



## The Rate-Limiting Step of O<sub>2</sub> Activation in the $\alpha$ -Ketoglutarate Oxygenase Factor Inhibiting Hypoxia Inducible Factor

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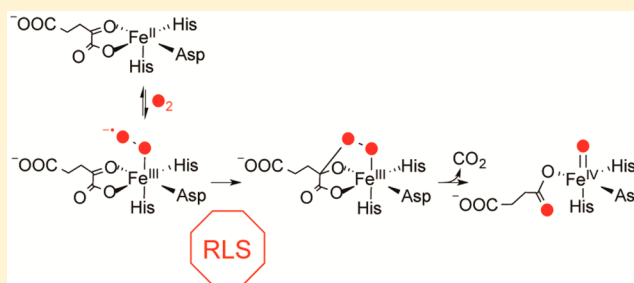
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### S Supporting Information

**ABSTRACT:** Factor inhibiting HIF (FIH) is a cellular O<sub>2</sub>-sensing enzyme, which hydroxylates the hypoxia inducible factor-1 $\alpha$ . Previously reported inverse solvent kinetic isotope effects indicated that FIH limits its overall turnover through an O<sub>2</sub> activation step (Hangasky, J. A., Saban, E., and Knapp, M. J. (2013) *Biochemistry* 52, 1594–1602). Here we characterize the rate-limiting step for O<sub>2</sub> activation by FIH using a suite of mechanistic probes on the second order rate constant  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ . Steady-state kinetics showed that the rate constant for O<sub>2</sub> activation was slow ( $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}} = 3500 \text{ M}^{-1} \text{ s}^{-1}$ ) compared

with other non-heme iron oxygenases, and solvent viscosity assays further excluded diffusional encounter with O<sub>2</sub> from being rate limiting on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ . Competitive oxygen-18 kinetic isotope effect measurements ( $^{18}k_{\text{cat}}/K_{\text{M}(\text{O}_2)} = 1.0114(5)$ ) indicated that the transition state for O<sub>2</sub> activation resembled a cyclic peroxohemiketal, which precedes the formation of the ferryl intermediate observed in related enzymes. We interpret this data to indicate that FIH limits its overall activity at the point of the nucleophilic attack of Fe-bound O<sub>2</sub><sup>−</sup> on the C-2 carbon of  $\alpha$ KG. Overall, these results show that FIH follows the consensus mechanism for  $\alpha$ KG oxygenases, suggesting that FIH may be an ideal enzyme to directly access steps involved in O<sub>2</sub> activation among the broad family of  $\alpha$ KG oxygenases.



Mammalian cells respond to decreased cellular pO<sub>2</sub> levels through the enzyme-catalyzed reaction of O<sub>2</sub> with the hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$  or HIF).<sup>1</sup> HIF mediates the transcription of hundreds of genes in response to hypoxia<sup>2</sup> with the functions of the gene products ranging from glucose and iron metabolism to cell proliferation and angiogenesis.<sup>3,4</sup> Factor inhibiting HIF (FIH) is a non-heme Fe(II)/ $\alpha$ KG oxygenase that turns-off the transcriptional activity of HIF<sup>5,6</sup> by hydroxylating the  $\beta$ -carbon of Asn<sup>803</sup> within the C-terminal activation domain (CTAD) of HIF (Scheme 1).<sup>7–9</sup> Because O<sub>2</sub> activation chemistry is central to hypoxia sensing by HIF, identifying the chemical steps involved in O<sub>2</sub> activation may point the way to methods for perturbing HIF-controlled gene expression.

FIH is proposed to follow the consensus mechanism for Fe(II)/ $\alpha$ KG oxygenases (Scheme 1) for which the steps are supported to varying degrees by spectroscopic, computational, and kinetic studies.<sup>10–15</sup> VTVH MCD methodologies have been used to spectroscopically identify the release of the aquo ligand upon substrate binding to FIH<sup>16</sup> and other Fe(II)/ $\alpha$ KG oxygenases including TauD and CAS.<sup>17,18</sup> O<sub>2</sub> is thought to bind as a ferric superoxide at the open coordination site and then attacks the C-2 carbonyl of  $\alpha$ KG to ultimately form succinate and a ferryl intermediate. The molecular details following O<sub>2</sub> activation, including isolation of the ferryl intermediate and

observation of HAT have been characterized in the Fe(II)/ $\alpha$ KG oxygenases TauD<sup>15,19–24</sup> and P4H<sup>25</sup> and the related Fe(II)/ $\alpha$ KG halogenases CytC3<sup>26</sup> and SyrB2.<sup>27</sup>

In contrast to the steps following ferryl formation, those steps of O<sub>2</sub> activation are poorly understood. Computational studies suggest that nucleophilic attack on the C-2 carbonyl of  $\alpha$ KG is the rate-limiting step on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ , with a cyclic peroxohemiketal proposed as the transition state.<sup>28–30</sup> Although this reaction sequence is supported by pre-steady-state kinetics and an oxygen-18 kinetic isotope effect (<sup>18</sup>O KIE) study of TauD,<sup>31</sup> insight into O<sub>2</sub> activation is limited because HAT or product release is rate-limiting in TauD and other well-characterized  $\alpha$ KG oxygenases.<sup>20,32,33</sup> Consequently, O<sub>2</sub> activation is too rapid to allow for the identification of any intermediates prior to the ferryl.

Recent studies showed that the rate-limiting step for FIH differed from that of other characterized  $\alpha$ KG oxygenases.<sup>16,34</sup> Upon FIH binding to CTAD, there is partial retention of the aquo ligand<sup>16</sup> suggesting that aquo release may be less facile in FIH than in other enzymes. The inverse SKIE on  $k_{\text{cat}}$ <sup>34</sup> for FIH

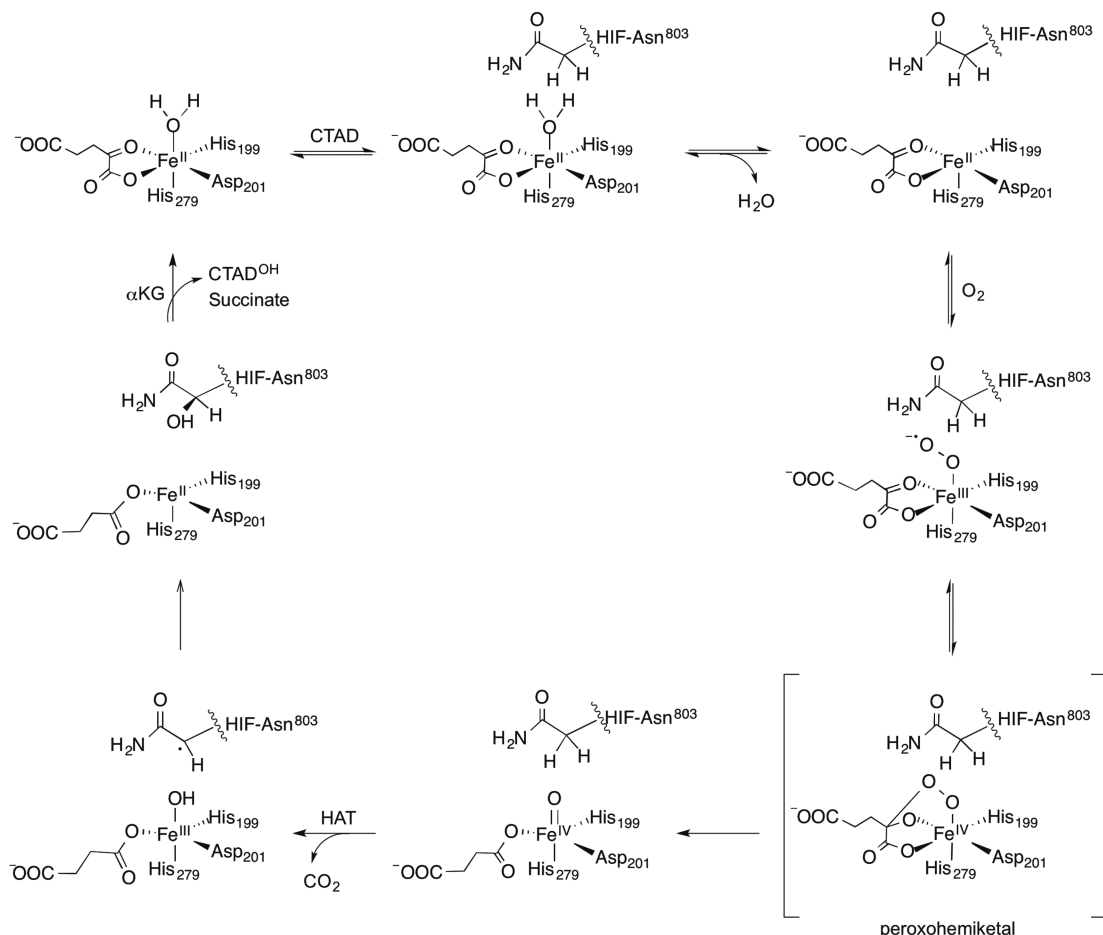
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Scheme 1. Consensus Chemical Mechanism of  $\alpha$ KG Oxygenases, Adapted for FIH



indicates that the aquo release reaches equilibrium prior to an irreversible step that is the overall rate-limiting step in FIH; in other words, the overall rate limiting step either precedes or coincides with the formation of the ferryl. This suggests that FIH either deviates from the consensus chemical mechanism or could provide a unique system to access other steps of mechanistic interest. In this work, we probe  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ , which focuses on the limited subset of steps that are involved in binding and reacting with  $\text{O}_2$ , to understand  $\text{O}_2$  activation by FIH. Steady-state kinetics under conditions of varied solvent viscosity indicated that diffusional encounter of  $\text{O}_2$  with FIH was not rate limiting on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ . Furthermore, we determined the  $^{18}\text{O}$  KIE on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  ( $^{18}k_{\text{cat}}/K_{\text{M}(\text{O}_2)} = 1.0114(5)$ ), identifying the rate-limiting step as formation of the peroxohemiketal. This showed that the chemical steps of  $\text{O}_2$  activation on FIH followed the consensus mechanism, indicating that FIH only differs from other  $\alpha$ KG oxygenases in that this  $\text{O}_2$  activation step is the overall rate-limiting step during turnover.<sup>34</sup>

## MATERIALS AND METHODS

**Materials.** All reagents were purchased from commercial sources and used as received unless noted. The sequences of the synthetic 19- and 39-mer CTAD peptides corresponded to the C-terminal activation domain (CTAD) of HIF-1 $\alpha$ <sup>788–806</sup> and HIF-1 $\alpha$ <sup>788–826</sup>, respectively, with a Cys<sup>800</sup> → Ala point mutation. Peptides were purchased from EZBiolab (Carmel,

Indiana, USA) with free N- and C-termini. The CTAD<sup>788–806</sup> peptide (purity >95%) was used without further purification; however CTAD<sup>788–826</sup> was purchased as a desalted peptide and purified to >95% purity using RP-HPLC as previously described.<sup>34</sup>

**Protein Expression and Purification.** FIH was overexpressed in *Escherichia coli* and purified as previously reported.<sup>34,35</sup> Thrombin cleavage of the His<sub>6</sub> tag led to three additional residues preceding the native sequence of FIH on the N-terminus (NH<sub>2</sub>-Gly-Ser-His-). The purity of the protein (>95%) was assessed through SDS-PAGE.

**Steady-State Kinetic Assays with Varying  $\text{O}_2$ .** All assays were performed in an AtmosBag (Sigma-Aldrich) with the  $\text{O}_2$  concentration of the reaction buffers equilibrated to the  $\text{O}_2$  partial pressure within the bag. The atmosphere of the bag was equilibrated for 30 min with a controlled mixture of  $\text{N}_2$  and  $\text{O}_2$ . HEPES, pH 7.00 (50 mM) was gently stirred for 5 min in a 37.0 °C water bath to equilibrate the reaction buffer with the atmosphere, and then the  $\text{O}_2$  concentration was measured using a Clarke electrode.

Steady-state assays in which  $\text{O}_2$  was the varied substrate (0.020–1 mM) utilized a fixed CTAD<sup>788–826</sup> concentration of either 80  $\mu\text{M}$  ( $\sim K_{\text{M}(\text{CTAD})}$ ) or 150  $\mu\text{M}$  ( $\sim 2K_{\text{M}(\text{CTAD})}$ ) and saturating concentrations of  $\text{FeSO}_4$  (25  $\mu\text{M}$ ),  $\alpha$ KG (100  $\mu\text{M}$ ), and ascorbate (2 mM), prepared in 50 mM HEPES, pH 7.00. Upon addition of all reagents except FIH, the reaction mixture (45  $\mu\text{L}$ ) was incubated at 37.0 °C for an additional 2 min. The enzyme stock was equilibrated to the atmosphere by gently

pipetting the solution down the side of the microcentrifuge tube, before injecting an aliquot (5  $\mu$ L) to initiate the assays. Reaction aliquots were removed throughout a 3 min time course, quenched in 75% acetonitrile/0.2% TFA (20  $\mu$ L) saturated with 3,5-dimethoxy-4-hydroxycinnamic acid and analyzed for the initial rate of formation of CTAD<sup>OH</sup> using a Bruker microFlex MALDI-TOF-MS. Initial rates were determined as previously described<sup>34</sup> and fit to the Michaelis–Menten equation resulting in the apparent kinetic parameters  $k_{\text{cat}}$ ,  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ , and  $K_{\text{M}(\text{O}_2)}$ .

**Solvent Viscosity Effect.** Assays to test for rate-limiting diffusional encounter of O<sub>2</sub> utilized a fixed CTAD<sup>788–826</sup> concentration of 80  $\mu$ M ( $\sim K_{\text{M}(\text{CTAD})}$ ) and saturating concentrations of FeSO<sub>4</sub> (25  $\mu$ M),  $\alpha$ KG (100  $\mu$ M), and ascorbate (2 mM), with the exception of the addition of sucrose (25% w/w) to the 50 mM HEPES, pH 7.00, to give a relative viscosity of  $\eta/\eta_0 = 2.4$ .<sup>36</sup> Reactions were performed as described above to determine initial rates with O<sub>2</sub> as the varied substrate, which were then fit to the Michaelis–Menten equation.

**Steady-State Kinetic Assays with CTAD<sup>788–806</sup>.** Assays in which CTAD<sup>788–806</sup> was varied (0.10–4.6 mM) were performed at 37.0 °C in 50 mM HEPES, pH 7.00, and contained ascorbate (2 mM),  $\alpha$ KG (1 mM), FeSO<sub>4</sub> (50  $\mu$ M), and an ambient O<sub>2</sub> concentration (217  $\mu$ M). Assays in which  $\alpha$ KG was varied (0.005–1 mM) were also performed at 37.0 °C in 50 mM HEPES, pH 7.00, and contained ascorbic acid (2 mM), FeSO<sub>4</sub> (50  $\mu$ M), and CTAD<sup>788–806</sup> (750  $\mu$ M), with an ambient O<sub>2</sub> concentration (217  $\mu$ M). Reagents were mixed and incubated at 37.0 °C for 2 min before initiating turnover with enzyme (5–20  $\mu$ M). At predetermined time points, aliquots were quenched in 75% acetonitrile/0.2% TFA (20  $\mu$ L) saturated with  $\alpha$ -cyano-4-hydroxycinnamic acid. Initial rates were determined as described above and fit to the Michaelis–Menten equation resulting in the apparent kinetic parameters  $k_{\text{cat}}$ ,  $k_{\text{cat}}/K_{\text{M}}$ , and  $K_{\text{M}}$ .

**<sup>18</sup>O KIE Sample Preparation and Analysis.** Assays used to determine the <sup>18</sup>O KIE contained  $\alpha$ KG (1.0 mM), CTAD<sup>788–806</sup> (250  $\mu$ M), FeSO<sub>4</sub> (50  $\mu$ M), and O<sub>2</sub> (280  $\mu$ M) in 50 mM HEPES, pH 7.00. Buffer was equilibrated to ambient O<sub>2</sub> concentration (280  $\mu$ M) by gently stirring for 2 days at 21 °C. All reagents were prepared freshly using the equilibrated buffer and gently mixed to make a common reaction mixture. This reaction mixture was injected into a 10 mL crimp vial sealed with a butyl rubber stopper (Geo-Microbial Technologies, Inc.; Ochelata, OK), ensuring all air was removed. After a 3 min incubation of the vial at 37.0 °C, each reaction was initiated with an injection (20  $\mu$ L) of a high concentration FIH stock that had been equilibrated to room temperature (21 °C). Reactions were quenched using 6 M HCl, 3.5 M ZnCl<sub>2</sub> (40  $\mu$ L) after an extended reaction time such that the fractional conversion based on O<sub>2</sub> was as high as 35%. An aliquot (5  $\mu$ L) was removed to determine the reaction progress by measuring CTAD<sup>OH</sup> formation using a Bruker MALDI-TOF-MS. The quantity of CTAD<sup>OH</sup> produced was used to determine the fractional conversion of O<sub>2</sub> for each quenched reaction. The sealed crimp vials containing the quenched reactions were stored inverted and submerged in water until analysis by isotope-ratio mass spectrometry (IRMS).

The <sup>18</sup>O KIE samples were carefully transferred into pre-evacuated 25 mL glass vessels fitted with a glass high vacuum stopcock (Chemglass). The filling procedure is described in

detail by Emerson et al.<sup>37</sup> but briefly consisted of flushing the neck of the bottle with a gentle stream of CO<sub>2</sub> to displace air followed by introduction of sample water from a small diameter tubing ( $\sim 3$  mm) to the bottleneck. Upon opening the stopcock slowly, sample water is drawn into the evacuated bottle until the bottle is approximately half full. The headspace gases and water are then equilibrated by gently shaking in a 25 °C water bath overnight. Before IRMS analysis, the sample water was removed using aspiration, leaving  $\sim 0.5$  mL of sample in the bottle. Headspace gases in the bottle were then analyzed for the  $\delta^{18}\text{O}$  of O<sub>2</sub> using a gas chromatograph interfaced to an Elementar Isoprime IRMS.<sup>38</sup> All  $\delta^{18}\text{O}$  isotopic values were reported using standard delta notation relative to the Vienna standard mean ocean water (VSMOW).<sup>39</sup> Equation 1 was used to convert the  $\delta^{18}\text{O}$  to an  $R$  value (<sup>18</sup>O/<sup>16</sup>O isotopic ratio), where  $R_{\text{std}}$  is the standard isotopic value for O<sub>2</sub> in air (0.0020531)<sup>40</sup> and  $R_f$  is the <sup>18</sup>O/<sup>16</sup>O isotopic ratio at O<sub>2</sub> fractional conversion  $f$ .

$$R_f = \left( \frac{\delta^{18}\text{O}}{1000} + 1 \right) R_{\text{std}} \quad (1)$$

To determine the <sup>18</sup>O/<sup>16</sup>O isotopic ratio at  $t = 0$  ( $R_0$ ), a sample was prepared from the common reaction stock containing  $\alpha$ KG (1.0 mM), CTAD<sup>788–806</sup> (250  $\mu$ M), FeSO<sub>4</sub> (50  $\mu$ M), and O<sub>2</sub> (280  $\mu$ M) in 50 mM HEPES, pH 7.00, and injected into a sealed crimp vial. After incubation for 3 min at 37.0 °C, an aliquot of 50 mM HEPES, pH 7.00 (20  $\mu$ L), was injected into the vial immediately followed by an injection (40  $\mu$ L) of 6 M HCl, 3.5 M ZnCl<sub>2</sub> to quench the reaction.

The <sup>18</sup>O KIE was determined by fitting  $R_f/R_0$  vs  $f$  to eq 2, where  $f$  is the fractional conversion of O<sub>2</sub> in the reaction aliquot,  $R_f$  is the <sup>18</sup>O/<sup>16</sup>O isotope ratio of the aliquot, and  $R_0$  is the <sup>18</sup>O/<sup>16</sup>O isotope ratio of the blank.

$$\frac{R_f}{R_0} = (1 - f)^{(1/^{18}\text{O-KIE}) - 1} \quad (2)$$

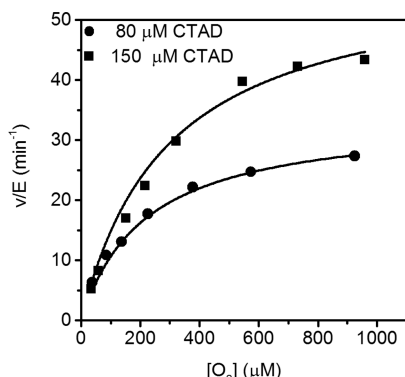
## RESULTS AND DISCUSSION

The O<sub>2</sub> activation mechanisms of Fe(II)/ $\alpha$ KG oxygenases are of enormous interest due to the biomedical significance of these enzymes.<sup>12,41,42</sup> FIH hydroxylates the HIF transcription factor for hypoxia sensing, and other members are involved in processes such as DNA and RNA repair and histone demethylation,<sup>12,43,44</sup> placing some of these enzymes into biological roles that are more concerned with regulation than with bulk turnover of metabolites. A crucial mechanistic feature of these enzymes is O<sub>2</sub> activation to form an active oxidant, identified in several enzymes as a ferryl species.<sup>19,25,26</sup> Despite the importance of the chemical steps leading up to formation of the ferryl, these steps remain largely uncharacterized. Because the overall rate-limiting step for FIH either precedes or coincides with ferryl formation,<sup>34</sup> FIH could be an excellent enzyme to interrogate steps involved in O<sub>2</sub> activation that are common to other  $\alpha$ KG oxygenases, provided that FIH follows the consensus mechanism.

**Steady-State Kinetics with Varying O<sub>2</sub>.** To characterize the steps limiting the rate of O<sub>2</sub> activation by FIH, steady-state kinetic assays with O<sub>2</sub> as the varied substrate were performed using a fixed CTAD<sup>788–826</sup> concentration. Because our assays used subsaturating CTAD concentration due to reagent expenses, these steady-state assays used two different

CTAD<sup>788–826</sup> concentrations to measure the second order rate constant,  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ .

The initial rate data using 80  $\mu\text{M}$  CTAD<sup>788–826</sup> was fit to the Michaelis–Menten equation with kinetic parameters of  $k_{\text{cat}}^{\text{app}} = 33 \pm 3 \text{ min}^{-1}$  and  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}} = 0.17 \pm 0.03 \mu\text{M}^{-1} \text{ min}^{-1}$  (Figure 1, Table 1). The steady-state assays with varying  $\text{O}_2$



**Figure 1.** Steady-state kinetics with varying  $\text{O}_2$ . Reactions contained FIH (0.25–0.5  $\mu\text{M}$ ), ascorbate (2 mM),  $\alpha\text{KG}$  (100  $\mu\text{M}$ ),  $\text{FeSO}_4$  (25  $\mu\text{M}$ ), and CTAD<sup>788–826</sup> (80  $\mu\text{M}$ , ●, or 150  $\mu\text{M}$ , ■) in 50 mM HEPES, pH 7.00, 37.0 °C.

**Table 1.** Apparent Kinetic Parameters for FIH with Varied  $\text{O}_2$  Concentration<sup>a</sup>

[CTAD <sup>788–826</sup> ] ( $\mu\text{M}$ )	$k_{\text{cat}}^{\text{app}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$ ( $\mu\text{M}^{-1} \text{ min}^{-1}$ )	$K_{\text{M}(\text{O}_2)}^{\text{app}}$ ( $\mu\text{M}$ )
80	$33 \pm 3.0$	$0.17 \pm 0.03$	$200 \pm 40$
150	$54 \pm 4.0$	$0.21 \pm 0.04$	$270 \pm 50$

<sup>a</sup>Reactions contained ascorbate (2 mM),  $\alpha\text{KG}$  (100  $\mu\text{M}$ ),  $\text{FeSO}_4$  (25  $\mu\text{M}$ ), and CTAD<sup>788–826</sup> in 50 mM HEPES, pH 7.00, 37.0 °C.

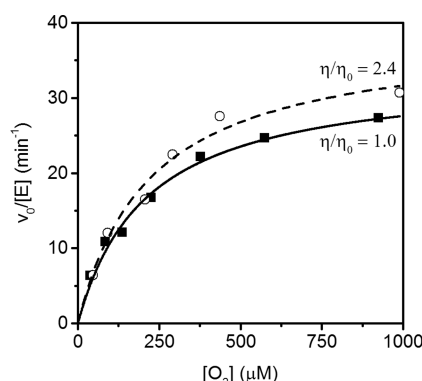
using an increased CTAD concentration (150  $\mu\text{M}$ ) resulted in  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}} = 0.21 \pm 0.04$ , which is statistically equivalent to the  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$  at 80  $\mu\text{M}$  CTAD<sup>788–826</sup>. These results indicated that  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  was independent of CTAD concentration as expected for the sequential consensus mechanism (Scheme 1). The high value for the Michaelis constant ( $K_{\text{M}(\text{O}_2)}^{\text{app}} > 200 \mu\text{M}$ ) was in agreement with previous reported values (90–237  $\mu\text{M}$ ) obtained using oxygen consumption assays and  $^{14}\text{CO}_2$  capture assays<sup>45,46</sup> and is thought to be essential for a proportionate sensory response by FIH to increasing  $\text{pO}_2$ .

When converted into standard units,  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}} = 3.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ , it was clear that the rate constant for  $\text{O}_2$  activation in FIH was significantly slower than those for non-heme iron oxygenases that are not involved in  $\text{O}_2$  concentration sensing, such as TauD ( $1.5 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ),<sup>33,47</sup> tyrosine hydroxylase ( $6.0 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ ),<sup>48</sup> and lipoxygenase ( $\sim 5.0 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ ).<sup>49,50</sup> In contrast, the slow rate constant for  $\text{O}_2$  activation and the high Michaelis constant for  $\text{O}_2$  found for FIH are found in those non-heme iron oxygenases implicated in  $\text{O}_2$  sensing, such as PHD2<sup>51</sup> and Jumonji C domain-containing histone demethylases.<sup>52</sup> The small magnitude of  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  for FIH is intriguing, raising the potential for FIH and these putative  $\text{O}_2$  sensors to use an unusual strategy for  $\text{O}_2$  activation. Although it is likely that a slow chemical step limits  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  and  $\text{O}_2$

activation, it was necessary to test diffusional encounter as a possibility for the rate-limiting step.

**Solvent Viscosity Effect.** To test for diffusional encounter with  $\text{O}_2$  as a partially rate-limiting step on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ , we performed steady-state assays under varied solvent viscosity. Although  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  is orders of magnitude slower than expected for a diffusional process (ca.  $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ), we could not dismiss the possibility that there was an unfavorable pre-equilibrium leading to a very small fraction of FIH being competent for reaction upon collision. For diffusion controlled processes,  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  would decrease in the presence of added viscosogen due to a lower diffusion rate as observed for other enzymes such as superoxide dismutase<sup>53,54</sup> and carbonic anhydrase.<sup>55,56</sup>

Our assays were performed as described above in the presence and absence of the viscosogen sucrose, giving a final relative viscosity ( $\eta/\eta_0$ ) of 1.0 and 2.4, respectively (Figure 2).



**Figure 2.** Steady-state kinetics with varying  $\text{O}_2$ : 0% sucrose (solid line,  $\eta/\eta_0 = 1$ ) and 25% sucrose (dashed line,  $\eta/\eta_0 = 2.4$ ). Assays contained ascorbate (2 mM),  $\alpha\text{KG}$  (100  $\mu\text{M}$ ),  $\text{FeSO}_4$  (25  $\mu\text{M}$ ), CTAD<sup>788–826</sup> (80  $\mu\text{M}$ ), and sucrose (0% or 25%) in 50 mM HEPES, pH 7.00, 37.0 °C.

At increased solvent viscosity, the resulting kinetic parameter  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}} = 0.18 \pm 0.04 \mu\text{M}^{-1} \text{ min}^{-1}$  was indistinguishable from the kinetic parameter collected in the absence of viscosogen (Table 2). The resulting insignificant solvent viscosity effect on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  indicated that diffusional encounter with  $\text{O}_2$  did not limit the rate constant for  $\text{O}_2$  activation in FIH.

**Table 2.** Solvent Viscosity Effect on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$ <sup>a</sup>

$\eta/\eta_0$	$k_{\text{cat}}^{\text{app}}$ ( $\text{min}^{-1}$ )	$k_{\text{cat}}/K_{\text{M}(\text{O}_2)}^{\text{app}}$ ( $\mu\text{M}^{-1} \text{ min}^{-1}$ )	$K_{\text{M}(\text{O}_2)}^{\text{app}}$ ( $\mu\text{M}$ )
1.0	$33 \pm 3.0$	$0.17 \pm 0.03$	$200 \pm 40$
2.4	$39 \pm 3.0$	$0.18 \pm 0.04$	$220 \pm 43$

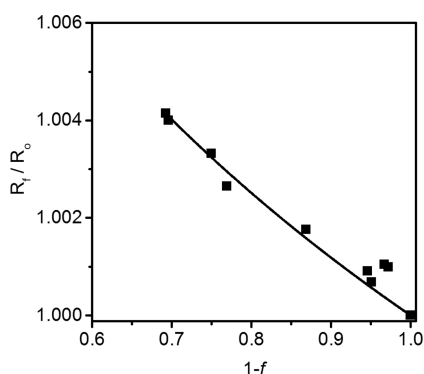
<sup>a</sup>Assays contained ascorbate (2 mM),  $\alpha\text{KG}$  (100  $\mu\text{M}$ ),  $\text{FeSO}_4$  (25  $\mu\text{M}$ ), CTAD<sup>788–826</sup> (80  $\mu\text{M}$ ), and sucrose (0% or 25% w/w) in 50 mM HEPES, pH 7.00, 37.0 °C.

Diffusion limited rate constants may be found with enzymes that have achieved catalytic perfection, reflecting a physiological role that requires bulk turnover of a large quantity of substrate. For example, diffusion limited rate constants fit well for SOD's cellular function to scavenge superoxide to minimize oxidative damage.<sup>57</sup> As an  $\text{O}_2$  sensor, one would imagine that FIH turnover could be limited by collisional encounter. However, the absence of a viscosity effect on  $k_{\text{cat}}/K_{\text{M}(\text{O}_2)}$  and  $k_{\text{cat}}$  showed



that FIH was not diffusionally limited under varied  $O_2$  concentration. This implicates a chemical step as rate-limiting under conditions of low  $O_2$  concentration, consistent with prior results indicating that  $k_{cat}$  was limited by a step that followed aquo release but preceded the HAT.<sup>34</sup>

**Competitive  $^{18}O$  Kinetic Isotope Effect.** Because  $k_{cat}/K_{M(O_2)}$  encompasses all steps between diffusional collision of FIH with  $O_2$  through the subsequent irreversible step, the  $^{18}O$  heavy atom isotope effect on this rate constant is an ideal reporter of the rate-limiting step. We employed competitive  $^{18}O/^{16}O$  KIE measurements using  $O_2$  at natural isotopic abundance to identify the rate limiting step on  $k_{cat}/K_{M(O_2)}$  in FIH. The  $^{18}O/^{16}O$  isotopic abundance of residual  $O_2$  was measured by IRMS from quenched reactions of FIH, which were fit to eq 2 resulting in a  $^{18}k_{cat}/K_{M(O_2)} = 1.0114(5)$  (Figure 3). Because the typical range of values for  $^{18}k_{cat}/K_{M(O_2)}$  is 1.00–

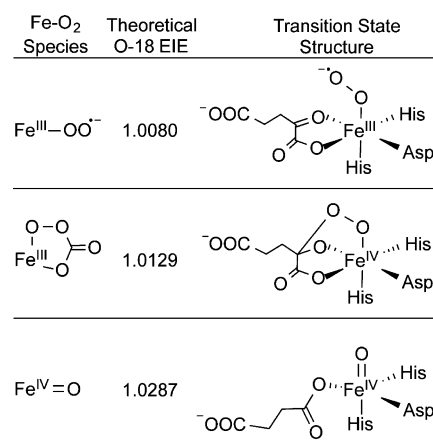


**Figure 3.**  $^{18}O$  fractionation ( $R_f/R_0$ ) versus the fractional conversion of  $O_2$  in quenched reactions of FIH. Each reaction contained  $\alpha$ KG (1.0 mM), CTAD<sup>788–806</sup> (250  $\mu$ M),  $FeSO_4$  (50  $\mu$ M), and  $O_2$  (280  $\mu$ M) in 50 mM HEPES, pH 7.00, 37.0  $^{\circ}C$ . Data are fit to eq 2;  $^{18}k_{cat}/K_{M(O_2)} = 1.0114(5)$ .

1.03, this places  $O_2$  activation by FIH in a clear context when considered next to the mechanisms followed by other non-heme Fe enzymes.<sup>31</sup>

$^{18}k_{cat}/K_{M(O_2)}$  reflects the changes in O–O bonding between molecular  $O_2$  and the transition state of the kinetically irreversible step on  $k_{cat}/K_{M(O_2)}$ .<sup>58,59</sup> Because the  $^{18}O$  equilibrium isotope effect ( $^{18}O$  EIE) provides an upper limit for the  $^{18}O$  KIE,<sup>60</sup> previously calculated  $^{18}O$  EIEs for the equilibrium  $Fe^{2+} + O_2 \rightleftharpoons X$  provide an excellent yardstick for the transition state structure based upon the value of  $^{18}k_{cat}/K_{M(O_2)}$  (Chart 1).<sup>31</sup> For example, the calculated  $^{18}O$  EIE for  $X = Fe^{3+}(O_2^-)$  is small ( $^{18}O$  EIE = 1.0080),<sup>31</sup> meaning that rate-limiting formation of this intermediate would lead to a correspondingly small value for  $^{18}k_{cat}/K_{M(O_2)}$ . A larger  $^{18}O$  EIE is calculated for  $X =$  ferric peroxo-carbonate ( $^{18}O$  EIE = 1.0129),<sup>31</sup> a structure resembling the putative peroxohemiketal, which is in good agreement with the observed  $^{18}k_{cat}/K_{M(O_2)}$  for FIH, suggesting that the rate-limiting step for FIH proceeds through a transition state that resembles this structure. In contrast, the observed  $^{18}k_{cat}/K_{M(O_2)}$  is inconsistent with the  $^{18}O$  EIE calculated for  $X =$  ferryl ( $^{18}O$  EIE = 1.0287),<sup>31</sup> indicating that the ferryl intermediate is formed after the rate-limiting step on  $k_{cat}/K_{M(O_2)}$  in FIH.

**Chart 1. Proposed Transition State Structures from  $^{18}O$  EIE<sup>a</sup>**



<sup>a</sup> $^{18}O$  EIE values were calculated in ref 31.

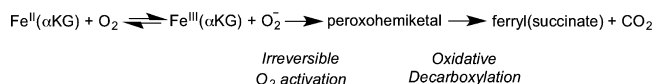
$^{18}k_{cat}/K_{M(O_2)}$  has been utilized to study the  $O_2$  activation pathways of other non-heme iron enzymes including soluble methane monooxygenase,<sup>61</sup> ACCO,<sup>62</sup> TauD,<sup>31</sup> HppE,<sup>31</sup> and tyrosine hydroxylase.<sup>63</sup> In each case, the magnitude of  $^{18}k_{cat}/K_{M(O_2)}$  provided important insight into the chemical strategy followed for  $O_2$  activation. For those enzymes in which  $O_2$  binds at  $Fe^{2+}$ , the initial step is the reversible formation of a  $Fe^{3+}(O_2^-)$  adduct, which subsequently requires electrons from cofactor or (co)substrate to activate the O–O bond for chemistry. In the case of TauD, this activation takes the form of a nucleophilic attack of the  $Fe^{3+}(O_2^-)$  on the C-2 keto position of  $\alpha$ KG.<sup>31</sup>

The  $^{18}k_{cat}/K_{M(O_2)}$  for FIH (1.0114(5)) is very similar to that observed for TauD ( $^{18}k_{cat}/K_{M(O_2)} = 1.0102$ ),<sup>31</sup> indicating a common transition state structure for  $\alpha$ KG decarboxylation in these two enzymes. The larger value for the  $^{18}k_{cat}/K_{M(O_2)}$  for FIH than for TauD is likely due to the slower turnover rate of FIH. The observed  $^{18}k_{cat}/K_{M(O_2)}$  will approach the  $^{18}O$  EIE when the forward commitment, which is the ratio of the forward and reverse rate constants for disappearance of the species immediately preceding the rate-limiting step, is small as might be expected for the slower chemistry in FIH.

The chemical strategy for  $O_2$  activation in  $\alpha$ KG oxygenases was predicted by DFT calculations to proceed through the nucleophilic attack on the  $\alpha$ KG cofactor<sup>28,29</sup> and is supported by the  $^{18}O$  KIE results. The self-consistent field (SCF) calculations predicted that decomposition of the initially formed cyclic peroxohemiketal intermediate was barrierless<sup>29</sup> leading to decarboxylation with the formation of a  $Fe^{2+}$ (peroxysuccinate) intermediate prior to formation of the ferryl(succinate). Because the decarboxylation is irreversible,  $k_{cat}/K_{M(O_2)}$  only reports on steps between the collision with  $O_2$  and this decarboxylation step (Scheme 2).

Studies to date that address  $O_2$  activation chemistry in  $\alpha$ KG oxygenases have relied on steady-state mechanistic probes and

**Scheme 2.  $O_2$  Activation in  $\alpha$ KG Oxygenases**



point mutagenesis<sup>22,45,64–67</sup> and suggest that hydrogen bonding contacts to the  $\alpha$ KG play an important role in facilitating decarboxylation. Further insight into oxidative decarboxylation has been hampered by the identity of the rate limiting steps in TauD and other  $\alpha$ KG oxygenases. In the cases of TauD,<sup>20</sup> P4H,<sup>25</sup> CytC3,<sup>32</sup> and by analogy DAOCS<sup>68</sup> and the histone demethylase KDM4E,<sup>69</sup> HAT by the ferryl or product release are partially rate-limiting on turnover at elevated O<sub>2</sub> concentration, preventing the accumulation of any species involved in O<sub>2</sub> activation during the pre-steady-state. A crucial difference between these other enzymes and FIH is that several lines of evidence indicate that decarboxylation is rate limiting in FIH, suggesting that FIH may allow direct access to steps involved in O<sub>2</sub> activation.

## CONCLUSIONS

We have used multiple kinetic probes to characterize O<sub>2</sub> activation by FIH. Unlike other previously characterized Fe(II)/ $\alpha$ KG enzymes, turnover in FIH is fully limited by the rate of O<sub>2</sub> activation. This kinetic feature is consistent with the function of FIH as an O<sub>2</sub> sensor; strong oxidants such as the ferryl would be short-lived, ensuring tight coupling between O<sub>2</sub> activation and CTAD hydroxylation. It may be possible that other biomedically important Fe(II)/ $\alpha$ KG enzymes such as the JmjC and JmjD domain-containing hydroxylases and PHD2 employ a similar mechanistic strategy to regulate their function. If so, rate-limited O<sub>2</sub> activation may be a more common mechanistic feature among Fe(II)/ $\alpha$ KG oxygenases than is currently appreciated.

## ASSOCIATED CONTENT

### Supporting Information

Control experiments involving steady-state kinetics with the CTAD<sup>788–806</sup> peptide, as well as steady-state kinetics in the presence and absence of ascorbate. The material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

## ABBREVIATIONS

ACCO, 1-aminocyclopropane-1-carboxylic acid oxidase;  $\alpha$ KG,  $\alpha$ -ketoglutarate; CAS, clavaminic synthase; CTAD, C-terminal transactivation domain; DFT, density functional theory; EIE, equilibrium isotope effect; FIH, factor-inhibiting HIF; HAT, hydrogen atom transfer; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF, hypoxia inducible factor-1 $\alpha$ ; HppE, (S)-2-hydroxypropyl-1-phosphonate epoxidase; IRMS, isotope ratio mass spectrometry; KIE, kinetic isotope effect; MALDI-TOF-MS, matrix assisted laser desorption ionization-time-of-flight-mass spectrometry; MCD, magnetic circular dichroism; P4H, prolyl-4-hydroxylase; PHD2, prolyl hydrox-

ylase domain 2; SKIE, solvent kinetic isotope effect; SCF, self-consistent field; SOD, superoxide dismutase; TauD, taurine dioxygenase; TFA, trifluoroacetic acid; VTVH, variable temperature variable field

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