The Rate-Limiting Step of O₂ Activation in the α-Ketoglutarate Oxygenase Factor Inhibiting Hypoxia Inducible Factor

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Supporting Information

ABSTRACT: Factor inhibiting HIF (FIH) is a cellular O₂-sensing enzyme, which hydroxylates the hypoxia inducible factor-1α. Previously reported inverse solvent kinetic isotope effects indicated that FIH limits its overall turnover through an O₂ activation step (Hangasky, J. A., Saban, E., and Knapp, M. J. (2013) Biochemistry 52, 1594–1602). Here we characterize the rate-limiting step for O₂ activation by FIH using a suite of mechanistic probes on the second order rate constant k₉/O₂. Steady-state kinetics showed that the rate constant for O₂ activation was slow (k₉/K₉ = 3500 M⁻¹ s⁻¹) compared with other non-heme iron oxygenases, and solvent viscosity assays further excluded diffusional encounter with O₂ from being rate limiting on k₉/K₉. Competitive oxygen-18 kinetic isotope effect measurements (18k₉/K₉ = 1.01(1)) indicated that the transition state for O₂ 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 O₂ on the C-2 carbon of αKG. Overall, these results show that FIH follows the consensus mechanism for αKG oxygenases, suggesting that FIH may be an ideal enzyme to directly access steps involved in O₂ activation among the broad family of αKG oxygenases.

Mammalian cells respond to decreased cellular pO₂ levels through the enzyme-catalyzed reaction of O₂ with the hypoxia inducible factor-1α (HIF-1α or HIF).¹ HIF mediates the transcription of hundreds of genes ranging from glucose and iron metabolism to cell proliferation and angiogenesis.²,³ Factor inhibiting HIF (FIH) is a non-heme Fe(II)/αKG oxygenase that turns-off the transcriptional activity of HIF⁵⁶ by hydroxylating the α-carbon of Asn⁹²³ within the C-terminal activation domain (CTAD) of HIF (Scheme 1).⁷⁻⁹ Because O₂ activation chemistry is central to hypoxia sensing by HIF, identifying the chemical steps involved in O₂ activation may point the way to methods for perturbing HIF-controlled gene expression.

FIH is proposed to follow the consensus mechanism for Fe(II)/αKG oxygenases (Scheme 1) for which the steps are supported to varying degrees by spectroscopic, computational, and kinetic studies.¹⁰⁻¹⁵ VTVH MCD methodologies have been used to spectroscopically identify the release of the aquo ligand upon substrate binding to FIH¹⁶ and other Fe(II)/αKG oxygenases including TauD and CAS.¹⁷,¹⁸ O₂ is thought to bind as a ferric superoxide at the open coordination site and then attacks the C-2 carboxyl of αKG to ultimately form succinate and a ferryl intermediate. The molecular details following O₂ activation, including isolation of the ferryl intermediate and observation of HAT have been characterized in the Fe(II)/αKG oxygenases TauD¹⁵,¹⁹⁻二十四 and P4H²⁵ and the related Fe(II)/αKG halogenases CytC³²⁶ and SyrB².²⁷

In contrast to the steps following ferryl formation, those steps of O₂ activation are poorly understood. Computational studies suggest that nucleophilic attack on the C-2 carboxyl of αKG is the rate-limiting step on k₉/K₉ with a cyclic peroxohemiketal proposed as the transition state.²⁸⁻⁻³⁰ Although this reaction sequence is supported by pre-steady-state kinetics and an oxygen-18 kinetic isotope effect (¹⁸O KIE) study of TauD,³¹ insight into O₂ activation is limited because HAT or product release is rate-limiting in TauD and other well-characterized αKG oxygenases.²⁰,³²,³³ Consequently, O₂ 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 αKG oxygenases.¹⁶,³⁴ Upon FIH binding to CTAD, there is partial retention of the aquo ligand¹⁶ suggesting that aquo release may be less facile in FIH than in other enzymes. The inverse SKIE on k₉ 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 $k_{\text{cat}}/K_M(O_2)$, which focuses on the limited subset of steps that are involved in binding and reacting with $O_2$ to understand $O_2$ activation by FIH. Steady-state kinetics under conditions of varied solvent viscosity indicated that diffusional encounter of $O_2$ with FIH was not rate limiting on $k_{\text{cat}}/K_M(O_2)$. Furthermore, we determined the $^{18}O$ KIE on $k_{\text{cat}}/K_M(O_2)$ ($^{18}k_{\text{cat}}/K_M(O_2) = 1.0114(5)$), identifying the rate-limiting step as formation of the peroxohemiketal. This showed that the chemical steps of $O_2$ activation on FIH followed the consensus mechanism, indicating that FIH only differs from other $\alpha$KG oxygenases in that this $O_2$ activation step is the overall rate-limiting step during turnover.34

**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-1α788−806 and HIF-1α788−826, respectively, with a Cys800 → Ala point mutation. Peptides were purchased from EZBiolab (Carmel, Indiana, USA) with free N- and C-termini. The CTAD788−806 peptide (purity >95%) was used without further purification; however CTAD788−826 was purchased as a desalted peptide and purified to >95% purity using RP-HPLC as previously described.34

**Protein Expression and Purification.** FIH was overexpressed in *Escherichia coli* and purified as previously reported.34,35 Thrombin cleavage of the His$_6$ tag led to three additional residues preceding the native sequence of FIH on the N-terminus (NH$_2$-Gly-Ser-His-). The purity of the protein (>95%) was assessed through SDS-PAGE.

**Steady-State Kinetic Assays with Varying $O_2$.** All assays were performed in an AtmosBag (Sigma-Aldrich) with the $O_2$ concentration of the reaction buffers equilibrated to the $O_2$ partial pressure within the bag. The atmosphere of the bag was equilibrated for 30 min with a controlled mixture of $N_2$ and $O_2$. 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 $O_2$ concentration was measured using a Clarke electrode.

Steady-state assays in which $O_2$ was the varied substrate (0.020−1 mM) utilized a fixed CTAD788−826 concentration of either 80 μM ($\sim K_M(\text{CTAD})$) or 150 μM ($\sim 2K_M(\text{CTAD})$) and saturating concentrations of FeSO$_4$ (25 μM), αKG (100 μM), and ascorbate (2 mM), prepared in 50 mM HEPES, pH 7.00. Upon addition of all reagents except FIH, the reaction mixture (45 μL) was incubated at 37.0 °C for an additional 2 min. The enzyme stock was equilibrated to the atmosphere by gently

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

![Scheme 1](image-url)
Steady-State Kinetic Assays with CTAD\textasciitilde{788–806}. Assays in which CTAD\textasciitilde{788–806} 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), αKG (1 mM), and an ambient O2 concentration (217 μM). Assays in which αKG 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), FeSO4 (50 μM), and CTAD\textasciitilde{788–806} (750 μM), with an ambient O2 concentration (217 μM). Reagents were mixed and incubated at 37.0 °C for 2 min before initiating turnover with enzyme (5–20 μM). At predetermined time points, aliquots were quenched in 75% acetonitrile/0.2% TFA (20 μL) saturated with α-cyano-4-hydroxycinnamic acid. Initial rates were determined as described above and fit to the Michaelis–Menten equation resulting in the apparent kinetic parameters $k_{cat}$, $k_{cat}/K_M(O_2)$, and $K_M(O_2)$.

**Solvent Viscosity Effect.** Assays to test for rate-limiting diffusional encounter of O2 utilized a fixed CTAD\textasciitilde{788–828} concentration of 80 μM ($K_M(CTAD)$) and saturating concentrations of FeSO4 (25 μM), αKG (100 μM), 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 $\eta/\eta_0 = 2.4^{36}$. Reactions were performed as described above to determine initial rates with O2 as the varied substrate, which were then fitted to the Michaelis–Menten equation.

**18O KIE Sample Preparation and Analysis.** Assays used to determine the $^{18}$O KIE contained αKG (1.0 mM), CTAD\textasciitilde{788–806} (250 μM), FeSO4 (50 μM), and O2 (280 μM) in 50 mM HEPES, pH 7.00. Buffer was equilibrated to ambient O2 concentration (280 μM) 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 μL) 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 ZnCl2 (40 μL) after an extended reaction time such that the fractional conversion based on O2 was as high as 35%. An aliquot (5 μL) was removed to determine the reaction progress by measuring CTADOH formation using a Bruker MALDI-TOF-MS. The quantity of CTADOH produced was used to determine the fractional conversion of O2 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 $^{18}$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.\textsuperscript{37} but briefly consisted of flushing the neck of the bottle with a gentle stream of CO2 to displace air followed by introduction of sample water from a small diameter tubing (~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 a needle, leaving ~0.5 mL of sample in the bottle. Headspace gases in the bottle were then analyzed for the $^{18}$O/16O isotope ratio using a gas chromatograph interfaced to a Varian 1210 IRMS.\textsuperscript{38} All $^{18}$O/16O isotopic values were reported using standard delta notation relative to the Vienna Standard Mean Ocean Water (VSMOW).\textsuperscript{39} Equation 1 was used to convert the $^{18}$O/16O to an R value ($^{18}$O/16O isotopic ratio), where $R_{std}$ is the standard isotopic value for O2 in air (0.0020531)\textsuperscript{40} and R is the $^{18}$O/16O isotopic ratio at O2 fractional conversion $f$.

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

To determine the $^{18}$O/16O isotopic ratio at $t = 0$ ($R_0$), a sample was prepared from the common reaction stock containing αKG (1.0 mM), CTAD\textasciitilde{788–806} (250 μM), FeSO4 (50 μM), and O2 (280 μM) 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 μL), was injected into the vial immediately followed by an injection (40 μL) of 6 M HCl, 3.5 M ZnCl2 to quench the reaction. The $^{18}$O KIE was determined by fitting $R_f/R_0$ vs $f$ to eq 2, where $f$ is the fractional conversion of O2 in the reaction aliquot, $R_f$ is the $^{18}$O/16O isotope ratio of the aliquot, and $R_0$ is the $^{18}$O/16O isotope ratio of the blank.

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

**RESULTS AND DISCUSSION**

The O2 activation mechanisms of Fe(II)/αKG oxygenases are of enormous interest due to the biomedical significance of these enzymes.\textsuperscript{12,41,42} 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.\textsuperscript{12,43,44} 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 O2 activation to form an active oxidant, identified in several enzymes as a ferryl species.\textsuperscript{19,25,26} 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,\textsuperscript{34} FIH could be an excellent enzyme to interrogate steps involved in O2 activation that are common to other αKG oxygenases, provided that FIH follows the consensus mechanism.

**Steady-State Kinetics with Varying O2.** To characterize the steps limiting the rate of O2 activation by FIH, steady-state kinetic assays with O2 as the varied substrate were performed using a fixed CTAD\textasciitilde{788–828} concentration. Because our assays used saturating CTAD concentration due to reagent expenses, these steady-state assays used two different
using a increased CTAD concentration (150 μM) resulted in $k_{cat}/K_{M(O2)} = 0.21 ± 0.04$, which is statistically equivalent to the $k_{cat}/K_{M(O2)}$ at 80 μM CTAD788–826. These results indicated that $k_{cat}/K_{M(O2)}$ was independent of CTAD concentration as expected for the sequential consensus mechanism (Scheme 1). The high value for the Michaelis constant ($K_{M(O2)} > 200$ μM) was in agreement with previous reported values (90–237 μM) obtained using oxygen consumption assays and $^{14}$CO$_2$ capture assays45,46 and is thought to be essential for a proportionate sensory response by FIH to increasing pO$_2$.

When converted into standard units, $k_{cat}/K_{M(O2)} = 3.5 \times 10^{5}$ M$^{-1}$ s$^{-1}$, it was clear that the rate constant for O$_2$ activation in FIH was significantly slower than those for non-heme iron oxygenases that are not involved in O$_2$ concentration sensing, such as TauD (1.5 \times 10^{6} M^{-1} s^{-1}), 33,47 tyrosine hydroxylase (6.0 \times 10^{4} M^{-1} s^{-1}), 48 and lipoxygenase (~5.0 \times 10^{3} M^{-1} s^{-1}). 49,50 In contrast, the slow rate constant for O$_2$ activation and the high Michaelis constant for O$_2$ found for FIH are consistent with non-heme iron oxygenases implicated in O$_2$ sensing, such as PHD2 23 and Jumonji C domain-containing histone demethylases. 52 The small magnitude of $k_{cat}/K_{M(O2)}$ for FIH is intriguing, raising the potential for FIH and these putative O$_2$ sensors to use an unusual strategy for O$_2$ activation. Although it is likely that a slow chemical step limits $k_{cat}/K_{M(O2)}$ and 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 O$_2$ as a partially rate-limiting step on $k_{cat}/K_{M(O2)}$, we performed steady-state assays under varied solvent viscosity. Although $k_{cat}/K_{M(O2)}$ is orders of magnitude slower than expected for a diffusional process (ca. 1 \times 10^{8} M^{-1} 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, $k_{cat}/K_{M(O2)}$ would decrease in the presence of added viscosogen due to a lower diffusion rate as observed for other enzymes such as superoxide dismutase$^{53,54}$ and carbonic anhydrase$^{55,56}$.

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

**Table 1. Apparent Kinetic Parameters for FIH with Varied O$_2$ Concentration**

| [CTAD$^{788-826}$] (μM) | $k_{cat}^{app}$ (μM) | $k_{cat}/K_{M(O2)}^{app}$ (μM$^{-1}$ min$^{-1}$) | $K_{M(O2)}^{app}$ (μM) |
|------------------------|---------------------|---------------------------------|---------------------|
| 80                     | 53 ± 3.0            | 0.17 ± 0.03                     | 200 ± 40            |
| 150                    | 54 ± 4.0            | 0.21 ± 0.04                     | 270 ± 50            |

*Reactions contained ascorbate (2 mM), αKG (100 μM), FeSO$_4$ (25 μM), and CTAD$^{788-826}$ (80 μM, ■, or 150 μM, ○) in 50 mM HEPES, pH 7.00, 37.0 °C.*

At increased solvent viscosity, the resulting kinetic parameter $k_{cat}/K_{M(O2)} = 0.18 ± 0.04$ μM$^{-1}$ min$^{-1}$ was indistinguishable from the kinetic parameter collected in the absence of viscosogen (Table 2). The resulting insignificant solvent viscosity effect on $k_{cat}/K_{M(O2)}$ indicated that diffusional encounter with O$_2$ did not limit the rate constant for O$_2$ activation in FIH.

**Table 2. Solvent Viscosity Effect on $k_{cat}/K_{M(O2)}$**

| $\eta/\eta_o$ | $k_{cat}^{app}$ (μM$^{-1}$ min$^{-1}$) | $k_{cat}/K_{M(O2)}^{app}$ (μM$^{-1}$ min$^{-1}$) | $K_{M(O2)}^{app}$ (μM) |
|--------------|-------------------------------------|-----------------------------------------------|---------------------|
| 1.0          | 33 ± 3.0                            | 0.17 ± 0.03                                   | 200 ± 40            |
| 2.4          | 39 ± 3.0                            | 0.18 ± 0.04                                   | 220 ± 43            |

*Assays contained ascorbate (2 mM), αKG (100 μM), FeSO$_4$ (25 μM), CTAD$^{788-826}$ (80 μM) 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. 57 As an O$_2$ sensor, one would imagine that FIH turnover could be limited by collisional encounter. However, the absence of a viscosity effect on $k_{cat}/K_{M(O2)}$ and $k_{cat}$ showed
that FIH was not diffusionally limited under varied O₂ concentration. This implicates a chemical step as rate-limiting under conditions of low O₂ concentration, consistent with prior results indicating that k_cat was limited by a step that followed aquo release but preceded the HAT. ³⁴

**Competitive ¹⁸O Kinetic Isotope Effect.** Because k_cat/K_M(O₂) encompasses all steps between diffusional collision of FIH with O₂ through the subsequent irreversible step, the ¹⁸O heavy atom isotope effect on this rate constant is an ideal reporter of the rate-limiting step. We employed competitive ¹⁸O/¹⁶O KIE measurements using O₂ at natural isotopic abundance to identify the rate limiting step on k_cat/K_M(O₂) in FIH. The ¹⁸O/¹⁶O isotopic abundance of residual O₂ was measured by IRMS from quenched reactions of FIH, which were fit to eq 2 resulting in a ¹⁸k_cat/K_M(O₂) = 1.0114(S) (Figure 3). Because the typical range of values for ¹⁸k_cat/K_M(O₂) is 1.00–1.03, this places O₂ activation by FIH in a clear context when considered next to the mechanisms followed by other non-heme Fe enzymes. ³¹

¹⁸k_cat/K_M(O₂) reflects the changes in O–O bonding between molecular O₂ and the transition state of the kinetically irreversible step on k_cat/K_M(O₂).³⁸,³⁹ Because the ¹⁸O equilibrium isotope effect (¹⁸O EIE) provides an upper limit for the ¹⁸O KIE,⁶⁰ previously calculated ¹⁸O EIEs for the equilibrium Fe²⁺ + O₂ ⇔ X provide an excellent yardstick for the transition state structure based upon the value of ¹⁸k_cat/K_M(O₂) (Chart 1).³¹ For example, the calculated ¹⁸O EIE for X = Fe³⁺(O₂⁻) is small (¹⁸O EIE = 1.0080),³¹ meaning that rate-limiting formation of this intermediate would lead to a correspondingly small value for ¹⁸k_cat/K_M(O₂). A larger ¹⁸O EIE is calculated for X = ferric peroxy-carbonate (¹⁸O EIE = 1.0129),³¹ a structure resembling the putative peroxyhemiketal, which is in good agreement with the observed ¹⁸k_cat/K_M(O₂) for FIH, suggesting that the rate-limiting step for FIH proceeds through a transition state that resembles this structure. In contrast, the observed ¹⁸k_cat/K_M(O₂) is inconsistent with the ¹⁸O EIE calculated for X = ferryl (¹⁸O EIE = 1.0287),³¹ indicating that the ferryl intermediate is formed after the rate-limiting step on k_cat/K_M(O₂) in FIH.

**Chart 1. Proposed Transition State Structures from ¹⁸O EIE**

| Species          | Theoretical O-18 EIE | Transition State Structure |
|------------------|----------------------|---------------------------|
| FeIII−O₂⁻         | 1.0080               | [Chart 1]                 |
| O−O−              | 1.0129               | [Chart 1]                 |
| FeIV=O            | 1.0287               | [Chart 1]                 |

¹⁸O EIE values were calculated in ref 31.

¹⁸k_cat/K_M(O₂) has been utilized to study the O₂ activation pathways of other non-heme iron enzymes including soluble methane monoxygenase,⁶¹ ACCO,⁶² TauD,⁶¹ HppE,³¹ and tyrosine hydroxylase.⁶³ In each case, the magnitude of ¹⁸k_cat/K_M(O₂) provided important insight into the chemical strategy followed for O₂ activation. For those enzymes in which O₂ binds at Fe²⁺, the initial step is the reversible formation of a Fe³⁺(O₂⁻) 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 Fe³⁺(O₂⁻) on the C-2 keto position of αKG.³¹

The ¹⁸k_cat/K_M(O₂) for FIH (1.0114(S)) is very similar to that observed for TauD (¹⁸k_cat/K_M(O₂) = 1.0102)³¹ indicating a common transition state structure for αKG decarboxylation in these two enzymes. The larger value for the ¹⁸k_cat/K_M(O₂) for FIH than for TauD is likely due to the slower turnover rate of FIH. The observed ¹⁸k_cat/K_M(O₂) will approach the ¹⁸O 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 O₂ activation in αKG oxygenases was predicted by DFT calculations to proceed through the nucleophilic attack on the αKG cofactor²⁸,²⁹ and is supported by the ¹⁸O KIE results. The self-consistent field (SCF) calculations predicted that decomposition of the initially formed cyclic peroxyhemiketal intermediate was barrierless²⁹ leading to decarboxylation with the formation of a Fe⁵⁺(peroxysuccinate) intermediate prior to formation of the ferryl(succinate). Because the decarboxylation is irreversible, k_cat/K_M(O₂) only reports on steps between the collision with O₂ and this decarboxylation step (Scheme 2).

Studies to date that address O₂ activation chemistry in αKG oxygenases have relied on steady-state mechanistic probes and
point mutagenesis\textsuperscript{22,45,64--67} and suggest that hydrogen bonding contacts to the αKG 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 αKG oxygenases. In the cases of TauD,\textsuperscript{20} P4H,\textsuperscript{32} CytC,\textsuperscript{32} and by analogy DAOCS\textsuperscript{68} and the histone demethylase KDM4E,\textsuperscript{69} HAT by the ferryl or product release are partially rate-limiting on turnover at elevated O$_2$ concentration, preventing the accumulation of any species involved in O$_2$ 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$_2$ activation.

**CONCLUSIONS**

We have used multiple kinetic probes to characterize O$_2$ activation by FIH. Unlike other previously characterized Fe(II)/αKG enzymes, turnover in FIH is fully limited by the rate of O$_2$ activation. This kinetic feature is consistent with the function of FIH as an O$_2$ sensor; strong oxidants such as the ferryl would be short-lived, ensuring tight coupling between O$_2$ activation and CTAD hydroxylation. It may be possible that other biomedically important Fe(II)/αKG 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$_2$ activation may be a more common mechanistic feature among Fe(II)/αKG oxygenases than is currently appreciated.

**ASSOCIATED CONTENT**

1. Supporting Information
Control experiments involving steady-state kinetics with the CTAD\textsuperscript{78--80} 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**

ACC0, 1-aminocyclopropane-1-carboxylic acid oxidase; αKG, α-ketoglutarate; CAS, clavamine 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-1α; HppE, (S)-2-hydroxypropyl-l-phenylphosphonic epoxide; 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, prolly-4-hydroxylase; PHD2, prolly hyrox-

**REFERENCES**

(1) Taabazuing, C. Y., Hangsanky, J. A., and Knapp, M. J. (2014) Oxygen sensing strategies in mammals and bacteria. J. Inorg. Biochem. 133, 63–72.

(2) Mole, D. R., Blancher, C., Copley, R. R., Pollard, P. J., Gleadle, J. M., Raguossius, J., and Ratcliffe, P. J. (2009) Genome-wide Association of Hypoxia-inducible Factor (HIF)-1α and HIF-2α DNA Binding with Expression Profiling of Hypoxia-inducible Transcripts. J. Biol. Chem. 284, 16767–16775.

(3) Ke, Q., and Costa, M. (2006) Hypoxia-Inducible Factor-1 (HIF-1). Mol. Pharmacol. 70, 1469–1480.

(4) Semenza, G. L. (2003) Targeting HIF-1 for cancer therapy. Nat. Rev. Cancer 3, 721–732.

(5) Metzen, E., and Ratcliffie, P. J. (2004) HIF hydroxylation and cellular oxygen sensing. Biol. Chem. 385, 223–230.

(6) Schofield, C. J., and Ratcliffe, P. J. (2004) Oxygen sensing by HIF-hydroxylases. Nat. Rev. Mol. Cell. Biol. 5, 343–354.

(7) Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L., and Bruck, R. K. (2002) FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. Genes Dev. 16, 1466–1471.

(8) McNeill, L. A., Hewitson, K. S., Claridge, T. D., Seibel, J. F., Horsfall, L. E., and Schofield, C. J. (2002) Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses hydroxylation at the beta-carbon of asparagine-803. Biochem. J. 367, 571–575.

(9) Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J., and Whitelaw, M. L. (2002) Asparagine hydroxylation of the HIF transcriptional activation domain is a hivoxic switch. Science 295, 858–861.

(10) Solomon, E. I., Light, K. M., Liu, L. V., Snesec, M., and Wong, S. D. (2013) Geometric and Electronic Structure Contributions to Function in Non-heme Iron Enzymes. Acc. Chem. Res. 46, 2725–2739.

(11) Blomberg, M. R. A., Borowski, T., Himo, F., Liao, R. Z., and Siegbahn, P. E. M. (2014) Quantum Chemical Studies of Mechanisms for Metalloenzymes. Chem. Rev. 114, 3601–3658.

(12) Haisinger, R. P. (2004) Fe(II)/α-Ketoglutarate-dependent hydroxylases and related enzymes. Crit. Rev. Biochem. Mol. Biol. 39, 21–68.

(13) Aik, W., McDonough, M. A., Thalhammer, A., Chowdhury, R., and Schofield, C. J. (2012) Role of the jelly-roll fold in substrate binding by 2-oxoglutarate oxygenases. Curr. Opin. Struct. Biol. 22, 691–700.

(14) Bollinger, J. M., Price, J. C., Hoffart, L. M., Barr, E. W., and Krebs, C. (2005) Mechanism of Taurine:α-Ketoglutarate Dioxygenase (TauD) from Escherichia coli. Eur. J. Inorg. Chem. 2005, 4245–4254.

(15) Grzyska, P. K., Appelman, E. H., Hausinger, R. P., and Proshlyakov, D. A. (2010) Insights into the mechanism of an iron dioxygenase by resolution of steps following the FeIV=O species. Proc. Natl. Acad. Sci. U. S. A. 107, 3982–3987.

(16) Light, K. M., Hangsanky, J. A., Knapp, M. J., and Solomon, E. I. (2013) Spectroscopic Studies of the Mononuclear Non-Heme Fe-II Enzyme FIH: Second-Sphere Contributions to Reactivity. J. Am. Chem. Soc. 135, 9665–9674.

(17) Neidig, M. L., Brown, C. D., Light, K. M., Fujimori, D. G., Nolan, E. M., Price, J. C., Barr, E. W., Bollinger, J. M., Krebs, C., Walsh, C. T., and Solomon, E. I. (2007) CD and MCD of CytC3 and taurine dioxygenase: role of the facial triad in alpha-KG-dependent oxygenases. J. Am. Chem. Soc. 129, 14224–14231.

(18) Zhou, J., Kelly, W. L., Bachmann, B. O., Gunsior, M., Townrsnd, C. A., and Solomon, E. I. (2001) Spectroscopic studies of substrate interactions with clavamine synthase 2, a multifunctional alpha-KG-dependent non-heme iron enzyme: Correlation with mechanisms and reactivities. J. Am. Chem. Soc. 123, 7388–7398.
(19) Price, J. C., Barr, E. W., Tirupati, B., Bollinger, J. M., and Krebs, C. (2003) The First Direct Characterization of a High-Valent Iron Intermediate in the Reaction of a Kctoglutarate-Dependent Dioxygenase: A High-Spin Fe(IV) Complex in Taurine/a-Ketoglutarate Dioxygenase (Taud). J. Am. Chem. Soc. 125, 13008–13009.

(20) Price, J. C., Barr, E. W., Glass, T. E., Krebs, C., and Bollinger, J. M. (2003) Evidence for Hydrogen Abstraction from C1 of Taurine by the High-Spin Fe(IV) Intermediate Detected during Oxygen Activation by Taurine:a-Ketoglutarate Dioxygenase (Taud). J. Am. Chem. Soc. 125, 8108–8109.

(21) Gryska, P. K., Ryle, M. J., Monterosso, G. R., Liu, J., Ballou, D. P., and Hausinger, R. P. (2005) Steady-State and Transient Kinetic Analyses of Taurine/a-Ketoglutarate Dioxygenase: Effects of Oxygen Concentration, Alternative Sulfonates, and Active-Site Variants on the FeIV-oxo Intermediate. Biochemistry 44, 3854–3855.

(22) L. M., Wolbach, T. A., Henshaw, T. F., Monterosso, G. R., Ryle, M. J., and Hausinger, R. P. (2004) Direct Detection of Oxygen Intermediates in the Non-Heme Fe Enzyme Taurine/a-Ketoglutarate Dioxygenase. J. Am. Chem. Soc. 126, 1022–1023.

(23) Ryle, M. J., Padmakumar, R., and Hausinger, R. P. (1999) Stopped-Flow Kinetic Analysis of Escherichia coli Taurine/a-Ketoglutarate Dioxygenase: Interactions with α-Ketoglutarate, Taurine, and Oxygen. Biochemistry 38, 15278–15286.

(24) Hoffart, L. M., Barr, E. W., Geyer, R. B., Bollinger, J. M., and Krebs, C. (2006) Direct spectroscopic detection of a C-H-cleaving high-spin Fe(IV) complex in a prolyl-4-hydroxylase. Proc. Natl. Acad. Sci. U. S. A. 103, 14738–14743.

(25) Galonic, D. P., Barr, E. W., Matthews, M. L., Koch, G. M., Yonce, J. R., Walsh, C. T., Bollinger, J. M., Jr., Krebs, C., and Riggs-Gelasco, P. J. (2007) Spectroscopic evidence for a high-spin Br-Fe(IV)-Oxo intermediate in the α-Ketoglutarate-dependent halogenase SyrB2. J. Am. Chem. Soc. 129, 1031–1032.

(26) Mirica, L. M., McCusker, K. P., Munos, J. W., Liu, H., and Klinman, J. P. (2008) 18O kinetic isotope effects in non-heme iron enzymes: Probing the nature of Fe/O intermediates. J. Am. Chem. Soc. 130, 8122–8123.

(27) Brookes, C., Barr, E. W., Walsh, C. T., Bollinger, J. M., Jr., and Krebs, C. (2007) Two interconverting Fe(IV) intermediates in aliphatic chlorination by the halogenase CytC3. Nat. Chem. Biol. 3, 113–116.

(28) Price, J. C., Barr, E. W., Hoffart, L. M., Krebs, C., and Bollinger, J. M. (2005) Kinetic dissection of the catalytic mechanism of taurine:α-ketoglutarate dioxygenase (Taud) from Escherichia coli. Biochemistry 44, 8138–8147.

(29) Hanagata, J. A., Saban, E., and Knapp, M. J. (2013) Inverse Solvent Isotope Effects Arising from Substrate Triggering in the Factor Inhibiting Hypoxia Inducible Factor. Biochemistry 52, 1594–1602.

(30) Chen, Y. H., Comeaux, L. M., Herbst, R. W., Saban, E., Kennedy, D. C., Maroney, M. J., and Knapp, M. J. (2008) Coordination changes and auto-hydroxylation of FIH-1: Uncoupled O-2-activation in a human hypoxia sensor. J. Inorg. Biochem. 102, 2120–2129.

(31) Mirica, L. M., McGusker, K. P., Munos, J. W., Liu, H., and Klinman, J. P. (2008) 18O kinetic isotope effects in non-heme iron enzymes: Probing the nature of Fe/O intermediates. J. Am. Chem. Soc. 130, 8122–8123.

(32) Galonic, D. P., Barr, E. W., Walsh, C. T., Bollinger, J. M., Jr., and Krebs, C. (2007) Two interconverting Fe(IV) intermediates in aliphatic chlorination by the halogenase CytC3. Nat. Chem. Biol. 3, 113–116.

(33) Price, J. C., Barr, E. W., Hoffart, L. M., Krebs, C., and Bollinger, J. M. (2005) Kinetic dissection of the catalytic mechanism of taurine:α-ketoglutarate dioxygenase (Taud) from Escherichia coli. Biochemistry 44, 8138–8147.
(54) Rotilio, G., Bray, R. C., and Fielden, E. M. (1972) A pulse radiolysis study of superoxide dismutase. Biochim. Biophys. Acta, Enzymol. 268, 605–609.

(55) Hasinoff, B. B. (1984) Kinetics of Carbonic-Anhydrase in Solvents of Increased Viscosity - A Partially Diffusion-Controlled Reaction. Arch. Biochem. Biophys. 233, 676–681.

(56) Pocker, Y., and Janjic, N. (1987) Enzyme-Kinetics in Solvents of Increased Viscosity - Dynamic Aspects of Carbonic-Anhydrase Catalysis. Biochemistry 26, 2597–2606.

(57) Perry, J. J. P., Shin, D. S., Getzoff, E. D., and Tainer, J. A. (2010) The structural biochemistry of the superoxide dismutases. Biochim. Biophys. Acta, Proteins Proteomics 1804, 245–262.

(58) Tian, G., and Klinman, J. P. (1993) Discrimination between 16O and 18O in Oxygen Binding to the Reversible Oxygen Carriers Hemoglobin, Hemerythrin, and Hemocyanin: A new Probe for Oxygen Binding and Reductive Activation by Proteins. J. Am. Chem. Soc. 115, 8891–8897.

(59) Tian, G., Berry, J., and Klinman, J. (1994) Oxygen-18 Kinetic Isotope Effects in the Dopamine β-Monoxygenase Reaction: Evidence for a New Chemical Mechanism in Non-Heme Metalloenzyme. Biochemistry 33, 226–234.

(60) Roth, J. P. (2007) Advances in studying bioinorganic reaction mechanisms: Isotopic probes of activated oxygen intermediates in metalloenzymes. Curr. Opin. Chem. Biol. 11, 142–150.

(61) Stahl, S. S., Francisco, W. a, Merks, M., Klinman, J. P., and Lippard, S. J. (2001) Oxygen kinetic isotope effects in soluble methane monoxygenase. J. Biol. Chem. 276, 4549–4553.

(62) Mirica, L. M., and Klinman, J. P. (2008) The nature of O2 activation by the ethylene-forming enzyme 1-aminocyclopropane-1-carboxylic acid oxidase. Proc. Natl. Acad. Sci. U. S. A. 105, 1814–1819.

(63) Francisco, W. A., Tian, G. C., Fitzpatrick, P. F., and Klinman, J. P. (1998) Oxygen-18 kinetic isotope effect studies of the tyrosine hydroxylase reaction: Evidence of rate limiting oxygen activation. J. Am. Chem. Soc. 120, 4057–4062.

(64) Hotopp, J. C. D., and Hausinger, R. P. (2002) Probing the 2,4-dichlorophenoxyacetate/α-ketoglutarate dioxygenase substrate-binding site by site-directed mutagenesis and mechanism-based inactivation. Biochemistry 41, 9787–9794.

(65) Thornburg, L. D., Lai, M. T., Wishnok, J. S., and Stubbe, J. (1993) A non-heme iron protein with heme tendencies: An investigation of the substrate specificity of thymine hydroxylase. Biochemistry 32, 14023–14033.

(66) Saban, E., Chen, Y. H., Hangasky, J, Taabazuing, C., Holmes, B., and Knapp, M. (2011) The second coordination sphere of FIH controls hydroxylation. Biochemistry 50, 4733–4740.

(67) Flagg, S. C., Giri, N., Pektas, S., Maroney, M. J., and Knapp, M. J. (2012) Inverse Solvent Isotope Effects Demonstrate Slow Aquo Release from Hypoxia Inducible Factor-Prolyl Hydroxylase (PHD2). Biochemistry 51, 6654–6666.

(68) Tarhonskaya, H., Szöllössy, A., Leung, I. K. H., Bush, J. T., Henry, L., Chowdhury, R., Iqbal, A., Claridge, T. D. W., Schofield, C. J., and Flashman, E. (2014) Studies on Deacetoxycephalosporin C Synthase Support a Consensus Mechanism for 2-Oxoglutarate Dependent Oxygenases. Biochemistry 53, 2483–2493.

(69) Sanchez-Fernandez, E. M., Tarhonskaya, H., Al-Qahtani, K., Hopkinson, R. J., McCullagh, J. S., Schofield, C. J., and Flashman, E. (2013) Investigations on the oxygen dependence of a 2-oxoglutarate histone demethylase. Bioch. J. 449, 491–496.