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Investigations on the role of a solvent tunnel in the α -ketoglutarate dependent oxygenase Factor Inhibiting HIF (FIH)

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Abstract

Non-heme Fe(II)/ α -ketoglutarate (α KG)-dependent oxygenases catalyze a wide array of reactions through coupling oxidative decarboxylation of α KG to substrate oxygenation. This class of enzymes follows a sequential mechanism in which O₂ reacts only after binding primary substrate, raising questions over how protein structure tailors molecular access to the Fe(II) cofactor. The enzyme “factor inhibiting hypoxia inducible factor” (FIH) senses pO₂ in human cells by hydroxylating the C-terminal transactivation domain (CTAD), suggesting that structural elements limiting molecular access to the active site may limit the pO₂ response. In this study, we tested the impact of a solvent-accessible tunnel in FIH on molecular access to the active site in FIH. The size of the tunnel was increased through alanine point mutagenesis (Y93A, E105A, and Q147A), followed by a suite of mechanistic and spectroscopic probes. Steady-state kinetics varying O₂ or CTAD indicated that O₂ passage through the tunnel was not affected by Ala substitutions, allowing us to conclude that this narrow tunnel did not impact pO₂ sensing by FIH. Steady-state kinetics with varied α KG concentrations revealed increased substrate inhibition for the Ala variants, suggesting that a second α KG molecule may bind near the active site of FIH. If this solvent-accessible tunnel is the O₂ entry tunnel, it may be narrow in order to permit O₂ access while preventing metabolic intermediates, such as α KG, from inhibiting FIH under physiological conditions.

Graphical Abstract Synopsis

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The authors declare no competing financial interest.

ASSOCIATED CONTENT

Anaerobic UV-Vis spectra of FIH variants bound with (Fe+ α KG), visualization of internal cavities of FIH, dose-response curves for citrate inhibition, SDS-PAGE and ESI data tables for the variants can be found in the supplementary data for this article online at

Substrate binding forms a narrow solvent-accessible tunnel, allowing O_2 to react with FIH while filtering out potential inhibitory compounds such as α -ketoglutarate.

Introduction

The "factor inhibiting hypoxia inducible factor" (FIH) is part of a large class of non-heme Fe(II) α KG-dependent oxygenases. These enzymes oxidize a highly diverse set of primary substrates through varied modifications, leading to protein side chain hydroxylation, demethylation of nucleic acids, biosynthesis of antibiotics and secondary metabolites [1]. Although this class of enzymes performs a variety of chemical modifications, the reactions can be explained through a consensus mechanism with the important feature of sequential substrate binding triggering the enzyme to permit O_2 binding (Scheme 1) [2–5]. The binding of primary substrate (RH in Scheme 1) severely limits access to the active site; this raises questions over how limited solvent accessibility impacts O_2 reactivity, and has implications for selective inhibition of α KG oxygenases.

FIH is a particularly intriguing α KG-dependent oxygenase to test the accessibility of the active site for two reasons. First, FIH is a target for modulating the hypoxic response in humans [6] due to its role regulating the hypoxia-inducible factor (HIF1 α) [7]. FIH exerts its effect on hypoxia sensing by hydroxylating the β -carbon of Asn⁸⁰³ within the C-terminal transactivation domain (CTAD) domain of HIF1 α [8]. Second, the FIH active site is solvent exposed until CTAD binds, leading to the formation of a narrow solvent accessible tunnel formed by residues from both FIH and CTAD (Figure 1) [9]. This tunnel likely serves as an access route for O_2 and other small molecules to the Fe cofactor, leading to a number of implications for function including high fidelity of pO_2 sensing either by limiting the rate of O_2 entry or by filtering out other molecules. Additionally, crystallographic studies of FIH in complex with inhibitors indicate that the dominant mode of FIH inhibition is via competition with α KG [10]; therefore, this tunnel may impact inhibition of FIH by altering the accessibility of α KG or inhibitors to the Fe²⁺ cofactor. As a tunnel is also formed when FIH binds to a consensus ankyrin repeat domain (ARD) peptide, this may also play a role in defining the reaction between FIH and alternative substrates [11, 12].

Solvent accessible tunnels impact oxygen access to deeply buried active sites in metalloenzymes either by directing the regiochemistry of oxygenation or by limiting diffusional cross-section for entry to the active site. Lipoyxygenase [13, 14], membrane-bound respiratory oxidase [15], cholesterol oxidase [16], copper-amine oxidases [17], and

myoglobin [18] all have solvent accessible tunnels that impact protein function. Although the kinetic parameter for the O₂ reaction of FIH ($k_{cat}/K_{M(O_2)} = 3.5 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$) [19] is much lower than predicted for a diffusional controlled reaction (ca. $1 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$), there is precedence for protein structures playing a role in O₂ chemistry even when these reactions are not diffusion limited [13, 20]. Based on the importance of molecular access to the FIH active site and the narrow tunnel, we tested the functional significance of this structural tunnel through a combination of alanine mutagenesis, mechanistic probes, and electronic absorption spectra. This work revealed that the main outcome of expanding the size of this tunnel was to increase substrate inhibition by α KG which we attribute to a second α KG molecule binding either to the Fe, or to an allosteric site. Our findings suggest that the functional significance of this narrow tunnel is not to tailor the O₂ response, but rather to prevent larger molecules from reaching the Fe cofactor after CTAD is present.

Methods

Mutagenesis, Protein Expression and Purification

WT-FIH was overexpressed in *Escherichia coli* (BL21 (DE3) strain) then purified by chromatography as previously described [21]. Quickchange mutagenesis was used to prepare the plasmids for the Y93A, E105A, and Q147A variants, which were then confirmed by DNA sequencing and ESI-MS. Thrombin was used to cleave the hexa-His tag, leaving a final purified protein which had a leading Gly-Ser-His- sequence ahead of the starting Met residue. Protein purification was followed using SDS-PAGE.

Peptide Purification

The crude 39-residue CTAD peptide was purchased from EZ BioLabs (Sequence: DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN), then purified by RP-HPLC. The crude peptide was dissolved in 25% acetonitrile and 0.1% trifluoroacetic acid, TFA). The sample was loaded onto a C-18 column (Higgins Analytical), and a gradient elution method was used starting at 30% acetonitrile 0.1% TFA and ending with 95% acetonitrile 0.1% TFA. Typical retention times for the peptide ranged from 19–22 minutes. MALDI-TOF-MS was used to test the final purity of the peptide.

Activity Assays

Initial rate measurements were performed to measure steady state kinetics for the FIH variants. These initial-rate data sets were fit using the Michaelis Menten equation (Origin Pro) to determine the apparent kinetic parameters k_{cat} and k_{cat}/K_M , as well as the K_M for each FIH substrate (α KG, CTAD and O₂). All assays were carried out at 37.0 °C, in 50 mM HEPES buffer (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) at pH 7.00 at 37.0 °C, under saturating concentrations of FeSO₄ (50 μ M) and ascorbate (2 mM). Reagents were incubated at 37.0 °C for 2 minutes before initiating the reaction with the addition of enzyme. When CTAD was the varied substrate, oxygen was fixed at ambient dissolved [O₂] (217 μ M) and α KG (α -ketoglutarate) was saturating at 100 μ M. When α KG was the varied substrate, O₂ was fixed at 217 μ M, and CTAD was fixed at 100 μ M.

All steady state kinetics assays were based on a common set of quenched reactions using MALDI-TOF-MS to measure the relative peak intensities of the CTAD ($m/z = 4156$) and hydroxylated CTAD ($m/z = 4172$) [21] to determine initial rates. Reaction aliquots were quenched in 75% acetonitrile/0.2% TFA saturated with 3,5-dimethoxy-4-hydroxycinnamic acid. The reported values are an average of each assay performed in triplicate.

Activity Assays Varying Oxygen

To determine the kinetic parameters k_{cat} (apparent) and K_M for O_2 , all reactions were performed in an Atmosbag (Sigma Aldrich). The ratio of O_2 and N_2 was varied using gas flow meters to produce different oxygen concentrations. A Clark electrode (Yellow Springs Inc.) was used to determine the oxygen concentration dissolved in 50 mM HEPES buffer (pH 7.00 at 37.0 °C). When O_2 was the varied substrate, the final concentration of αKG was fixed at 100 μM ($> 5 \times K_M$), and the final concentration of CTAD was fixed at 80 μM (roughly equal to the K_M). Although CTAD was not saturating, this concentration was used to economize on the peptide reagent. The assays were incubated for 2 minutes at 37.0 °C before adding 5 μL of 0.3 μM –0.6 μM of enzyme to initiate the reaction. In order to be sure that the buffer was fully equilibrated to the oxygen concentration in the bag, buffer was allowed to equilibrate for 30 minutes under the O_2/N_2 atmosphere prior to each reaction.

UV-Visible Spectroscopy

UV-vis spectroscopy was used to assess the electronic structure of WT-FIH and the FIH variants. All experiments were performed in an anaerobic glove box at 22 °C. 50 mM HEPES buffer (pH 7.0 at 22 °C), 300 μM enzyme and 300 μM FeSO_4 , forming (Fe)FIH, were added to a 100 μL cuvette with a 1 cm path length, and were used as a blank for the experiment. αKG was added into the cuvette using a Hamilton syringe. An Avantes AvaSpec-2048 Fiber Optic spectrometer with a 2048 pixel charge-coupled device detector array recorded all spectra. The (Fe)FIH spectrum was subtracted from the (Fe+ αKG)FIH and (Fe+ αKG +CTAD)FIH spectra to record the changes in spectral features.

Results and Discussion

Crystal structures of (Fe+ αKG)FIH/CTAD reveal a solvent accessible tunnel extending from the surface to the buried active site [9]. Due to the sequential mechanism of the αKG -dependent dioxygenases (Scheme 1), the binding of CTAD leads to the mutually exclusive needs of permitting O_2 entry to complete turnover while avoiding other molecules that could lead to enzyme inactivation [22–25] or inhibition [10]. As similar tunnels impact O_2 reactivity in other oxygenases [13–18] we tested the effect of increasing tunnel size on FIH catalysis.

Steady-State Kinetics Varying O_2

Diffusional encounter between O_2 and FIH is not a rate-limiting step, as shown by lack of a solvent viscosity effect on $k_{\text{cat}}/K_{M(\text{O}_2)}$ [19]. However migration of O_2 from the protein surface to the active site could be impeded by the tunnel, as seen for a series of cavities that impede O_2 reactivity in the T201S variant of soluble methane monooxygenase (sMMO) [20] or that control regiochemistry in lipoxygenase [13, 14]. Steady state kinetic parameters with

varied $[O_2]$ were determined under saturating ascorbate (2mM), $FeSO_4$ (25 μ M), and α KG (100 μ M) to test for changes in the O_2 reactivity upon tunnel substitution. The CTAD concentration was fixed at 80 μ M ($\sim K_{M(CTAD)}$ for WT FIH) due to reagent expense, however the use of sub-saturating levels of CTAD provide certain advantages in later assays in which α KG is the varied substrate. The kinetic parameters determined by fitting initial rate data to the Michaelis-Menten equation showed minor differences in O_2 reactivity for each variant (Figure 2 and Table 1). The apparent substrate specificity for WT FIH ($k_{cat}/K_{M(O_2)} = 0.15(0.02) \mu M^{-1} min^{-1}$) was essentially unchanged in the E105A variant ($k_{cat}/K_{M(O_2)} = 0.17(0.02) \mu M^{-1} min^{-1}$). Although the Y93A and Q147A variants exhibited diminished activity ($k_{cat}/K_{M(O_2)} = 0.09(0.03) \mu M^{-1} min^{-1}$ and $0.04(0.02) \mu M^{-1} min^{-1}$ respectively), this was less than a 5-fold variation in $k_{cat}/K_{M(O_2)}$ compared with WT-FIH.

Steady-state kinetics varying O_2 tested whether expanding the width the tunnel affected oxygen reactivity. While the modest decrease in $k_{cat}/K_{M(O_2)}$ upon increasing the tunnel size could reflect changes in protein stability or Fe binding, it is nevertheless a small enough effect that we feel confident in stating that O_2 passage through this tunnel was unlikely to be rate-limiting. As neither diffusional encounter with O_2 [19], nor O_2 passage through the tunnel was rate-limiting, this led us to conclude that the hypoxia sensing function of FIH is not determined by this narrow solvent-accessible tunnel.

Steady-State Kinetics Varying CTAD

As O_2 is the third substrate to bind sequentially in the consensus mechanism, we decided to step back through each substrate addition to check for any impact of the tunnel variants upon reactivity. Steady-state kinetic parameters with varied CTAD were measured under saturating conditions of ascorbate (2mM), $FeSO_4$ (25 μ M), and α KG (100 μ M). The assays were performed using ambient levels of oxygen (217 μ M), which caused the apparent $k_{cat}/K_{M(CTAD)}$ kinetic parameter to report on steps between FIH/CTAD encounter up through the irreversible chemical step of oxidative decarboxylation. The apparent $k_{cat}/K_{M(CTAD)}$ for all variants spanned a relatively narrow range of values (Figure 3). Correcting for the sub-saturating $[O_2]$ allowed us to estimate the maximal $k_{cat}/K_{M(CTAD)}$ by reference to the relative level of saturation with O_2 for each variant as follows: $V_{max} = v \{([O_2] + K_{M(O_2)}) / [O_2]\}$, in which V_{max} is the true value for $k_{cat}/K_{M(O_2)}$ whereas v is the apparent $k_{cat}/K_{M(O_2)}$, and ambient dissolved $[O_2] = 217 \mu M$. The corrections for sub-saturating O_2 were relatively modest: WT-FIH (2), Y93A (3), E105A, (2), Q147A (4.5) (Table 1). This correction led to estimates for the true value of $k_{cat}/K_{M(CTAD)}$ for each variant ($k_{cat}/K_{M(CTAD)} = 0.7 - 1.5 \mu M^{-1} min^{-1}$); in each case, this value is nearly equal to that of WT-FIH. Despite the absence of thermodynamic binding constant data, we interpret the kinetic data to indicate that widening the cavity did not lead to a large effect on steps between CTAD binding and O_2 activating chemistry, indicating that the structural alignment of substrates within the active site had not changed for the tunnel variants.

Steady-State Kinetics Varying α KG

Steady state parameters with varied $[\alpha KG]$ were measured using saturating levels of ascorbate (2mM) and $FeSO_4$ (25 μ M). The concentration of CTAD was fixed at 90 μ M, and the concentration of O_2 was fixed at 217 μ M (ambient O_2 concentration at 37.0 $^{\circ}C$), which

are roughly equal to the K_M for each substrate with WT-FIH. The apparent kinetic parameters obtained under these conditions were composites of the rate constants for steps ranging from the α KG/FIH encounter up through the irreversible oxidative decarboxylation step. This is crucial, as it allowed us to learn about steps after CTAD is already bound to FIH, and the enzyme had become triggered for reaction with O_2 [5]. When initial rate data was plotted versus the α KG concentration, substrate inhibition was observed at increased α KG concentrations. The initial rates reached a maximum at approximately 100 μ M α KG (Figure 4) before decreasing as a function of increased α KG. The data was fit with an equation that assumed simple substrate inhibition (Eq. 1). The initial rate ($v_o/[E]$) was a function of the maximal velocity (k_{cat}), and kinetic parameters relative to the α KG concentration ($k_{cat}/K_{M(\alpha KG)}$, $K_{M(\alpha KG)}$) and an inhibition constant, K_I .

$$v_o/[E] = k_{cat}[S]/(K_M + [S](1 + [S]/K_I)) \quad (\text{Eq. 1})$$

When allowed to float freely, the $K_{M(\alpha KG)}$ for each variant was very similar to that from WT-FIH ($K_{M(\alpha KG)} = 17 \mu$ M), but there was a covariance of the fit parameters leading to poor convergence and large uncertainties. By fixing $K_{M(\alpha KG)}$ at the value for WT-FIH (17 μ M), we were able to obtain good fits to the data and reasonable estimates of the inhibition constant for each of the FIH variants (Figure 4). There was weak inhibition observed for WT ($K_I > 1$ mM), with the inhibition constant roughly 100-fold greater than the Michaelis constant. In contrast, the K_I for each of the tunnel variants was moderate ($K_I \sim 300 \mu$ M) and roughly 20 -fold greater than the Michaelis constant, leading to significantly lower rates of turnover at even moderate α KG levels. The relative magnitude of the inhibition constants and the Michaelis constants (Table 2), suggested that the inhibitory mode for α KG binding was much weaker than the catalytic mode of binding (Scheme 2).

α KG Binding and electronic absorption spectrum

The electronic absorption spectrum of WT-FIH and the FIH variants was measured in an anaerobic glovebox to test for any changes in the electronic environment of Fe^{2+} in the tunnel variants. These samples used an equimolar concentration of FIH (300 μ M) and $FeSO_4$ (300 μ M), with α KG concentrations that were sub-stoichiometric (250 μ M) or 2-fold excess (660 μ M) relative to enzyme. When α KG was present in sub-stoichiometric levels, WT-FIH and each variant exhibited the expected broad absorbance peak ($\lambda_{max} \sim 500$ nm) that is due to a $d(Fe^{2+}) \rightarrow \pi^*(keto)$ metal to ligand charge transfer in the $(Fe+\alpha KG)$ FIH form of enzyme [21, 26]. By reference to both the consensus mechanism and to MCD spectra for WT-FIH [27], the Fe^{2+} in these protein samples is coordinated by a N_2O_4 ligation shell from the 2His-1Asp facial triad from the protein, a bidentate α KG ligand, and an aquo ligand [26].

We tested for binding of a second α KG ligand to the variant proteins, as one mode for the inhibition by excess α KG would be for Fe^{2+} to bind two α KG ligands. Addition of a greater than two-fold excess of α KG (660 μ M) to the (Fe) FIH samples (300 μ M FIH and Fe^{2+}) did not substantially shift λ_{max} in the electronic spectrum of WT-FIH or of any variant. Although this did not show a second α KG bound to the Fe^{2+} in the variants, we cannot

definitively exclude this as the weaker binding predicted for the inhibitory α KG (K_I 300 μ M) made it unlikely that the putative binding site would be saturated. Further, as the normal ligation shell for the Fe^{2+} in FIH is N_2O_4 , an additional α KG may not lead to significant electronic changes at the Fe^{2+} if the α KG were to simply displace the aquo and Asp²⁰¹ ligands.

Addition of CTAD (300 μ M) to each of the (Fe+ α KG)FIH variants led to a red-shifted absorption band ($\lambda_{\text{max}} \sim 500$ nm), as expected for the release of this aquo ligand in the triggered enzyme form [3–5, 26]. Loss of the aquo ligand in WT-FIH was shown by the appearance of a 5-coordinate geometry via magneto-circular dichroism spectroscopy upon binding CTAD to form the (Fe+ α KG)FIH/CTAD form of enzyme [27], which is essential to trigger the Fe^{2+} to react with O_2 in the consensus mechanism [26, 28, 29]. The similarity of the spectra for each variant to that of WT-FIH indicated that the primary coordination sphere around the Fe^{2+} was not altered by the mutations in the solvent-accessible tunnel, and that each variant was triggered by CTAD binding.

Implications for FIH function

Substrate inhibition of α KG-dependent oxygenases by α KG is rare but there are a few reports of α KG inhibition [30, 31], including one report in which α KG competed with O_2 for the active site [31], providing precedent for substrate inhibition in this class of enzyme. As the level of α KG in healthy brain[32] and liver[33] tissues are in the range of 0.4 – 2 mM, greatly exceeding the $K_{M(\alpha\text{KG})} \sim 10$ μ M for FIH, modest substrate inhibition may be found in select tissues. This suggests that the normal function of WT-FIH may require a structural filter to disfavor inhibitory α KG binding, such as a narrow tunnel to limit molecular access to the active site. It is quite certain that α KG can reach the active site under normal turnover, and that compounds larger than α KG can compete with α KG to bind Fe^{2+} [6, 10, 34, 35]. In several cases, FIH/inhibitor structures are known (reviewed by Rose, et. al[10]), revealing much about the size and shape of the active site pocket. Notably, one structure (PDB ID 2WA4) showed two aromatic hydroxamates bound directly to the Fe^{2+} in the active site [36], with one hydroxamate displacing α KG and the other displacing the D201 residue. This structure provides a structural precedent for two ligands binding to FIH, and suggests that the α KG inhibition reported here could arise from an additional α KG molecule binding near the active site.

A subsequent screen of common carboxylic acids, including succinate and intermediates in the tricarboxylic acid (TCA) cycle, was undertaken to test the susceptibility of the FIH and its variants to inhibition (Fig. 6). This screen was a very simple screen using a high concentration of compound (1 mM) and a sub-saturating concentration of α KG (20 μ M) in order to test whether or not the decreased bulk of the tunnel variants led to differential inhibition. Both citrate and oxaloacetate inhibited WT-FIH and each of the variants. As citrate suggested differential inhibition of the variants, this compound was selected for dose-response assays.

Dose-response curves for citrate inhibition (7 – 1000 μ M) were collected from initial rate data using sub-saturating concentrations of α KG (20 μ M), with IC_{50} values reported in Table 3. WT-FIH exhibited weak dose-response inhibition by citrate, as did the E105A

variant. In contrast, citrate inhibition of the Y93A and Q147A variants was markedly concentration-dependent ($IC_{50} = 140 \mu M$, supplemental material). As these two variants contain less bulky residues near the bottom of the substrate tunnel, it appears that these variants create sufficient volume near the cofactor for inhibition by citrate (Figure 1).

We propose that a functional role for the narrow tunnel in FIH is to allow O_2 access while limiting αKG and metabolite access. WT-FIH was weakly inhibited by elevated αKG levels ($K_I > 1.3 \text{ mM}$). Decreasing the bulk of residues in the Y93A, E105A, and Q147A variants led to increased substrate inhibition by αKG (Figure 4), which we attribute to the binding of a second αKG molecule to the enzyme (Scheme 2). As physiological levels of αKG are in the low millimolar range, such inhibition could seriously impede hypoxia sensing. Carboxylic di-acids from metabolism, including fumarate and succinate, are also present at $\sim 1 \text{ mM}$ levels in normal tissues, and these and related compounds such as 2-hydroxyglutarate[37] are elevated in many proliferative cells. As these TCA intermediates are weak inhibitors of FIH, HIF-prolyl hydroxylase-2, and other αKG -dependent dioxygenases by competing with αKG for binding to the Fe^{2+} within the active site [38], there is precedent for metabolite inhibition in this family of enzymes.

Our screen of TCA intermediates led to the interesting observation that citrate, but not other TCA intermediates, differentially inhibited the two tunnel variants with decreased bulk near the cofactor, Y93A and Q147A. This suggests that both the specific structure of the metabolite as well as the accessible volume near the active site are factors in inhibition. This is consistent with the observation that αKG and structurally related molecules can be moderate-affinity inhibitors of αKG -dependent oxygenases, albeit with K_I ranging from $110 \mu M$ to greater than 10 mM [38]. In this context, inhibition of the FIH tunnel variants by αKG but not by other TCA intermediates should not be surprising. Two possible explanations for αKG inhibition are that αKG binds within the tunnel (such as by binding to the Fe), or that αKG binds at a more remote allosteric site. These two options are challenging to test directly, as neither is expected to lead to large spectroscopic changes in the FIH.

The activities of FIH and other αKG -dependent oxygenases are correlated with cancer and proliferation as shown by the Warburg effect, in which cancerous cells convert from aerobic respiration over to anaerobic glycolysis, even when pO_2 is normal [39]. The Warburg effect involves a variety of gene expression changes, including an upregulation of HIF-1 α controlled genes, which is likely due to reduced activity from FIH and prolyl hydroxylases, suggesting that there is a complex relationship between FIH activity and metabolism [39], although causality is unclear. Avoiding this inhibition under normal physiological levels of carboxylic acids, such as by minimizing molecular access to the FIH active site by virtue of a narrow access tunnel, would be crucial for hypoxia sensing in healthy tissues.

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Abbreviations

ARD	ankyrin repeat domain
CTAD	C-terminal transactivation domain of HIF-1 α
E. coli	Escherichia coli
FIH	factor-inhibiting HIF-1
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF-1α	hypoxia inducible factor-1 α
MALDI/TOF MS	matrix assisted laser desorption ionization/time-of-flight mass spectrometry
MCD	magnetic circular dichroism
sMMO	soluble methane mono-oxygenase
TCA	tricarboxylic acid cycle
TFA	trifluoroacetic acid
αKG	α -ketoglutarate

Highlights

Substrate binding creates a narrow solvent-accessible tunnel in *factor inhibiting HIF*

Access of O₂ is not diffusionally limited, nor is it altered in tunnel variants.

Substrate inhibition by α -ketoglutarate depends on the bulk of residues lining the tunnel

The narrow solvent-accessible tunnel controls molecular access to the active site.

Figure 1.

Side view of the solvent accessible tunnel in FIH. FIH secondary structure (wheat), CTAD (cyan), Fe (orange) and N-oxalylglycine (NOG, an isostructural α KG mimic) are shown, with the internal tunnel surface (grey) calculated for a 1.2 Å solvent sphere (PDB ID: 1H2L [10]) using PyMol. Tunnel surface formed by Y93, E105, and Q147 shown (red); CTAD and CTAD-N803(cyan) form part of the solvent accessible tunnel.

Figure 2.

Steady-state kinetics of the FIH variants with O₂ as varied substrate. WT (black, ■), Y92A (green, ◆), E105 (blue, ●), Q147A (red, ▲). Reactions were conducted in 50 mM HEPES (pH 7.00) containing FIH (0.3–0.6 μM), ascorbate (2 mM), αKG (100 μM), FeSO₄ (25 μM), and CTAD (80 μM).

Figure 3.

Steady-state kinetics of the FIH variants with CTAD as varied substrate. WT (black, ■), Y92A (green, ◆), E105 (blue, ●), Q147A (red, ▲) Reaction mixtures contained FIH (0.3–0.6 μM), ascorbate (2 mM), αKG (100 μM), FeSO₄ (25 μM), and CTAD (20–275 μM) in 50 mM HEPES (pH 7.00).

Figure 4.

Steady-state kinetics of the FIH variants with α KG as varied substrate. WT-FIH (black squares), Y93A (green, $\blacklozenge$), E105 (blue, $\bullet$), Q147A (red, $\blacktriangle$). Reaction mixtures contained FIH (0.3-0.6 μ M), ascorbate (2 mM), α KG (10-800 μ M), FeSO_4 (25 μ M), and CTAD (90 μ M) in 50 mM HEPES (pH 7.00).

Figure 5. UV-Vis spectra for Y93A with 250 μ M α KG (solid black) and 660 μ M α KG (dashed gray); FIH (300 μ M), α KG (250 or 660 μ M), and FeSO_4 (300 μ M) in 50 mM HEPES (pH 7.00). The spectra were blanked against (Fe)FIH-Y93A.

Figure 6.

Inhibition screens with FIH variants and various TCA cycle intermediates. WT-FIH (black), E105A (white), Q147A (grey), Y93A (slashed). Reactions were conducted in 50 mM HEPES (pH 7.00) containing FIH (0.3–0.6 μ M), ascorbate (2 mM), α KG (20 μ M), FeSO_4 (25 μ M), CTAD (80 μ M) and inhibitor (1 mM).

Scheme 1.

Consensus mechanism for FIH based on other α KG-dependent oxygenases.

Scheme 2.

Proposed model for α KG inhibition of tunnel variants of FIH, with the hydroxylated peptide (P) indicated.

Table 1

Apparent kinetic parameters for WT FIH and variants with O₂ or CTAD as the varied substrate.

Enzyme	k_{cat}^{app} (min ⁻¹) ^a	K_M (O ₂) (μM) ^a	k_{cat}/K_M (O ₂) ^{app} (min ⁻¹ μM ⁻¹) ^a	K_M (CTAD) (μM) ^b	k_{cat}/K_M (CTAD) ^{app} (min ⁻¹ μM ⁻¹) ^b
WT FIH	33 ± 2	221 ± 33	0.15 ± 0.02	67 ± 13	0.44 ± 0.09
Y93A	41 ± 5	440 ± 150	0.09 ± 0.03	126 ± 15	0.30 ± 0.04
E105A	42 ± 3	250 ± 44	0.17 ± 0.03	60 ± 6	0.70 ± 0.06
Q147A	31 ± 5	758 ± 250	0.04 ± 0.02	180 ± 29	0.16 ± 0.03

^a O₂ was the varied substrate, with other reagents as follows: FeSO₄ (50 μM) ascorbate (2 mM), αKG (100 μM), CTAD (80 μM) in 50 mM HEPES buffer (pH 7.00 at 37.0 °C).

^b CTAD was the varied substrate, with other reagents as follows: FeSO₄ (50 μM) ascorbate (2 mM), αKG (100 μM), O₂ (217 μM) in 50 mM HEPES buffer (pH 7.00 at 37.0 °C).

Table 2Apparent kinetic parameters determined from steady-state assays varying α KG.

Enzyme	k_{cat}^{app} (min^{-1})	$K_M(\alpha\text{KG})$ (μM)	$K_I(\alpha\text{KG})$ (μM)
WT FIH	33 ± 2	9 ± 2	> 1300
Y93A	15 ± 4	13 ± 5	350 ± 120
E105A	23 ± 3	9 ± 2	430 ± 80
Q147A	19 ± 9	38 ± 8	200 ± 80

^a α KG was the varied substrate, with other reagents as follows: FeSO_4 (50 μM) ascorbate (2 mM), CTAD (100 μM), O_2 (217 μM) in 50 mM HEPES buffer (pH 7.00 at 37.0 °C).

Table 3

Dose-dependent inhibition of FIH variants by citrate and oxaloacetate.

	Citrate IC ₅₀ , μ M	Oxaloacetate IC ₅₀
WT FIH	> 1000	> 1000
E105A	> 1000	> 1000
Q147A	140 $\pm$ 1	> 1000
Y93A	140 $\pm$ 1	> 1000

Reactions were conducted in 50 mM HEPES (pH 7.00) containing FIH (0.3–0.6 μ M), ascorbate (2 mM), α -KG (20 μ M), FeSO₄ (25 μ M), CTAD (80 μ M), dissolved O₂ (217 μ M), and inhibitor (7–1000 μ M).