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# Changes in regioselectivity of H atom abstraction during the hydroxylation and cyclization reactions catalyzed by hyoscyamine 6 $\beta$ -hydroxylase

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## Abstract

Hyoscyamine 6 $\beta$ -hydroxylase (H6H) is an  $\alpha$ KG-dependent nonheme iron oxidase that catalyzes the oxidation of hyoscyamine to scopolamine via two separate reactions: hydroxylation followed by oxidative cyclization. Both of these reactions are expected to involve H atom abstraction from each of two adjacent carbon centers (C6 versus C7) in the substrate. During hydroxylation there is a roughly 85 : 1 preference for H atom abstraction from C6 versus C7; however, this inverts to a 1 : 16 preference during cyclization. Furthermore, <sup>18</sup>O incorporation experiments in the presence of deuterated substrate are consistent with the catalytic iron(IV)-oxo complex being able to support the coordination of an additional ligand during hydroxylation. These observations suggest that subtle differences in the substrate binding configuration can have significant consequences for the catalytic cycle of H6H.

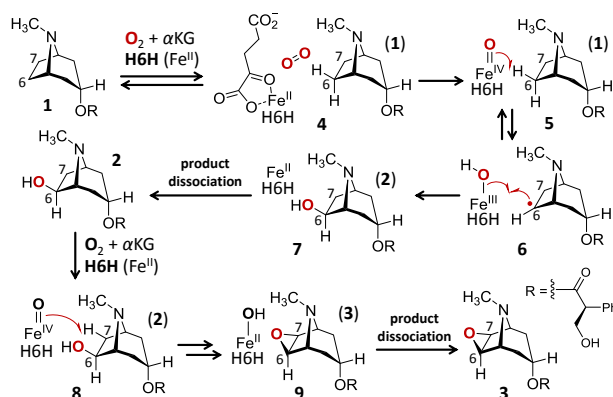


Figure 1: Mechanistic outline of the hydroxylation and cyclization reactions catalyzed by H6H.

## Introduction

Nonheme iron oxidases represent an important class of enzymes that catalyze a wide range of oxidative transformations.<sup>1–4</sup> The prototypical catalytic cycle of these enzymes involves the use of molecular oxygen to oxidize  $\alpha$ -ketoglutarate ( $\alpha$ KG) coordinated to an active site iron(II) center (**4**) to generate succinate,  $CO_2$  and an activated iron(IV)-oxo complex (see **5** & **8** in Fig. 1).<sup>1,5</sup> The iron(IV)-oxo complex then participates in the oxidation of the nominal substrate with the possible involvement of organic radicals confined to the active site. Despite the reactivity of iron(IV)-oxo complexes and radical intermediates, nonheme iron oxidases are able to exert considerable control over the course of the reaction such that they proceed in a regio- and stereoselective manner. Therefore, the mechanistic study of these enzymes can offer significant insights into how enzymes constrain the reactions they catalyze.

Hyoscyamine 6 $\beta$ -hydroxylase (H6H) is one such enzyme of particular interest. H6H is an  $\alpha$ KG-dependent nonheme iron oxidase from plants that catalyzes two separate and sequential oxidations during biosynthesis of the antimuscarinic natural product scopolamine (**3**).<sup>6–13</sup> The first of these reactions is the C6-hydroxylation of hyoscyamine (**1**) to generate 6 $\beta$ -hydroxyhyoscyamine (**1**→**2**, see Fig. 1). This is followed by a second reaction

where H6H catalyzes the dehydrogenation of **2** to yield the epoxide scopolamine (**2**→**3**). These two reactions are believed to involve H atom abstraction from the *exo*-C6 and *exo*-C7 positions of the corresponding substrates **1** and **2**, respectively, by an iron(IV)-oxo intermediary species (**5** & **8**) as shown in Fig. 1. However, H6H can also accept the regioisomer **10** (see Fig. 2) as a substrate for the cyclization reaction again yielding scopolamine.<sup>14</sup> These observations immediately raise questions regarding how the regiochemistry of the two reactions is controlled within the H6H active site.

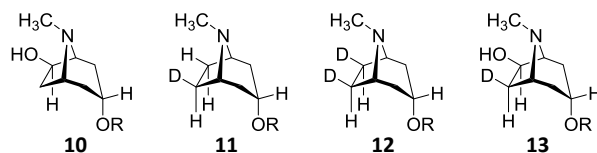


Figure 2: Additional H6H substrate and product analogs studied in the present work.

Analysis of the H6H-catalyzed hydroxylation reaction by LC-MS demonstrates that over 98% of the product is **2** consistent with previous reports.<sup>7</sup> However, as described in the *Supporting Information*, a minor side product can also be identified and was confirmed to be **10**. Therefore, while H atom abstraction from C7 is possible during hydroxylation, nearly all of the reaction flux proceeds via H atom abstraction from C6. It follows that from a biological standpoint **2** rather than **10** is the relevant biosynthetic substrate for cyclization to **3** and thus *exo*-C7-H rather than *exo*-C6-H is almost always the only option for H atom abstraction during cyclization. However, the regioselectivity of H6H-catalyzed hydroxylation suggests that the H6H iron(IV)-oxo intermediate bound with **2** (i.e., **8**) would actually undergo H atom abstraction and subsequent cyclization *more slowly* than the corresponding complex with **10**. In other words, the *preferred* site of H atom abstraction may in fact be C6 rather than C7 during cyclization as well. In contrast, *preferential* H atom abstraction from C7 during cyclization would indicate an inversion in the regioselectivity of H6H-catalyzed radical initiation via an unknown mechanism—the details of which underlie precisely how the iron(IV)-complex interacts with the substrate

thereby directing the course of the reaction.

## Results and Discussion

In order to properly address the question of regioselectivity during the H6H-catalyzed reactions, it is necessary to disentangle the kinetics of H atom abstraction from all other elementary reactions (e.g., substrate binding) in the two catalytic cycles of H6H. In the case of hydroxylation, H atom abstraction is product-determining, and thus at pseudo-steady-state the **10**/**2** product ratio is a constant under fixed concentrations of  $\alpha$ KG and O<sub>2</sub> and given by

$${}^{7/6}k'_{hyd} = \frac{k'_{hyd}}{k'_{hyd}} = \frac{[\mathbf{10}]}{[\mathbf{2}]} \quad (1)$$

In this expression,  $k'_{hyd}$  and  $k'_{hyd}$  represent the *net rate constants*<sup>15</sup> associated with the net decomposition of the iron(IV)-oxo complex bound to **1** via hydroxylation at C7 versus C6, respectively (i.e., **5**→**10** and **5**→**2**, see Fig. 3). In other words, the net rate constants are indicative of all steps from H atom abstraction up to and including the first “irreversible” downstream elementary reaction. Therefore,  ${}^{7/6}k'_{hyd}$  reports on the relative partitioning between the two hydroxylation pathways (see Fig. 3).

The partitioning ratio  ${}^{7/6}k'_{hyd}$  was measured by stirring 16  $\mu$ M H6H in a well-ventilated vial under air (0.21 atm O<sub>2</sub>) with 100  $\mu$ M **1** in the presence of 10 mM  $\alpha$ KG, 400  $\mu$ M FeSO<sub>4</sub>, 4 mM sodium ascorbate and 50 mM sodium 4-(2-hydroxyethyl)-1-piperazineethanesulfonate (HEPES buffer) at pH 6.9 and room temperature. Under these conditions, the concentrations of the cosubstrates  $\alpha$ KG and O<sub>2</sub> remain essentially unchanged as the substrate **1** is consumed. Aliquots were taken at three time points within 20 min of initiating the reaction and subjected to LC-MS analysis whereupon the product peaks in the extracted ion chromatograms (EICs) were integrated to provide the respective signal intensities. Samples were diluted to ensure that both the **2** (high intensity) and **10** (low intensity) signals were simultaneously within the linear dynamic range of the instrument; however,

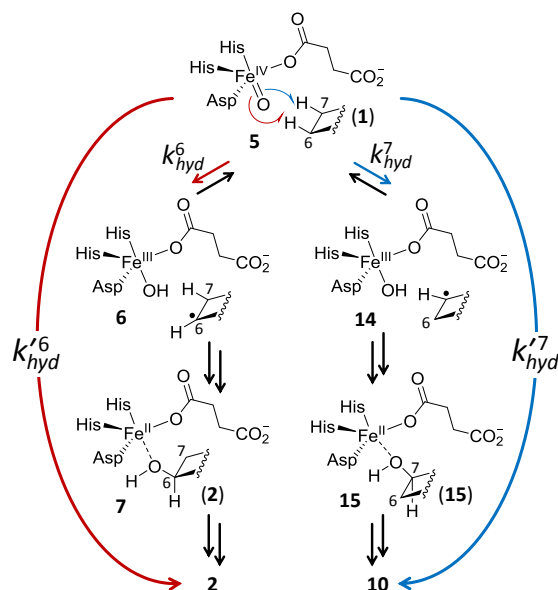


Figure 3: Partitioning model for the H6H-catalyzed hydroxylation of hyoscyamine (**1**) across the product determining step of H atom abstraction. The elementary reactions involving H atom abstraction from the *exo*-C6 versus *exo*-C7 sites are governed in the “forward” direction by the pseudo-first order rate constants  $k_{hyd}^6$  versus  $k_{hyd}^7$ , respectively. The steady-state decomposition of intermediate **5** to generate **2** versus **10** is governed by the net rate constants  $k'_{hyd}^6$  and  $k'_{hyd}^7$ . As discussed in the text, experimental results suggest that the  $5 \rightarrow 6$  and  $5 \rightarrow 14$  elementary reactions may be treated as irreversible.

an intercept correction was necessary to account for minimum detection cutoffs. This correction resulted in an approximately 15% increase in the observed ratios versus the uncorrected values. Both **1** and **2** were determined to have the same ionization efficiency, and scopolamine production was found to be negligible. The experiment was replicated five times yielding an average product ratio of  $0.0117 \pm 0.0003$  ( $\pm$  standard error of the mean,  $n = 5$ ). Experimental details including examination of controls are provided in the *Supporting Information*.

A similar experiment was also performed with the regio- and stereo-specifically deuterated substrate **11**, which was synthesized as described in the *Supporting Information*. In this case, the observed **13**/**2** ratio was found to be  $0.50 \pm 0.04$  ( $n = 5$ ) under the aforementioned reaction conditions. By analogy to Eqn. (1), this product ratio is indicative of the

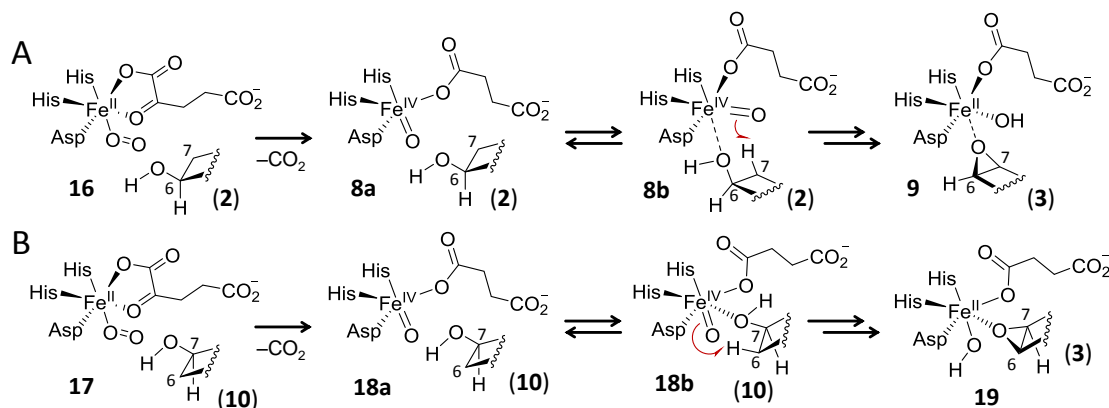


Figure 4: Mechanistic hypothesis for the reduction and inversion of the regioselectivity of H6H-catalyzed cyclization with respect to hydroxylation. This mechanism involves direct coordination of the substrate to the iron(IV)-oxo complex to facilitate dehydrogenation of the hydroxylated substrates **2** (A) and **10** (B).

net rate constant ratio given by<sup>16</sup>

$$^{7/6D}k'_{hyd} = \frac{k'^{7}_{hyd}}{k'^{6D}_{hyd}} = \frac{k'^{6}_{hyd}}{k'^{6D}_{hyd}} \cdot \frac{k'^{7}_{hyd}}{k'^{6}_{hyd}} = {}^Dk'^{6}_{hyd} \cdot ^{7/6}k'_{hyd}. \quad (2)$$

In Eqn. (2),  $k'^{6D}_{hyd}$  is the net rate constant for steady-state decomposition of the iron(IV)-oxo complex via D atom abstraction from C6 of **11**, and  ${}^Dk'^{6}_{hyd}$  is the corresponding kinetic isotope effect. Consequently, this KIE can be readily computed from the ratio

$${}^Dk'^{6}_{hyd} = ^{7/6D}k'_{hyd} / ^{7/6}k'_{hyd}, \quad (3)$$

yielding an observed value of  $43 \pm 3$ .

While the large primary deuterium KIE on  $k'^{6}_{hyd}$  exceeds the semiclassical limit,<sup>17</sup> it is consistent with previous reports of H atom abstraction catalyzed by other nonheme iron enzymes.<sup>18–21</sup> Moreover, it supports the hypothesis that the elementary reaction involving H atom abstraction (**5**→**6**) is most rate-limiting (i.e., has the greatest sensitivity index) with respect to decomposition of the iron(IV)-oxo species bound with substrate to hydroxylated product and may thus be reasonably modeled as “irreversible” in the

steady-state (i.e., it has a negligible reverse commitment along this sequence).<sup>22-26</sup> Assuming the same holds for H atom abstraction from C7 and decomposition proceeds first via H atom abstraction (see Fig. 3), we can make the approximations

$$^Dk_{hyd}^6 \approx ^Dk_{hyd}^7, \quad (4a)$$

$$k_{hyd}^7/k_{hyd}^6 \approx k_{hyd}^7/k_{hyd}^6, \quad (4b)$$

where  $k_{hyd}^6$  and  $k_{hyd}^7$  are the respective microscopic rate constants governing H atom abstraction from C6 (5→6) and C7 (5→14).<sup>26</sup> These conclusions are consistent with computational results on the mechanisms of non-heme iron oxidases in general.<sup>27</sup> Therefore,  $^{7/6}k_{hyd}^7/k_{hyd}^6$  not only reports on the relative partitioning between the two hydroxylation pathways but the regioselectivity of H atom abstraction itself.

In contrast to hydroxylation, the regioselectivity of H atom abstraction during cyclization cannot be inferred from the partitioning across a product-determining step. However, if H atom abstraction is effectively irreversible (see above) and the only elementary reactions to be significantly perturbed by deuteration or OH placement in the substrate are substrate binding and H atom abstraction, then the ratio of  $V$ -max for **1** versus that for H6H substrate  $Y = \mathbf{1}, \mathbf{12}, \mathbf{2}$  or **10** (see Fig. 2) can be approximated as

$$V_1/V_Y \approx \alpha_1 k_1/k_Y + (1 - \alpha_1). \quad (5)$$

Here,  $k_1/k_Y$  is the ratio of the microscopic rate constants governing H atom abstraction from **1** versus  $Y$ . The weighting term  $\alpha_1$  lies between 0 and 1 and depends only on the values of the rate constants in the catalytic cycle for **1**. The result (5) is trivial when  $Y = \mathbf{1}$  and follows immediately from an analysis by Northrop when  $Y = \mathbf{12}$ ,<sup>22,28</sup> which is generalized to the case where  $Y = \mathbf{2}, \mathbf{10}$  in the *Supporting Information*. Note that contributions to  $V_1/V_Y$  from substrate binding are eliminated, because the substrate concentrations are saturating. Therefore, once the weighting term  $\alpha_1$  is known, Eqn. (5) provides a means



to calculating the regioselectivity of H atom abstraction during cyclization from measurements of  $V$ -max.

Table 1: Measurements of  $V$ -max per unit concentration total enzyme ( $e_0$ ) for **1**, **12**, **2** and **10** in the presence of 10 mM  $\alpha$ KG and 0.21 atm  $O_2$  at room temperature and pH 6.9. Values are reported  $\pm$  one standard error of the estimate.

substrate	$V/e_0$ ( $\text{min}^{-1}$ )	$n$
<b>1</b>	$3.90 \pm 0.04$	3
<b>12</b>	$0.10 \pm 0.01$	3
<b>2</b>	$0.32 \pm 0.04$	3
<b>10</b>	$0.020 \pm 0.003$	3

Compounds **2** and **12** were prepared as described in the *Supporting Information*, and initial rates were measured for saturating **1**, **12**, **2** and **10** at fixed 10 mM  $\alpha$ KG and 0.21 atm  $O_2$ . In these experiments, reactions were initiated by the addition of 1.6  $\mu$ M H6H to solutions containing 100  $\mu$ M substrate, 10 mM  $\alpha$ KG, 400  $\mu$ M  $FeSO_4$ , 4 mM sodium ascorbate and 50 mM HEPES at pH 6.9 and room temperature in a well-ventilated vial under air. Reaction aliquots were taken at three time points ( $< 20\%$  conversion), quenched by addition to MeCN and analyzed by LC-MS to obtain the signal intensities of the product and residual substrate. Since the ionization efficiency of **1** is equal to that of **2** (see *Supporting Information*), the fraction of reaction in the aliquot could be calculated directly from peak integrations of the corresponding EICs. In contrast, the ionization efficiency of scopolamine (**3**) was found to be ca. 50% that of either hydroxylated substrate and was thus corrected accordingly prior to determining the fraction of reaction. From the fraction of reaction, the residual substrate concentration could be calculated and linearly regressed versus quench time to obtain the initial rate. The experiment was replicated three times for each substrate. Finally, decreasing or increasing the initial substrate concentration by 50% in all cases induced no significant change to the measured initial rates consistent with substrate saturation and no substrate inhibition at 100  $\mu$ M for all four substrates under the experimental conditions.

The  $V$ -max parameters measured for each of the four substrates are listed in Tbl. 1. We can solve for the weighting term  $\alpha_1$  from Eqn. (5) by using the previous result that  ${}^Dk_{hyd}^6 \approx {}^Dk_{hyd}'^6$  to obtain

$$\alpha_1 = \frac{V_1/V_{12} - 1}{{}^Dk_{hyd}^6 - 1} = 0.9 \pm 0.1.$$

As this does not differ significantly from unity, it implies that the primary deuterium isotope effect on H atom abstraction is almost fully expressed in the  $V$ -max parameter. This conclusion is consistent with the observation based on the values in Tbl. 1 that

$$V_1/V_{12} \approx 40 \approx {}^Dk_{hyd}^6.$$

Furthermore, since  $\alpha_1$  is measurably greater than 0, Eqn. (5) can also be used to get the ratio

$${}^{7/6}k_{cyc} = \frac{k_{cyc}^7}{k_{cyc}^6} = \frac{V_1/V_{10} + (\alpha_1 - 1)}{V_1/V_2 + (\alpha_1 - 1)}. \quad (6)$$

Substituting in the observed parameters, we thus obtain a value of  $16 \pm 3$  for  ${}^{7/6}k_{cyc}$ . Therefore, the preference for H atom abstraction from C7 versus C6 increases roughly 1400-fold in the H6H-catalyzed cyclization reaction compared to hydroxylation. However, whereas hydroxylation proceeds with a roughly 85 : 1 selectivity for H atom abstraction from C6 vs. C7, the magnitude of the selectivity for C7 vs. C6 during cyclization is reduced to ca. 16 : 1. Thus, while there is indeed an inversion in the site-selectivity between hydroxylation and cyclization, the effect is strongest during hydroxylation when no substrate hydroxyl group is present.

One possible hypothesis to help explain this result is that the hydroxyl groups of **2** and **10** coordinate the ferryl iron during the cyclization reaction as shown in Fig. 4. It has been established that the activated iron(IV)-oxo complex of a number of nonheme iron oxidases is pentacoordinate though computations suggest that octahedral complexes are also feasible.<sup>29–32</sup> Consequently, the coordination environment of the iron(IV)-oxo com-

plexes during H6H-catalyzed hydroxylation versus cyclization need not necessarily be the same. Thus, the complexes poised for H atom abstraction during cyclization (i.e., **8** & **18**) may resemble the putative product complexes expected to follow hydroxyl rebound during hydroxylation (i.e., **7** & **15**). Coordination of the substrate hydroxyl group would also permit inner-sphere electron transfer from the substrate to the iron center thereby facilitating the oxidative cyclizations of **2** and **10**. However, given that the ability of iron(IV)-oxo complexes to catalyze H atom abstraction appears to be highly sensitive to orientation,<sup>33</sup> direct coordination of a substrate to the ferryl iron may also serve to reorient the reactive oxo-ligand to favor H atom abstraction from the adjacent carbon (**8b**→**9** & **18b**→**19** in Fig. 4). In comparison with the unligated case during hydroxylation, the result may thus be a change in preference for abstraction of the C7-H atom but not without the observed reduction in overall regioselectivity.

As an initial test of this hypothesis, the ability of the iron(IV)-oxo complex to support an additional aquo or hydroxide ligand during the H6H-catalyzed hydroxylation reaction was investigated. When **1** was incubated with H6H and <sup>18</sup>O<sub>2</sub> in natural abundance water, the majority (87%) of the hydroxylated product was found to contain <sup>18</sup>O consistent with a previous report.<sup>9</sup> Likewise, in the converse experiment with natural abundance O<sub>2</sub> and <sup>18</sup>O-water, only 10% of the product contained <sup>18</sup>O. However, when the same experiments were conducted with the deuterated substrate **12**, 57% of the product contained <sup>18</sup>O with <sup>18</sup>O<sub>2</sub> and natural abundance water becoming 40% in the presence of natural abundance O<sub>2</sub> and <sup>18</sup>O-water (see *Supporting Information*). Hence, there appears to be significant exchange of the reactive oxo-ligand in the ferryl complex with solvent during the H6H-catalyzed hydroxylation reaction. This implies that the iron(IV)-oxo complex can indeed support an additional ligand,<sup>34,35</sup> and is thus consistent with the hypothesis that the substrate directly coordinates the iron(IV)-oxo complex during the H6H-catalyzed cyclization reaction while not doing so during hydroxylation.

## Conclusions

H6H catalysis represents a useful system for studying how the course of enzymatic reactions involving highly reactive intermediates can be controlled. During the H6H-catalyzed hydroxylation of hyoscyamine, nearly all of the reaction flux proceeds via H atom abstraction from the *exo*-C6 position. However, there is more than a thousand-fold inversion in this regioselectivity when a hydroxyl group is present at the adjacent carbon. One possible mechanism to explain this inversion involves direct coordination of the hydroxylated substrate to the Fe(IV)-oxo complex during cyclization (as depicted in **8b** and **18b**). Thus, in the absence of coordination, the oxo ligand may be directed so as to favor H atom abstraction and subsequent hydroxylation at C6. In the presence of coordination, however, the reactive oxo ligand may be shifted to abstract the H atom from the adjacent carbon being most optimized for abstraction of the *exo*-C7 H atom in particular. Nevertheless, other explanations for the inversion in regiochemistry that do not necessarily involve direct coordination of the substrate to the iron-center (e.g., substrate repositioning due to changes in H-bonding, steric interactions or solvent reorganization within the active site) remain very much open possibilities. Further evaluation of these and other mechanistic hypotheses regarding the two H6H-catalyzed reactions is thus expected to provide additional insights into how nonheme iron enzymes direct the course of the reactions they catalyze.

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**Supporting Information Available** The Supporting Information is available free of charge on the ACS Publications website DOI: xx.xxxx/jacs.xxxxxxxx. Details regarding H6H assays, analytical controls, synthesis of compounds and kinetic analysis.

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Graphical TOC Entry

