbon forces a reorientation of the side chain trimethylene bridge and brings C2 closest to the iron center (4.3 Å). C3, the HAT donor for the native reaction, moves to a more distant 5.4 Å from the cofactor and, more importantly, directs its C–H

bonds away. This analysis shows that the distinct outcome in the D-Arg reaction results from initial H[•] abstraction from a different site, C2 instead of C3.

Many Fe/2OG enzymes use a dynamic lid loop to permit substrate access to the active site. A comparison of previously reported VioC structures determined in the absence of a primary substrate to the complex with the native substrate, L-Arg, revealed that a lid region (residues 220–251) undergoes a disorder-to-order transition upon substrate binding (Figure 2F and Figure S3A).⁸ This phenomenon was originally reported in another amino acid-targeting Fe/2OG enzyme, the asparagine β -hydroxylase, AsnO,³⁷ and is observed in some form in most Fe/2OG enzymes that have been structurally characterized to date. Interestingly, the VioC/D-Arg reactant complex exhibits lid-loop disorder, even though the substrate is bound at full occupancy (Figure 2C and Figure S3B). Apparently, D-Arg is less efficient than the native enantiomer at driving proper closure of the lid, a step presumed to be part of the mechanism by which substrate binding “triggers” rapid reaction with O₂.^{8,15} This difference could account, at least in part, for the diminutions in substrate specificity (discussed above) and triggering efficacy (see below) of the D-enantiomer.

The identity of the D-Arg reaction product was further confirmed by determination of a structure of the VioC·Fe(II)·succinate·2O5GP product complex to 1.89 Å resolution (Figure 3A). The structure was obtained by exposing anoxic crystals of the VioC/D-Arg reactant complex to O₂ for multiple hours followed by flash freezing for data collection. Inspection of composite $2F_o - F_c$ electron density maps reveals a single, well-ordered product molecule, consistent with the conversion of α -amine to α -ketone. As for the substrate complex, the product omit map exhibits weakened density for C3 and C4, again suggesting some flexibility in the side chain. Around C2, the electron density is best modeled by the assumption of trigonal rather than tetrahedral geometry, as expected for the ketone product (Figure 3B,C). Interestingly, the carbonyl oxygen of the ketone appears to coordinate the cofactor, as do the newly installed O atoms in several crystal structures of other Fe/2OG hydroxylase product complexes (Figure S3).^{33,36} The α -ketoacid product remains fixed in the active site by the same hydrogen bonding interactions observed in both the L-Arg and D-Arg reactant complex structures. In association with the conversion, the lid region (residues 220–251) becomes almost entirely ordered, with only a shorter segment (residues 233–237) yielding weak electron density (Figure S4C).

Solvent Rather Than O₂ Is the Source of the Product Ketone Oxygen.

The facts that (1) VioC is natively a hydroxylase and (2) the altered outcome with its substrate enantiomer superficially resembles that of the Fe/2OG *N*-demethylases would perhaps suggest that the most likely mechanism for the oxidative deamination of D-Arg would involve hydroxylation at C2 followed by elimination of ammonia. In this mechanism, the newly installed ketone oxygen would be expected to originate from O₂, as has been demonstrated in a number of Fe/2OG oxygenase reactions.^{24,26,33,38} To test this hypothesis, we determined the masses of the deamination products from reactions performed with ¹⁸O₂-charged buffer in H₂¹⁶O solvent (¹⁸O₂/H₂¹⁶O) or ¹⁶O₂-charged buffer in H₂¹⁸O solvent (¹⁶O₂/H₂¹⁸O). To guard against potentially confounding exchange with solvent during the analytical procedure, we terminated the reaction at varying times by addition of NaBH₄,

which reduces the ketone to fix the C2 O atom (Figure 4). No peak at a (m/z) of +2, signifying formation of a product with ^{18}O incorporated, could be detected in the $^{18}\text{O}_2/\text{H}_2^{16}\text{O}$ reaction (red squares), whereas nearly 100% ^{18}O incorporation was seen in the $^{16}\text{O}_2/\text{H}_2^{18}\text{O}$ reaction (blue diamonds). The time at which the reaction was quenched with NaBH_4 (0–240 min) had no effect in either case.

In a control experiment, we tested for ^{18}O incorporation in the native hydroxylation of L-Arg, expected to yield an O_2 -derived, exchange-inert C3–OH substituent. Indeed, we detected almost 100% ^{18}O incorporation in the $^{18}\text{O}_2/\text{H}_2^{16}\text{O}$ reaction (purple triangles; see below for a more detailed analysis). As a final control, we determined the rate of solvent exchange of the ketone oxygen. We added H_2^{18}O to a complete reaction mixture ($^{16}\text{O}_2/\text{H}_2^{16}\text{O}$) and incubated the solution for varying times before treating with NaBH_4 to fix the oxygen isotope. The time dependence of ^{18}O solvent “wash-in” (black circles) was fit by an exponential function to obtain an exchange rate constant (k_{ex}) of 0.03 min^{-1} (black trace) (Table S2). The associated half-life of 23 min implies that incorporation of ^{18}O into the ketone from O_2 in the $^{18}\text{O}_2/\text{H}_2^{16}\text{O}$ reaction should readily have been detected at the shorter reaction times examined (<10 min), had any incorporation actually occurred.

Analysis of Solvent Exchange of the Ferryl Oxo Ligand.

Exchange of the initially O_2 -derived oxygen could, in principle, also occur in the ferryl intermediate state. Complete solvent exchange in the intermediate could, theoretically, also rationalize the observed solvent origin of the ketone oxygen, even within the framework of the canonical HAT-rebound hydroxylation mechanism. The aforementioned experiment establishes that the O_2 -derived ferryl oxo ligand does not exchange appreciably with solvent in the native hydroxylation of L-Arg. This result implies that solvent exchange is too slow in the ferryl state to compete with abstraction of protium [and, less surprisingly, too slow in the $\text{Fe(III)}\text{--OH}$ state to compete with facile rebound]. To slow ferryl decay to expand the temporal window for solvent exchange to occur, we determined the extent of ^{18}O incorporation in the hydroxylation of *per-d*₇-L-Arg, the substrate isotopologue with deuterium at all exchange-inert positions. Previous work has shown that deuterium substitution generally slows ferryl decay in this class of enzymes by 10–60-fold,^{16,33,39–42} owing to the ^2H KIE associated with the initiating HAT steps. We first assessed the lifetime of the ferryl complexes in the VioC reactions with L-Arg, *d*₇-L-Arg, and D-Arg in single-turnover reactions with limiting O_2 . Anoxic solutions of the VioC reactant complexes were rapidly mixed in a stopped-flow instrument with air-saturated buffer, and the kinetics of ferryl formation and decay were monitored by the change in absorbance at 320 nm [A_{320} (Figure 5)], as in previous studies of Fe/2OG enzymes.^{16,33,39–42} Interestingly, the apparent first-order rate constants for ferryl formation extracted by nonlinear regression analysis (as previously described)⁴³ are similar for all three reactions (Table S3), implying that the D-Arg enantiomer does trigger the reaction with O_2 . However, in experiments with excess O_2 , the maximum accumulation of the intermediate (as determined by the amplitude of the A_{320} -vs-time traces) is lower by a factor of ~6 in the D-Arg reaction than in the L-Arg reaction (Figure S5), implying that only ~15% of the VioC/D-Arg reactant complex is in the proper configuration to react rapidly with O_2 (perhaps because of the aforementioned incomplete lid-loop closure). The rate constants for decay of the intermediate are, remarkably, also

nearly identical for the reactions with the protium-containing L-Arg and D-Arg substrates (8.6 and 9.7 s⁻¹, respectively), whereas the *per-d*₇-L-Arg reaction exhibits a characteristically large, normal ²H KIE of ~40. Given the absence of solvent exchange in the ferryl state of the native reaction (with protium L-Arg), it seems unlikely that solvent exchange in the comparably short-lived ferryl state of the D-Arg oxidative deamination could be the reason for the strict solvent origin of the ketone oxygen. Consistent with this argument, the drastic slowing of ferryl decay in the *per-d*₇-L-Arg reaction does permit detectable solvent exchange in the ferryl state, but only to an extent of ~25% (Figure 4).

The potentially increased solvent exposure of the cofactor in the D-Arg complex associated with the inefficient lid-loop closure could, in principle, allow for an increased rate of exchange in the ferryl state. To evaluate this possibility, we examined a space-filling model of the VioC D-Arg complex (Figure S6A). The iron cofactor appears to be still largely occluded from solvent in this model. Together, the results thus imply that ketone formation occurs by hydrolysis of an imine/iminium intermediate rather than via hydroxylation and elimination of ammonia.

Evidence for an Iminium Intermediate in the D-Arg Oxidative Deamination.

We sought more direct chemical evidence of a hydrolytically labile C=N-bonded intermediate by (1) quenching the reaction with NaB²H₄ shortly (~ 10 s) after initiating it by mixing the anoxic reactant complex with air-saturated buffer and (2) then subjecting the quenched reaction solution to Fmoc derivatization and LC–MS analysis (Figure S7A). Recall that the only detected product at completion of the reaction, the 2-ketoacid, does not couple to Fmoc, because of the absence of the α-amine, and the product therefore elutes early and with a diminished *m/z* value during the LC–MS analysis. By contrast, any iminium intermediate present at 10 s should be reduced by NaB²H₄ to produce arginine (most likely racemic, if reduced following release from the active site) containing ²H at C2, which should then be susceptible to Fmoc attachment and subsequent LC–MS detection as a late-eluting species at *m/z* 398, + 1 relative to Fmoc-Arg. Indeed, the reductive quenching and derivatization led to development of a prominent LC–MS peak at the appropriate elution time and *m/z* (Figure 6, rear red trace). The new peak constituted 51% of the total integrated intensity attributable to Fmoc-arginine [sum of peak areas for *m/z* 397 (Figure 6, rear black trace) and *m/z* 398]. By comparison, a standard of Fmoc-D-Arg exhibited only 20% intensity for the *m/z* 398 peak (Figure 6, front), close to the value of ~23% predicted to arise from the 1.1% natural ¹³C abundance in the C₂₁ compound. The 31% increase in the intensity of the *m/z* 398 signal is thus the result of reductive trapping of the iminium intermediate with NaB²H₄ and subsequent derivatization of the (*m/z*) + 1 [²H]Arg. The detection of the iminium intermediate explains the solvent origin of the ketone oxygen: the reaction proceeds by C–N desaturation and hydrolysis.

We further tested the expectation that the arginine produced by this sequence of desaturation and borohydride reduction would be racemic (or at least contain some L-Arg) by subjecting the resultant product(s) to another round of the VioC reaction and testing for the 3-hydroxy-Arg product that forms only with L-Arg (Figure S7A). Indeed, we readily detected the expected peak at (*m/z*) = +17, consistent with 3-hydroxylation of 2-[²H]-L-Arg generated

by desaturation of D-Arg and reduction by NaB^2H_4 (Figure S7B). This observation is consistent with the conclusions that the product of D-Arg oxidation by VioC is the imine and its borohydride-mediated reduction back to Arg occurs in solution.

Analysis of the Mechanism of VioC-Mediated Cryptic C–N Desaturation of D-Arg.

As laid out in a recent study of two different C–C desaturation reactions catalyzed by Fe/2OG oxygenases, including one by VioC,³³ three conceivable mechanisms of desaturation exist in this catalytic manifold (Scheme 3). The $\text{C}^*/\text{Fe(III)}\text{--OH}$ intermediate produced by HAT from C2 to the ferryl complex could react by transfer of either (1) a second $\text{H}^\bullet$ (HAT, pathway A) or (2) an electron to the cofactor (ET, pathway B) or, alternatively, (3) by oxygen rebound and α -amine-enabled dehydration (pathway C). In pathway 2, deprotonation of the α -amine, either in a prior rapid equilibrium or concertedly with ET in a proton-coupled electron transfer (PCET) step, would be necessary for the α -nitrogen to drive the ET. Likewise, pathway 3 would also require deprotonation of the α -amine for it to assist in expulsion of the transiently installed hydroxyl group. In the previous study, the dispositive evidence for a sequential HAT mechanism was the observation that the presence of deuterium at the second donor site slows the second HAT and allows rebound to compete more effectively, thus resulting in a diminished yield of the desaturation product and an enhanced yield of the hydroxylation product. In the oxidative deamination of D-Arg by VioC, the second HAT donor would be the α -nitrogen, and its hydrogens readily exchange with solvent. A second distinction here is that increased flux through the rebound pathway by retardation of the competing step would not result in additional stable hydroxylation; rather, it would be expected to result in enhanced incorporation of ^{18}O from $^{18}\text{O}_2$ into the ketone product. Therefore, we tested the prediction of the sequential HAT mechanism that the reaction performed in $^2\text{H}_2^{16}\text{O}$ buffer charged with $^{18}\text{O}_2$ would yield ^{18}O product enrichment. The absence of such enrichment (Figure 4, green triangles) weighs against the sequential HAT pathway and in favor of one of the other two classes of mechanisms, as was also proposed for the C4–C5 desaturation of L-Arg by NapI. We favor the possibility of a PCET step over the rebound–dehydration pathway, because we would expect that the predominance of rebound would result in some competing elimination of ammonia and incorporation of oxygen from O_2 into the keto group of the product, an expectation contradicted by the experimental results. The identity of the posited base to accept the proton from the α -nitrogen to allow it to assist either ET or (less likely) dehydration after rebound is not clear, but one attractive candidate is E170. The E170 side chain hydrogen bonds to the substrate α -amine in all X-ray crystal structures of VioC. However, it is also an Fe(II) ligand, and this interaction could preclude its transient protonation. Interestingly, the partial disorder of the lid loop in the D-Arg reactant complex allows for the possibility that a water network could participate in proton transfer from the α -amine.

Relationship of the VioC C–N-Desaturation Mechanism to Those of Other Fe/2OG Enzymes.

The number of reported examples of alternative transformations of substrate analogues by Fe/2OG oxygenases is increasing; this study demonstrates a novel type. The structural comparison of VioC with L-Arg and D-Arg shows that the imperative to preserve enzyme–

substrate binding contacts, despite the inverted C2 configuration, enforces the presentation of a different carbon to the metal center for C–H activation. Just as surprisingly, the mechanism of the ensuing reaction appears also to diverge from the native, canonical hydroxylation pathway, proceeding through an iminium instead of (or, less likely, subsequent to) a hemiaminal intermediate. This mechanism is strongly reminiscent of all but the last step in the pathway recently proposed for the 4,5-desaturation of L-Arg by NapI.³³

The mechanistic dichotomy laid out in this study, rebound versus direct desaturation, is also potentially relevant to the histone demethylases, actively studied in recent years for their important roles in epigenetics.^{31,44} The mechanism of D-Arg oxidation by VioC is somewhat analogous to that employed by the lysine-specific demethylases 1 and 2 (LSD1 and –2, respectively), which use flavin adenine dinucleotide (FAD) as their cofactor.^{45,46} In demethylation of lysine 4 of histone subunit H3, the lysine *N*-methyl reduces the FAD cofactor, generating an iminium intermediate that is ultimately hydro-lyzed by water, as shown here for the VioC/D-Arg reaction. Fe/2OG histone demethylases have also been described and can, as the flavin-dependent enzymes do, act on mono- and dimethyllysine-containing substrates.³² A crucial distinction from LSD1 and –2, however, is the ability of a subset of the Fe/2OG enzymes to act on trimethyllysine substrates, a reaction that must, in the absence of a nonbonded electron pair on the heteroatom, necessarily proceed by the H[•] abstraction/oxygen-rebound pathway.⁴⁷ Thus, whereas demethylations of mono and dimethyl substrates by the Fe/2OG demethylases could, in principle, proceed by the alternative of direct C–N desaturation implicated here, it has generally been presumed that they follow the canonical oxygen-rebound pathway. Other Fe/2OG *N*-demethylases (AlkB, ALKBH1–8, and FTO) are also thought to utilize this mechanism.²⁸ Although some evidence for hydroxylated intermediates has been presented,⁴⁸ the dispositive O-isotope-tracer experiments described here have not, to the best of our knowledge, been reported, leaving open the question of whether iminium rather than hemiaminal intermediates might be on-pathway in some of these important reactions.

A growing body of work appears to underscore the surprising flexibility of Fe/2OG oxygenases involved in biosynthesis of complex secondary metabolites to accommodate a range of structurally related substrates and mediate alternative transformations thereupon. This promiscuity stands in apparent contradiction to the general expectation of exquisite specificity of enzymes in selection of substrate and direction of outcome. It seems likely that the enzymes that build such secondary metabolites have not been subject to the usual evolutionary pressure for efficiency. An active site that permits substrate and product diversity would enable rapid, combinatorial sampling of natural products for bioactivities that confer a competitive advantage. This inherent flexibility bodes well for the directed evolution of Fe/2OG enzymes for novel transformations in drug discovery and synthesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Extracted ion chromatograms (EICs) from LC-MS analysis of reactions of VioC with D-Arg: (front) control reactions lacking 2OG, (middle) full reactions allowed to proceed to completion, and (rear) full reactions allowed to proceed to completion and quenched with NaBH₄ prior to Fmoc derivatization. The traces shown are for *m/z* values corresponding to Fmoc-D-Arg (397, black traces), Fmoc-hydroxy-D-Arg (413, blue traces), 2OG (174, green traces), and 2-hydroxy-arginine (176, red traces).

Figure 2.

(A) X-ray crystal structure of the VioC-Fe(II)-2OG· D-Arg complex at 1.89 Å resolution. The $2F_o - F_c$ electron density map (black mesh) for selected active site components is contoured to 1.0σ . (D) Structure of the VioC-Fe(II)-2OG· L-Arg reactant complex (PDB entry 6ALM). Hydrogen bonding interactions between VioC and D-Arg (B) and L-Arg are identical (E). The lid region (blue, residues 220–251) in the VioC L-Arg structure (F) is well-ordered and closes over the active site. In the D-Arg structure (C), the lid region is disordered and could not be modeled.

Figure 3.

Views of the active site and comparison of the orientations of D-Arg and 2O5GP binding from X-ray crystal structures of the reactant and product complexes. (A) Structure of the VioC·Fe(II)·succinate-2O5GP complex at 1.89 Å resolution. Product-state crystals were generated by exposing crystals of the D-Arg reactant complex to O₂ for ~5 h and flash-freezing prior to data collection. (B) Overlay of the models for VioC-bound D-Arg and 2O5GP, showing distinct geometry about C2. (C) Comparison of the $2F_o - F_c$ electron density maps (black mesh) for D-Arg (top) and 2O5GP (bottom), contoured to 1.0σ .

Figure 4.

LC–MS determination of the origin of the appended oxygen in the transformations of D-Arg, L-Arg, and *per-d7*-L-Arg by VioC. The control samples (black circles) were prepared by incubating VioC, 2OG, and D-Arg in aerobic H₂O at ambient temperature until the reaction reached completion, injecting H₂¹⁸O into the samples, and allowing varying times for exchange of the ketone oxygen with the injected H₂¹⁸O (*x*-axis) before addition of NaBH₄ to halt the exchange. Fitting the equation for an exponential rise to the data gave a *k*_{ex} of 0.03 min^{−1} (black trace). Sample reactions are shown for D-Arg in ¹⁸O₂/H₂¹⁶O (red squares), D-Arg in ¹⁶O₂/H₂¹⁸O (blue diamonds), D-Arg in ¹⁸O₂/²H₂¹⁶O (green triangles), L-Arg in ¹⁸O₂/H₂¹⁶O (purple triangles), and *per-d7*-L-Arg in ¹⁸O₂/H₂¹⁶O (teal squares). The LC–MS analytical procedure is described in Experimental Details.

Figure 5.

Kinetics of formation and decay of the ferryl intermediates in the VioC reactions with L-Arg, *per-d₇*-L-Arg, and D-Arg monitored by the absorbance at 320 nm. An anoxic solution containing 1.2 mM VioC, 1.1 mM Fe(II), 6.0 mM 2OG, and 6.0 mM D-Arg (green), L-Arg (red), or *per-d₇*-L-Arg (blue) was mixed at 5 °C with an equal volume of air-saturated buffer (giving a final O₂ concentration of ~0.19 mM). Regression fits to the data are shown as black lines using parameters listed in Table S3. The equations for the D-Arg and *per-d₇*-L-Arg reactions give the absorbance as a function of time arising from the isosbestic reactant and product and more intensely absorbing intermediate in a sequence of two consecutive, irreversible, (pseudo)-first-order reactions. For the L-Arg reaction, a second intermediate species forms before re-formation of the reactant complex; thus, the fit corresponds to three consecutive, irreversible (pseudo)first-order reactions (see the Supporting Information for more details).

Figure 6.

EICs at m/z values of the Fmoc-D-Arg standard (397, black traces) and Fmoc-2-[²H]arginine produced by NaB²H₄ quenching of the VioC/D-Arg reactions at short reaction times (~10 s). The peak in the m/z 398 EIC also includes the contribution from Fmoc-¹³C₁-arginine arising from the 1.1% natural abundance of ¹³C in the C₂₁ product. Percentages given reflect the area of the m/z 398 peak relative to the sum of the areas of the m/z 397 and 398 peaks.

Scheme 1.

Possible Mechanisms by Which Hydroxylated Intermediates Produced in Reactions of Fe/2OG Oxygenases Could Be Further Processed To Yield Alternative Outcomes

Scheme 2.
VioC Reactions with L-Arg and D-Arg

Scheme 3. Possible Mechanisms for C–N Desaturation of D-Arg by VioC^a^a(A) Sequential HAT (blue), (B) electron transfer (red), and (C) rebound (green) pathways.

The electron transfer pathway could also proceed in a concerted fashion, with the iminium intermediate formed directly from the C2 radical state.