Communication

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J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/jacs.8b03729 • Publication Date (Web): 05 Jun 2018

Substrate Conformation Correlates with the Outcome of Hyoscyamine 6β-Hydroxylase Catalyzed Oxidation Reactions

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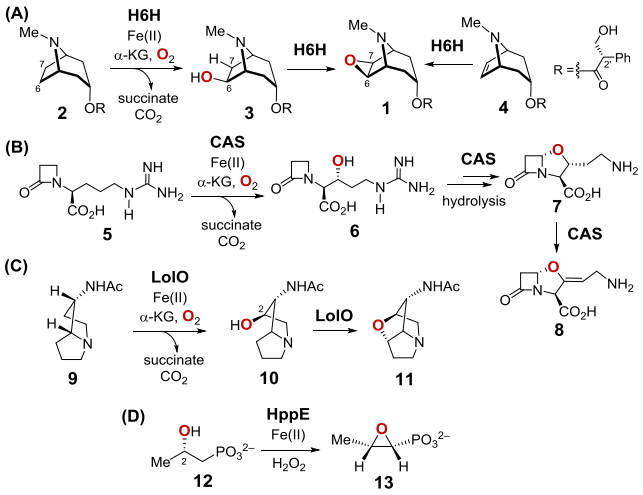
Supporting Information Placeholder

ABSTRACT: Hyoscyamine 6β–hydroxylase (H6H) is an α-ketoglutarate dependent mononuclear non-heme iron enzyme that catalyzes C6-hydroxylation of hyoscyamine and oxidative cyclization of the resulting product to give the oxirane natural product scopolamine. Herein, the chemistry of H6H is investigated using hyoscyamine derivatives with modifications at the C6 or C7 position as well as substrate analogues possessing a 9-azabicyclo[3.3.1]-nonane core. Results indicate that hydroxyl rebound is unlikely to take place during the cyclization reaction and that the hydroxylase versus oxidative cyclase activity of H6H is correlated with the presence of an *exo*-hydroxy group having *syn*-periplanar geometry with respect to the adjacent H-atom to be abstracted.

The mononuclear non-heme iron dependent oxidases are an important class of enzymes that catalyze a diverse array of reaction types including hydroxylation, desaturation, epimerization, halogenation and epoxidation.[1–4] Members of this enzyme family typically require α-ketoglutarate (α-KG) as a co-substrate for catalysis and possess a highly conserved His/His/Asp(Glu) facial triad that coordinates the catalytic iron center.[1–4] Despite significant progress in understanding this class of enzymes, questions remain regarding how these enzymes are able to catalyze specific transformations thereby preventing alternative reaction outcomes.

An atypical member of this enzyme family is hyoscyamine 6β-hydroxylase (H6H), which is involved in the biosynthesis of the anticholinergic alkaloid scopolamine (**1**) in the Solanaceae family of plants.[5–13] What makes H6H unusual is its versatility, since it can catalyze hydroxylation (**2** → **3**), dehydrogenation (**3** → **1**), and *in vitro* epoxidation (**4** → **1**) reactions as shown in Scheme 1.[7–10,11–13] Two other enzymes with similar catalytic properties are clavaminate synthase (CAS)[14] and LolO,[15,16] which catalyze the conversion of deoxyguanidino proclavaminate (**5**) to clavaminate (**8**) and *exo*-1-acetamidopyrrolizidine (**9**) to *N*-acetylnorloline (**11**), respectively (Scheme 1). Particularly notable are the cyclization reactions (**3** → **1**, **6** → **7** & **10** → **11**), which are also dehydrogenations.[9,13,17] The conversion of (*S*)-2-hydroxypropylphosphonate (HPP, **12**) to fosfomycin (**13**) is another example of this chemistry also catalyzed by a mononuclear non-heme iron enzyme, HPP epoxidase (HppE), which uses H_2O_2 rather than α-KG as the oxidant.[18,19]

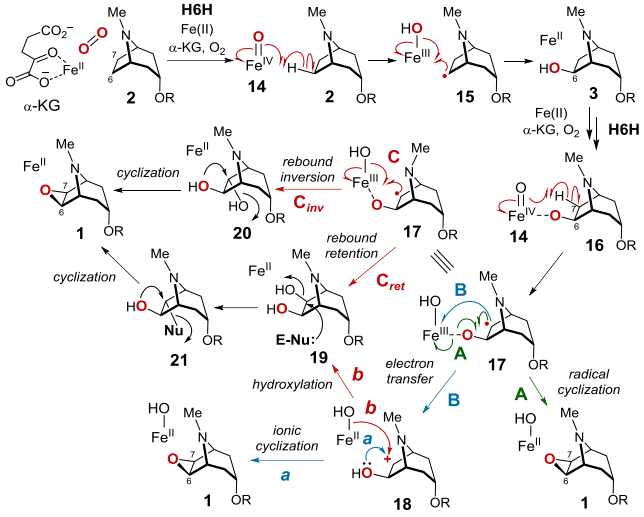
Scheme 1. Reactions catalyzed by (A) H6H, (B) CAS, (C) LolO, and (D) HppE



Similar to other α-KG-dependent non-heme iron enzymes,[1–4] the hydroxylation reaction catalyzed by H6H is predicted to involve H-atom abstraction from C6 of hyoscyamine (**2**) to give **15** by a high valent iron-oxo species (**14**) that is generated via the reaction of the α-KG-Fe(II)-substrate complex with O_2 (see Scheme 2). Subsequent rebound of the metal-coordinated hydroxide then yields the *exo*-6–hydroxylated product (**15** → **3**). A similar iron-oxo intermediary species could also play a key role in formation of the oxirane ring (**3** → **1**). However, the reasons behind the conversion of H6H to an oxidative cyclase rather than a hydroxylase when **3** is the substrate have yet to be established. We report here the investigation of four substrate analogues as mechanistic probes to delineate those features that determine the outcome of H6H-catalyzed reactions. The results

indicate that cyclization does not involve a diol intermediate and that hydroxylation versus cyclization correlates with the degree to which the abstracted *exo*-hydrogen eclipses the adjacent hydroxy group in the unbound substrate.

Scheme 2. Possible mechanisms for the epoxidation of 2.



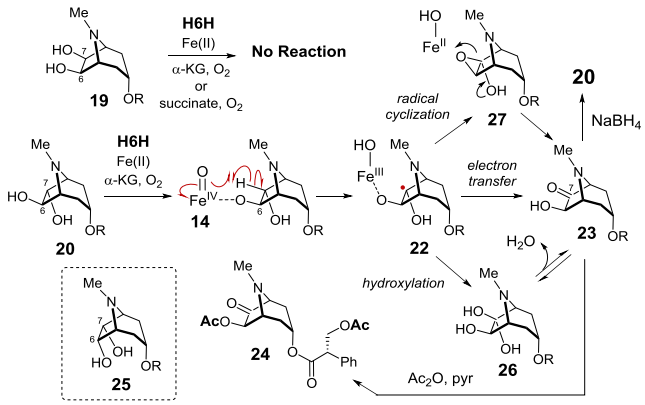
Several possible mechanisms for the conversion of **3** to **1** are shown in Scheme 2. The 6-OH of **3** may coordinate the iron center in the active site of H6H similar to substrate binding in the HppE-catalyzed epoxidation reaction (**12** → **13**, Scheme 1).[20–23] Such an interaction could facilitate H-atom abstraction from the *exo*-C7 position in **3** by the Fe(IV)=O complex (**14**) to generate the radical intermediate **17**[10] prior to radical-mediated cyclization (route A). Ring formation may also result from intramolecular nucleophilic addition of the *exo*-C6-OH to the C7 carbocation in **18**, which would be produced via electron transfer from **17** to the Fe(III)OH complex (route B-*a*). Alternatively, hydroxyl rebound to **17** could yield a 1,2-diol (**19** or **20**), which can then be converted to **1** through nucleophilic displacement of the C7 hydroxy group (route C). The dihydroxylation could also occur via a cation intermediate (**18**, route B-*b*).

To test whether a 1,2-diol intermediate is formed during the catalytic cycle, **19** and **20** were synthesized (the latter as a 3:2 mixture of 2′*S* and 2′*R* diastereomers) and incubated with H6H from *Hyoscyamus niger* heterologously expressed and purified using *Escherichia coli* (see *Supporting Information*). No consumption of **19** (1 mM) was detected under standard assay conditions (aerobic, 68 μM H6H, 0.40 mM $FeSO_4$, 5.0 mM α-KG, 4.0 mM ascorbate, 50 mM tris(hydroxymethyl)aminomethane (Tris) buffer, pH 7.4). Replacing α-KG with succinate, which is the by-product derived from α-KG upon formation of the iron-oxo species,[1–4] did not change the outcome (Figure S2). These results indicated that **19** is an unlikely intermediate in the conversion of **3** to **1** (Scheme 3).

On the contrary, consumption of (2′*S*)-**20** was observed under the standard assay conditions with α-KG, and a new peak was detected by HPLC different from that of **1**; however, no reaction was observed with succinate or (2′*R*)-**20** (see Figure S3). ESI-MS analysis of the reaction product suggested that one of the hydroxy groups in **20** had been oxidized to a carbonyl (calcd *m/z* for $C_{17}H_{22}NO_5^+$ $[M+H]^+$: 320.1492, obsd: 320.1500, see Figure S12). Isolation and ESI-MS analysis of the reaction product following treatment with Ac_2O in pyridine (80 °C, 15 min) was consistent with formation of a diacetylated species (e.g., **24**, calcd *m/z* for $C_{21}H_{26}NO_7^+$ $[M+H]^+$: 404.1704, obsd: 404.1717, see Figure S12). Furthermore, reduction of the reaction product with sodium borohydride gave a compound that co-eluted with (2′*S*)-**20**, but not with 6α,7α-dihydroxyhyoscyamine (**25**, Figure S4). This implies that the keto functionality of the reaction product is at C7 rather than C6, since reduction of the keto group of **23** with $NaBH_4$ should occur from the less hindered *exo* face to regenerate **20**.[24] Based on these observations, the reaction product of **20** with H6H was assigned as 7-keto-6β-hydroxyhyoscyamine (**23**).

As shown in Scheme 3, the 7-keto product **23** could be produced either by rebound of the hydroxyl group from Fe(III)–OH to the radical intermediate (**22** → **26**), the direct oxidation of **22** via electron transfer to the ferric iron (**22** → **23**), or oxidative cyclization of **22** to **27** followed by ring opening. When the reaction of **20** (as a 2′*S*/2′*R* mixture) was conducted under $^{18}O_2$, no ^{18}O incorporation was found in **23** (Figure S5). Incorporation of ^{18}O into **23** was observed when the reaction was run in $H_2{}^{18}O$; however, this could be fully explained by hydration of the resulting ketone (Figure S5). While these results do not rule out the possibility for stereoselective elimination of the *exo*-OH from **26**, they do suggest a mechanism involving either electron transfer (**22** → **23**) or an oxirane intermediate (**22** → **27** → **23**). In either case, neither **19** nor **20** appears to be an intermediate during the cyclization of **3** to **1** arguing against the formation of a 1,2-diol intermediate and effectively ruling out routes C and B-*b* (Scheme 2).

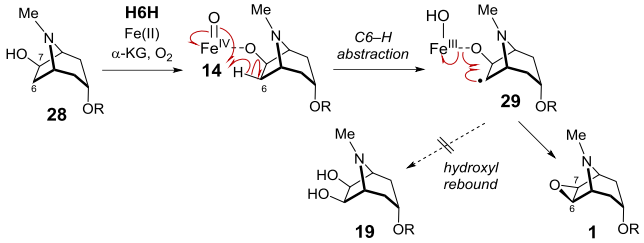
Scheme 3. H6H-catalzyed oxidation of 20.



In contrast to **2**, which is hydroxylated in the presence of H6H, compounds **3** and **20** undergo dehydrogenation with the former known to result in an oxirane. These observations raised the question as to what determines hydroxylation versus dehydrogenation. One possibility is that the presence of an *exo*-hydroxy functionality vicinal to the site of H-atom abstraction plays a role in redirecting reaction flux to dehydrogenation. As a test of this hypothesis, 7β-hydroxyhyoscyamine (**28**)[25,26] was prepared and incubated with H6H

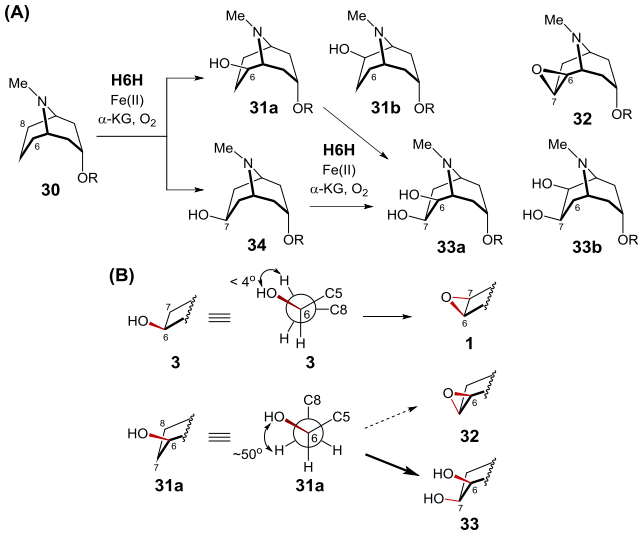
(Scheme 4). In this experiment, formation of the C6-hydroxylated product **19** from **28** was expected assuming the presence of the *exo* C7-OH leaves the H6H catalytic cycle essentially unperturbed compared to that for **2**. Instead, however, **28** was slowly cyclized to **1** in the presence of H6H under standard conditions (Scheme 4), which was confirmed by ESI-MS spectroscopy (calcd *m*/*z* for $C_{17}H_{22}NO_4^+$ $[M+H]^+$: 304.1543, obsd: 304.1554) and co-elution with a standard (Figures S6 & S12). No dihydroxylated product (e.g., **19**) could be detected. Hence, the presence of an *exo*-hydroxy group at either C6 or C7 indeed influences the course of the reaction. Consistent with previous reports,[10] the observation that **3**, **20** and **28** are all substrates for H6H whereas **19** is not implies that H-atom abstraction can occur from either the *exo*-C6 or *exo*-C7 position but not from the corresponding *endo* positions.

Scheme 4. H6H-catalyzed oxidation of 28.



To explore the relationship between substrate structure and reaction course, an analogue with a 9-azabicyclo[3.3.1]nonane core (**30**) was synthesized (Scheme 5A). Two new products in an approximately 3:2 ratio were detected by HPLC when **30** was incubated with H6H for 10 min under standard conditions (Figure S9). These were assigned as the mono-hydroxylated species **31a** (or its isomer **31b**) and **34** based on NMR and ESI-MS (calcd *m*/*z* for $C_{18}H_{26}NO_4^+$ $[M+H]^+$: 320.1856, obsd: 320.1848 and 320.1849 for **31** and **34**, respectively, see *Supporting Information*). The identity of the major product was confirmed to be **34** by co-elution with a synthetic standard (Figure S10). Upon further incubation, these products are consumed with concomitant formation of two new species in a roughly 3:1 ratio both having masses consistent with dihydroxylated derivatives of **30** (calcd *m*/*z* for $C_{18}H_{26}NO_5^+$ $[M+H]^+$: 336.1805, obsd: 336.1799 and 336.1795, see Figures S9 & S12). The major dihydroxylated species was identified as the 6β,7β-dihydroxylated compound **33a** (or its isomer **33b**) by HPLC co-elution with a mixed synthetic standard of **33a** & **33b** (Figure S11). Purification of the mono-hydroxylated products (**31** & **34**) followed by reincubation with H6H showed that both could be converted to **33** (Figure S10). While the structure of the minor dihydroxylated species could not be determined, it does not coelute with the mixed standard of **33a** & **33b** by HPLC. The only evidence for cyclization was an LCMS signal consistent with **32** or a C6/C8-keto derivative of **30** (calcd *m*/*z* for $C_{18}H_{24}NO_4^+$ $[M+H]^+$: 318.1700, obsd: 318.1695, see Figure S12) when purified **30** was incubated with H6H. However, the MS signal intensity of this species was less than 5% relative to that of the major dihydroxylation product (**33**). Therefore, if cyclization does take place, then it is only a very minor side reaction.

Scheme 5. (A) H6H-catalyzed oxidation of 30; (B) Correlation of H6H-catalyzed reaction outcome with substrate conformation.



The observation that H6H catalyzes cyclization of hydroxy-azabicyclo[3.2.1]octanes (**3** & **28**) and hydroxylation of hydroxy-azabicyclo[3.3.1]nonanes (**31** & **34**) suggests that the orientation of the *exo*-C–H bond to be broken versus the adjacent *exo*-C–O bond is correlated with the subsequent reaction course. The X-ray crystal structure of **30** shows that it adopts a staggered conformation with an H–C6–C7–H dihedral angle of 49° (Figure S8). Furthermore, gas phase RB3LYP/6-31G* computations of models of **31a** (with R = CH_3) implied that a similar HO–C6–C7–H dihedral angle is retained in **31a** (see Scheme 5B and Figure S42), which is consistent with characterization by NMR (see *Supporting Information*). Likewise, computational models of **34** (R = CH_3) show H–C6–C7–OH dihedral angles ranging from 35° to 52° with respect to the *exo*-C6–H bond (see Figure S40). This continues a general trend of staggered substrate conformations observed among other α-KG-dependent nonheme iron enzymes catalyzing the β-hydroxylation of alcohols to produce vicinal diols (e.g., OrfP,[27] PolL,[28] GA 2β oxidase,[29] KdoO,[30] BcmG,[31,32] and RbtG[33]). In contrast, gas phase models of **3** (R = CH_3) exhibit dihedral angles less than 4° versus the *exo*-C7–H bond (see Scheme 5B & Figure S38).

One possible explanation for this correlation is that a similar geometric arrangement is maintained between the *exo*-C–OH bond and the adjacent partially filled *p*-orbital in the radical intermediate resulting from H-atom abstraction. This would then facilitate cyclization of radicals derived from **3** and **28** (i.e., **17** & **29**) as opposed to oxygen rebound for radicals derived from **31** and **34**. However, while gas phase UB3LYP/6-31G* computations indicated that the HO–C6–C7–*p* angle increases to no more than 22° in the modeled radicals derived from **3** (R = CH_3, see Figure S39), angles less than 15° (i.e., periplanar) could also be found among optimized conformers of radicals derived from **31** and **34** (see Figures S41 & S43). Therefore, interactions between the enzyme and bound substrate may be important to maintain the initial substrate geometry in order to explain the ob-

served correlation. Alternatively, a discrete radical intermediate susceptible to rebound may only form from substrates with staggered (**31** & **34**) rather than eclipsed alignments (**3** & **28**). In the latter case, cyclization may proceed in concert with H-atom abstraction resulting in a one-step-two-electron reduction of the Fe(IV)=O complex. While enzymatic Fe(IV)=O complexes are typically modeled as stepwise one-electron acceptors, Fe(IV)=O complexes have been implicated in two-electron processes,[34,35] and a similar chemistry may be at work in the catalytic cycle of H6H.

H6H represents an excellent system for studying the partitioning of radical-mediated catalytic cycles among different reaction paths. Herein, evidence is provided that the oxidative cyclization catalyzed by H6H does not involve rebound of the hydroxyl group following H-atom abstraction. Furthermore, cyclization versus rebound appears to require that an *exo*-OH not only be adjacent to the site of H-atom abstraction but also have the correct *syn*-periplanar configuration. Future investigation of these properties and the chemistry underlying them will provide new insights into the mechanics of nonheme iron biocatalysts.

ASSOCIATED CONTENT

The Supporting Information is available free of charge on the ACS Publications website at DOI: XXX. Details regarding H6H assays along with HPLC protocols, chemical synthesis of compounds and computational methods and results.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

We thank Dr. Takashi Hashimoto at Nara Institute of Science and Technology for generously providing the clone of *h6h*. This work was supported by grants from the National Institutes of Health (GM113106 and GM040541) and the Welch Foundation (F-1511).

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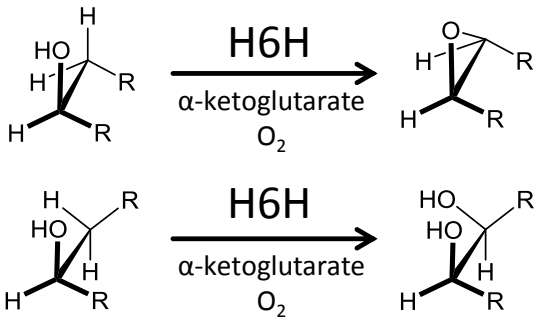
H6H
α-ketoglutarate
O2
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