Published in final edited form as:

Biochemistry. 2019 October 01; 58(39): 4047–4057. doi:10.1021/acs.biochem.9b00619.

Protein Flexibility of the α -Ketoglutarate-Dependent Oxygenase Factor-Inhibiting HIF-1: Implications for Substrate Binding, Catalysis, and Regulation

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Abstract

Protein dynamics are crucial for the mechanistically ordered enzymes to bind to their substrate in the correct sequence and perform catalysis. Factor-inhibiting HIF-1 (FIH) is a nonheme Fe(II) α -ketoglutarate-dependent oxygenase that is a key hypoxia (low p_{O_2}) sensor in humans. As these

hypoxia-sensing enzymes follow a multistep chemical mechanism consuming α -ketoglutarate, a protein substrate that is hydroxylated, and O_2 , understanding protein flexibility and the order of substrate binding may aid in the development of strategies for selective targeting. The primary substrate of FIH is the C-terminal transactivation domain (CTAD) of hypoxia-inducible factor 1 α (HIF) that is hydroxylated on the side chain of Asn803. We assessed changes in protein flexibility connected to metal and α KG binding, finding that (M+ α KG) binding significantly stabilized the cupin barrel core of FIH as evidenced by enhanced thermal stability and decreased protein dynamics as assessed by global amide hydrogen/deuterium exchange mass spectrometry and limited proteolysis. Confirming predictions of the consensus mechanism, (M+ α KG) increased the affinity of FIH for CTAD as measured by titrations monitoring intrinsic tryptophan fluorescence. The decreased protein dynamics caused by (M+ α KG) enforces a sequentially ordered substrate binding sequence in which α KG binds before CTAD, suggesting that selective inhibition may require inhibitors that target the binding sites of both α KG and the prime substrate. A consequence of the correlation between dynamics and α KG binding is that all relevant ligands must be included in binding-based inhibitor screens, as shown by testing permutations of M, α KG, and inhibitor.

Graphical Abstract

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The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acs.biochem.9b00619.

CTAD binding titration of (Mn+ α KG)FIH, CD spectra of FIH bound to α KG, and T_M values for FIH with different ligands (PDF)

Accession Codes

FIH, Q9NWT6.

Protein dynamics play a key role in slow time scale events involved in ligand and substrate binding and have been shown to couple to active site chemistry.^{1,2} The functional significance of these dynamics should be particularly high for enzymes with multisubstrate reactions and those involved in modifying other proteins. Hypoxia (low p_{O_2}) sensing is a rich area for such interesting enzymes, as it is typified by complex protein/protein recognitions and covalent modifications.³ The nexus in eukaryotic hypoxia sensing is hypoxia-inducible factor 1 α (HIF-1 α), which is regulated by several enzymes, including factor-inhibiting HIF (FIH; UniProt entry Q9NWT6).^{4,5} FIH is a nonheme Fe(II) α -ketoglutarate-dependent oxygenase that senses p_{O_2} in humans by hydroxylating Asn803 in the C-terminal transactivation domain (CTAD) of HIF-1 α , transcriptionally repressing HIF-1 under normal p_{O_2} .^{5,6} Due to the variety of reactions catalyzed by αKG -dependent oxygenases,⁷ their conserved protein fold,⁸ and the use of protein substrates,⁹ dynamics may play a significant role in determining the substrate binding order and active site chemistry for FIH and related enzymes.

Structures of FIH bound to varied substrates and cofactor reveal that the equilibrium structure of FIH does not change when binding varied transition metals ($\text{M} = \text{Fe}$ or Zn), αKG , CTAD, NO as a mimic of O_2 ,¹⁰ or some combination thereof.^{11–13} The use of a non-native metal permits structural studies while avoiding deleterious autoxidation reactions. In contrast, average B -factors in the refined structures show that thermal fluctuations of FIH decrease upon binding ($\text{M} + \alpha\text{KG}$),^{11,12} and a mechanistic study further revealed that the access of O_2 to the active site was not limited by constriction in a narrow channel.¹⁴ These kinetic and structural data suggest that protein dynamics may be essential for ligand binding in FIH, as seen for ligand binding in other metalloenzymes.^{15–17} Establishing the connection between protein dynamics and substrate binding may provide insight into the sequential chemical mechanism as well as providing hints for selectively targeting members of this important enzyme superfamily.^{7,18}

The consensus reaction mechanism for this family of nonheme iron αKG -dependent oxygenases is based on the HAG mechanism,¹⁹ which assumes that αKG is the first substrate to bind.^{20,21} Next, the prime substrate binds near the cofactor, triggering the subsequent reaction with O_2 and leading to tightly coupled hydroxylation.^{22–25} Research on

FIH^{24,26,27} and other α KG oxygenases indicates that triggering is due, at least in part, to the prime substrate causing the release of an aquo ligand from the Fe(II) to open a binding site for O₂.^{20,26–28} Although the body of evidence supporting a local change in coordination at Fe(II) as a determining factor for O₂ binding is growing, very few data concerning the order of substrate binding for α KG and the prime substrate exist.

X-ray crystallography identified three regions of FIH involved in CTAD binding.¹¹ Site I binds to an extended conformation of CTAD 795–801; residues within the cupin barrel of FIH contact residues in the γ -turn from CTAD 802–805, containing the Asn803 target residue (Figure 1); and site II on FIH binding to a helical stretch of CTAD 812–823. Truncated forms of CTAD consisting of residues 788–806 serve as substrates, albeit with a much weaker affinity ($K_M = 400 \mu\text{M}$),²⁹ indicating that site I and the target residue site are essential for the binding of CTAD to FIH. This leads to important predictions for CTAD binding.

First, the multiple points of contact between Asn803 and the Fe/ α KG center suggest that the affinity for CTAD will depend on the Fe coordination environment. When FIH was first identified as a protein that interacts with HIF-1 from yeast two-hybrid assays, it was noted that the short CTAD peptides (HIF-1 α 786–826) did not bind FIH under the GST pull-down assay conditions,³¹ but similar short peptides (HIF-1 α 788–826) were satisfactory stand-alone substrates under turnover conditions.³² These structural and biochemical observations suggested that the affinity for CTAD would increase in the presence of (M+ α KG).

Second, the constrained conformation of the γ -turn suggests that protein dynamics are coupled to CTAD binding and may impact the positioning of the CTAD Asn803 target residue. The substrate binding order for FIH can be viewed as a matter of the extent to which α KG binding leads to a tighter affinity for CTAD. Although the crystal structures for the apo¹² and (M+ α KG)FIH and (M+ α KG)FIH/CTAD enzyme forms^{11,13} showed a very small deviation in atomic positions [root-mean-square deviation of 1.38 Å for Protein Data Bank (PDB) entries 1IZ3 and 1H2K], analysis of the experimental *B*-factors revealed that binding (Fe+ α KG) decreased the flexibility of the outer rims of the cupin barrel core consisting of α -helices and loops.^{11,12} This reduced protein flexibility for the (Fe+ α KG)FIH enzyme form suggested that the CTAD binding site would be stabilized by coordination of α KG to Fe(II).

Here, we show that binding of (M+ α KG) to FIH decreases protein flexibility and increases the affinity for CTAD, indicating that sequential substrate binding is the result of an induced fit process caused by protein dynamics. We used the non-native metal Mn to avoid autoxidation reactions that can occur with the native Fe. This establishes changes in protein dynamics essential for the sequentially ordered mechanism of FIH, raising the potential that dynamics in other α KG-dependent oxygenases may be functionally significant.^{16,33} Decreased protein dynamics in FIH upon binding (M+ α KG) reveal metal is required for binding-based inhibitor screens, as shown by a thermal stability assay of the binding of the inhibitor to FIH. The correlation between (M+ α KG) binding and protein dynamics has important ramifications on the nature of substrate binding, catalysis, and the design and screening of selective inhibitors for FIH.

MATERIALS AND METHODS

Materials.

All reagents were purchased from commercial vendors and were used as received, with the exception of the 39-residue CTAD peptide substrate that was obtained from EZBiolab (Carmel, IN) as a desalted product. The CTAD sequence that was used corresponded to that of human HIF-1 α 788–826, with the exception of a C800A variation to prevent disulfide formation: (Asn803 underlined) DESG-LPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN with unmodified termini. The peptide was purified prior to use by reverse phase high-performance liquid chromatography (RP-HPLC) to at least 95% purity. The concentration of the peptide was determined by monitoring the absorbance of the Tyr residue at 293 nm ($\epsilon = 2400 \text{ M}^{-1} \text{ cm}^{-1}$) in 0.1 M NaOH.³⁴

FIH Expression and Purification.

BL21-DE3 *Escherichia coli* cells containing the wild-type FIH/pET28a plasmid were grown in 2X YT media (25 g/L) supplemented with 30 $\mu\text{g/mL}$ kanamycin at 37 °C with constant shaking. The cells were induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) and harvested by centrifugation. Cell lysis was facilitated by lysozyme (1 mg/mL) and sonication on ice for 30 min. The crude cell lysate was repeatedly dialyzed against 10 mM Tris (pH 8.0) at 4 °C. Purification of the His-tagged enzyme was carried out by Ni-affinity chromatography using a peristaltic pump system (Bio-Rad). The His tag attached to the N-terminus was then removed by incubation with thrombin (0.5 unit/mg of protein) for 36 h at 4 °C. Further overnight incubation with 50 mM EDTA (pH 8.0) at 4 °C was performed for metal removal. The protein sample was loaded onto a Sephadex G75 resin column to obtain the FIH dimer. The purity of the enzyme was checked by SDS-PAGE. The concentration of the protein was estimated by ultraviolet (UV) absorbance at 280 nm using an ϵ_{280} of 1.22 mL mg⁻¹ cm⁻¹.³⁴

Ligand Binding by Intrinsic Fluorescence Quenching.

The samples in the cuvette for fluorescence experiments contained the following components as indicated: 2 μM FIH, 50 μM MnSO₄, 500 μM α KG, and 200 μM HIF-1 α /CTAD in 50 mM HEPES (pH 7.00) with 100 mM NaCl. All samples were mixed thoroughly and then incubated at room temperature (23 °C) for at least 3 min prior to the measurement of fluorescence with a Photon Technology International Quantamaster-4 SE fluorimeter. The tryptophan fluorescence emission scan was done in the wavelength range of 300–375 nm using excitation and emission slit widths of 0.1 and 2.5 nm, respectively. The excitation wavelength used was 295 nm, and the emission intensity at 330 nm was monitored.

For binding experiments, the samples were prepared without the HIF-1 α /CTAD substrate. The titrant solution contained 2.8 mM HIF-1 α /CTAD, 2 μM FIH, 50 μM MnSO₄, and 500 μM α KG in 50 mM HEPES (pH 7.0) with the total ionic strength maintained at 100 mM using NaCl. The generated emission spectra were baseline-corrected with the buffer blank and smoothed using the adjacent averaging method in OriginPro. The titration data were

obtained by recording the magnitude of the emission intensity at 330 nm. The binding data were fitted to a Langmuir binding equation

$$Y = \frac{Y_{\max} [L]}{K + [L]} \quad (1)$$

where $Y_{\max}$ is normalized to unity and K is the binding affinity.

Thermal Stability Experiments.

Protein samples for the thermal melt curve experiments contained one or two or all of the following components: 5 μM FIH, 50 μM MnSO_4 , and 500 μM αKG in 50 mM HEPES (pH 7.0). For each sample, 45 μL of the master mix was distributed in each well of the 96-well quantitative polymerase chain reaction microplate and 5 μL of freshly prepared 5 $\times$ SYPRO Orange (Sigma-Aldrich, 5000 $\times$ stock diluted in dimethyl sulfoxide) was thoroughly mixed into it. Inhibitor screens used similar conditions, with the exception of 5 μM MnSO_4 and the addition of 100 μM inhibitor. The microplate was sealed with a Microseal adhesive film, briefly spun, and immediately loaded onto a Bio-Rad CFX Connect RT-PCR machine, and the samples were heated at a rate of 1 $^{\circ}\text{C}/\text{min}$ from 20 to 90 $^{\circ}\text{C}$. The fluorescence intensity (excitation wavelength range of 450–490 nm, emission wavelength range of 560–580 nm) was monitored as a function of temperature. The T_m was determined using the positive first-derivative plot of the data points generated. All thermal shift assay curves were fit to the Boltzmann equation from zero to maximum intensity to determine T_m .

$$y = \frac{A_1 - A_2}{1 + e^{(x - x_0)/dx}} + A_2 \quad (2)$$

Global Hydrogen/Deuterium Exchange with Mass Spectrometry.

For the HDX-MS experiments, a portable ice box equipped with an injection port and a C4 RP-HPLC column was used. It was connected to the HPLC pump on one end and the Turbo Ion Spray source of a QStarXL (AB Sciex, Framingham, MA) ESI-TOF mass spectrometer on the other end. All samples were initially prepared on ice prior to incubation at room temperature (26 $^{\circ}\text{C}$). Samples contained 20 μM FIH and one, two, or all of the following components upon final dilution into ammonium acetate buffer: 50 μM MnSO_4 , 500 μM αKG , and 200 μM CTAD in 50 mM HEPES (pH 7.0) with the total ionic strength maintained at 100 mM with NaCl. A master mix for each of the different samples was initially prepared, from which a 2 μL aliquot for each time point was incubated for 3 min at room temperature. Ammonium acetate buffer (10 mM) in H_2O (pH 7.1) (for the m_0 control) or in D_2O (pD 7.4) was then added to a final volume of 20 μL to initiate H/D exchange. Samples were quenched at varying time points by injection into the LC lines that had been equilibrated with 0.1% formic acid and kept on ice. A short gradient of 0.1% aqueous formic acid/0.1% formic acid in acetonitrile was immediately initiated through the column at a constant flow rate of 200 $\mu\text{L}/\text{min}$ with eluent for the first 3 min being directed to waste to avoid salt contamination of the ion source. The LC line was then connected to the mass

spectrometer, and ESI-MS data were acquired in positive mode over a mass range of m/z 200–2000. In total, 10 exchange time points were acquired from 25 s to 90 min.

To account for the back-exchange during data acquisition, a nondeuterated control (m_0) and a fully deuterated control (m_{100}) were obtained. The fully deuterated control was prepared by incubation of the protein in D₂O at 37 °C for 16 h and then for 5 min at 90 °C prior to injection. The relative deuterium uptake (D) was calculated using the equation³⁵

$$D = \frac{m_{\text{obs}} - m_0}{m_{100} - m_0} \quad (3)$$

The data points were fit to a triexponential equation where y_0 is the actual number of exchangeable amide protons under a given condition (%D multiplied by N , where N is the theoretical number of exchangeable amide protons; N is the total number of amino acid residues – the number of Pro residues – 1 for the amino terminus; for FIH, $N = 324$), and A–C correspond to the slow, medium, and fast exchangers with assigned average rate constants of k_1 (0.05 min^{−1}), k_2 (1 min^{−1}), and k_3 (20 min^{−1}), respectively:

$$y = y_0 - Ae^{-k_1 t} - Be^{-k_2 t} - Ce^{-k_3 t} \quad (4)$$

Limited Proteolysis with Mass Spectrometry.

Proteolysis experiments were carried out at 37.0 °C with 150 μg of FIH in 50 mM Tris (pH 7.5) in the presence or absence of 100 μM MnSO₄ and 500 μM αKG . Samples were initially incubated on ice for 10 min and then at 37.0 °C for 1 min prior to the addition of 0.5 mg/mL trypsin to a protease:substrate weight ratio of 1:300. Aliquots (9 μL) were quenched at varying time points into 1 μL of 10 mM AEBSF and stored at −80 °C until use. The samples were analyzed as soon as possible. For SDS-PAGE analysis in a 12% acrylamide gel, 1.5 μL of the quenched sample was mixed with 6 μL of 2 $\times$ gel loading buffer and diluted to a total volume of 10 μL with 50 mM Tris (pH 7.5). For MALDI-MS analysis, 3 μL of the quenched sample was mixed with 15 μL of 10 mg/mL sinapinic acid in 70% CH₃CN/0.03% TFA and spotted onto a MALDI target for analysis. MALDI-TOF mass spectra of the limited proteolysis products were recorded over an m/z range from 10000 to 60000. To verify the protein terminal sequence via MALDI-MSD (in-source decay), the samples were mixed in a 1:1 ratio with 10 mg/mL sinapinic acid in 30% CH₃CN/0.1% TFA. The air-dried spotted samples were briefly rinsed with cold water to remove excess salt and then air-dried again before analysis. MS/MS data were acquired in linear mode over a m/z range from 300 to 20000 with a Bruker (Billerica, MA) MicroFlex LRF MALDI-TOF instrument using at least 50% laser power to induce ISD.

Circular Dichroism Spectroscopy.

Protein samples for circular dichroism (CD) measurements were prepared in 10 mM KH₂PO₄ (pH 7.04) with 500 μM αKG and 50 μM MnSO₄. Protein concentrations ranged from 0.2 to 0.4 mg mL^{−1} as measured by UV absorbance at 280 nm ($\epsilon_{280} = 1.22 \text{ mL mg}^{-1} \text{ cm}^{-1}$). The spectra were recorded over the wavelength range of 190–250 nm at 37.0 °C

using a highly transparent quartz cell with a path length of 0.1 cm on a Jasco J-1500 CD spectropolarimeter. The instrument was blanked with the buffer solution containing all constituents except the protein. All measurements were taken at least three times to ensure that the signal is for that of a sample at equilibrium. The averages of these measurements were used to generate the CD spectra.

RESULTS AND DISCUSSION

Effect of α KG on CTAD Affinity.

The consensus mechanism for FIH and other α KG hydroxylases assumes sequential substrate binding in the following order: α KG, prime substrate, and then O_2 . While this makes sense from the perspective of the chemistry, it is hard to reconcile an obligate sequential order based on the crystallographic structures of FIH bound to various substrates. Early reports of FIH/CTAD binding in biological assays suggested that (M+ α KG) increased the binding affinity of FIH for the HIF-1 α /CTAD domain.³¹ However, protein crystallography indicated that the equilibrium structure of FIH did not change upon binding α KG^{10,11} and that the CTAD binding site was invariant in the structures.

To directly test the effect of α KG on the CTAD binding affinity, we monitored the binding of CTAD to FIH via changes in the intrinsic tryptophan fluorescence intensity in the presence and absence of α KG. Titration of 500 μ M CTAD into apoFIH did not lead to a lower-intensity fluorescence signal, even in the presence of 50 μ M Mn^{2+} , indicating a very weak affinity for CTAD ($K_D > 1$ mM). In contrast, titration of 500 μ M CTAD into (Mn+ α KG)FIH led to a smooth decrease in Trp fluorescence, indicating moderate affinity for CTAD. Nonlinear curve fitting of the data for (Mn+ α KG)FIH gave a K_D of 92 ± 9 μ M, which is similar to the K_M value (~ 90 μ M) for FIH previously observed,³⁴ indicating that α KG was essential for medium-affinity binding of CTAD to FIH. Notably, the K_D for binding of CTAD to (M+ α KG)FIH using varied metals (M = Mn^{2+} or Co^{2+}) closely corresponded to the Michaelis constant for CTAD ($K_M \sim 90$ μ M), while the native metal (M = Fe^{2+}) was used, confirming that the FIH protein structure is well ordered with the middle transition metals. This is consistent with the biophysical data using a variety of metals in the active site, including Fe, Co, and Zn.

The FIH/CTAD affinity increased >10-fold in the presence of α KG, indicating sequential binding of α KG followed by CTAD as assumed by the consensus mechanism. The origin of the increased affinity is unclear, as the FIH protein structure does not change upon binding α KG. This suggests that altered protein dynamics of FIH may impact the CTAD binding site (Figure 2).

Effect of α KG on Metal Binding.

Control titrations were performed to ensure full loading of the metal site of FIH. The isosteric Mn^{2+} was used in place of Fe^{2+} for binding assays to prevent complications due to autohydroxylation³⁶ or enzyme turnover. Titration of citrate-buffered Mn^{2+} into FIH containing 500 μ M α KG led to a monotonic decrease in the intrinsic tryptophan fluorescence of FIH, indicating the binding of metal to the active site. The normalized

fluorescence was fit to a dose–response equation, with a K_D of 13(2) μM for the equilibrium $\text{FIH}(\text{Mn}+\alpha\text{KG}) \rightleftharpoons \text{FIH} + \alpha\text{KG} + \text{Mn}^{2+}$. The binding affinity for Mn^{2+} is very similar to that for Co^{2+} [14(1) μM],³⁷ which has been shown to be a good substitute for Fe^{2+} .³⁴ A similar titration in which αKG was omitted did not lead to a change in fluorescence, indicating that high-affinity binding of metal to the FIH active site required αKG .

Effect of M+ αKG Binding on FIH Structure and Stability.

Although protein crystallography showed that the protein backbone structure of apoFIH could be superimposed upon that of (M+ αKG)FIH,¹¹ this reflects the equilibrium structure without revealing protein flexibility or dynamics and may be perturbed by crystal packing forces. To test for changes in the secondary structures of FIH upon (M+ αKG) binding, CD spectra of the apo and (Mn+ αKG)-bound enzyme forms were obtained. Each spectrum had two negative features around 210 and 219 nm and a positive feature at 191 nm, indicating a mixed α -helix/ β -strand structure. The similarity of each spectrum showed that the secondary structure of FIH in solution was not significantly impacted by binding (Mn+ αKG), supporting the retention of structure seen by crystallography.

The thermal stability of FIH was measured to test for stability changes upon cofactor and substrate binding (Figure 3). Addition of Mn^{2+} along with αKG increased the apparent melting temperature from 47 to 52 °C. This 5 °C increase in the enzyme's thermal stability was consistent with (Mn+ αKG) addition leading to a more tightly folded structure of the protein; a similar observation of the stabilizing effect of αKG was also seen for the other HIF hydroxylase, PHD2.^{38,39} Addition of only Mn^{2+} to FIH did not stabilize the protein (T_M = 47 °C), consistent with the very weak binding of Mn^{2+} by FIH in the absence of αKG .

Impact of (Mn+ αKG) on Global Amide Exchange (HDX-MS).

As αKG binding did not alter the average protein structure, the increase in protein stability suggested that FIH became less dynamic upon binding M+ αKG . Comparing the B -factors for apoFIH (PDB entry 1IZ3¹²) and (M+ αKG)FIH (PDB entry 1H2N¹¹) structures revealed a global decrease in these refinement parameters upon binding (M+ αKG), suggesting that backbone dynamics also decreased. As HIF-1 α /CTAD is unstructured in solution,⁴⁰ a preformed CTAD binding site on FIH would serve to select the correct conformation of the CTAD domain. We assessed the flexibility of the FIH protein via global amide H/D exchange experiments coupled to mass spectrometry (HDX-MS) (Figure 4). Global HDX measures the isotopic exchange of ^1H in backbone amide N–H protons with ^2H in solvent D_2O , leading to an increase in mass upon forming the amide N–D. For tightly folded proteins, amide HDX occurs via the transient unfolding of local protein structure, such that amide HDX measures backbone dynamics over the time scale of seconds to hours. Both kinetics and the number of amides undergoing amide HDX are observed, leading to an overall view of protein flexibility in solution.

Global amide HDX was measured for FIH over 100 min, in which the increase in mass was fit to eq 3 (Table 1) to account for amides exchanging over a wide time scale. The varied parameters were the numbers of exchanging amides, as the three rate constants were fixed to reduce variables by binning amides into three different exchange time scales. ApoFIH

exhibited >50% D uptake, with a broad distribution between slow ($A = 47$), medium ($B = 55$), and fast ($C = 84$) exchanging amides. The overall amide exchange distribution was similar for the FIH sample containing Mn^{2+} , which was expected as Mn^{2+} did not bind to FIH in the absence of αKG . In contrast, the $(Mn+\alpha KG)$ FIH sample achieved <50% D uptake over the time scale of measurement, indicating a more rigid protein structure. The distribution of amide exchangers for $(Mn+\alpha KG)$ FIH further differed from that of apoFIH in the significant decrease in the number of fast ($C = 51$) exchanging amides, again indicating that the overall protein became much more rigid upon binding αKG . A similar observation using the same global amide HDX method was also noted in our lab for the other HIF hydroxylase, PHD2.³⁹

The decreased flexibility of FIH upon binding αKG suggested that broad segments of FIH become less dynamic once bound to αKG . This experimental result is also consistent with a molecular dynamics study⁴¹ that predicted substantially reduced flexibility for FIH in the presence of αKG . When considered along with the decrease in the crystallographic B -factors upon binding $Fe+\alpha KG$,^{11,12} these results indicate a reduction in protein dynamics for FIH once αKG binds to the active site.

Limited Proteolysis: MALDI-TOF MS in Parallel with SDS-PAGE Analysis.

To gain better local insight into the decreased flexibility of $(Mn+\alpha KG)$ FIH, limited proteolysis was performed. Limited proteolysis is widely used for studies of protein dynamics as it relies on transient unfolding to expose proteolytic cleavage sites. Ligand binding can cause regions of the protein to change in conformation and become less solvent accessible, which can lead to a decreased rate of proteolytic cleavage.³⁸ We used trypsin due to its high specificity for Arg/Lys residues, which facilitates downstream identification of the generated proteolytic fragments, giving structural information about the protein regions that were stabilized upon αKG binding.

FIH is a homodimeric protein that dissociates under denaturing conditions to run as a single band at 41 kDa via SDS-PAGE. The SDS-PAGE of trypsinolysis products of apoFIH (Figure 5) revealed that the intact protein (band 1, ~41 kDa) was successively cleaved into three heavy fragments, the lightest of which (band 4, ~34 kDa) appeared to diminish in abundance at long time points presumably due to cleavage into smaller tryptic peptides. A similar pattern of fragmentation was observed for $(Mn+\alpha KG)$ FIH, but with two notable differences from the pattern for apoFIH. First, full-length $(Mn+\alpha KG)$ FIH was not cleaved as rapidly as apoFIH, as shown by the higher relative abundance of intact protein after digestion for 15 min (Figure 5B). Second, the 34 kDa band (band 4) accumulated for $(Mn+\alpha KG)$ FIH out to 240 min, whereas this band was visibly reduced from the apoFIH lanes (Figure 5A); an even greater difference was noted at 1440 min (Figure 5C).

The intensity of band 4 remained strong even after proteolytic treatment in $(Mn+\alpha KG)$ FIH for 24 h, whereas it completely disappeared for the apoenzyme under this longer incubation period (Figure 5C). This points to the 34 kDa fragment as corresponding to a stable core of FIH in the presence of Mn and αKG . Collectively, limited proteolysis, melting temperatures, and global amide HDX data indicated that αKG binding leads to a less dynamic and more

tightly structured protein core in (Mn+ α KG)FIH, which agreed with the predictions from molecular dynamics.⁴¹

Limited proteolysis samples were analyzed using MALDI-TOF mass spectrometry to identify the 34 kDa stabilized core. The MALDI data revealed the presence of proteolytic fragments with m/z values that were in excellent agreement with the bands observed by SDS-PAGE and could further be compared to the tryptic cleavage pattern predicted for FIH (Figure 6). The protein sequence of FIH (Figure 7) illustrated the three predicted cleavage sites at residues with high solvent accessibility. While sequential trypsin cleavage from either terminus of FIH could lead to similar pattern fragments, attention was focused on those Lys/Arg residues at the N-terminus because the FIH homodimer interface is at the C-terminus. Monomeric FIH is unstable, suggesting that residues found at the dimer interface are unlikely to be accessible for proteolysis, a suggestion supported by the MALDI data (see below).

The three trypsin sites at the N-terminus of FIH are Arg17, Arg44, and Arg51. Successive trypsin cleavage of the protein at these sites would generate fragments as follows (Table 2): cleavage of the intact protein at Arg17 (39 kDa fragment), cleavage at Arg44 (~36 kDa fragment), and cleavage at Arg51 (~34 kDa fragment). Importantly, each cleavage would result in a truncated protein with an intact cupin barrel for α KG binding.

Band 4 (~34 kDa fragment) was tentatively assigned as the cupin barrel core due to the observed stability of this fragment to trypsinolysis. Sequencing data were obtained via ISD using MALDI-TOF mass spectrometry (Figure 8) to positively identify this band. MALDI-ISD is a useful tool for top-down sequencing to verify the end termini of intact known proteins.⁴² The fragmentation process during ISD occurs via a radical pathway, so the major fragment ions expected are the c_n and $(z_n + 2)$ ions.⁴³ Peaks corresponding to the ladder of $(z_n + 2)$ ions as well as some c ions generated at 60% laser power were observed and identified for the parallel sample [(Mn+ α KG)FIH] quenched after trypsin incubation for 4 h. This particular sample was chosen due to the accumulation of the ~34 kDa fragment (Figures 5 and 6). A few y ions were also observed, which are less common than the c_n and z_n ions but further support the assignment.^{42,43} This analysis verified the N-terminal sequence of the fragment by virtue of the ladder of c ions, which indicated that Arg51 was the N-terminal residue. Similarly, the residues involved in FIH dimerization were also identified from the z ion ladder, indicating that the residues up through Asn349 were present in the ~34 kDa fragment (Table 3). This confirms that the stable enzyme core observed by SDS-PAGE as band 4 corresponds to FIH51–FIH349, the cupin barrel along with the dimerization interface.

MALDI-ISD sequencing showed that the 34 kDa stabilized core of (Mn+ α KG)FIH includes the cupin barrel and the dimerization interface, as well as the entire CTAD binding site (Figure 9). Upon binding α KG, FIH adopts a more rigid structure as shown by the lower extent of amide HDX and susceptibility to proteolysis induced by α KG. The stabilizing effect of α KG on FIH reflects the coordinate bonds formed to the metal cofactor, the salt bridges formed to α KG, and the numerous second-sphere contacts that serve to connect metal ligands to the β -strands across the cupin barrel. As it is common to find a similar

pattern of the hydrogen bonding within the cupin barrel,⁸ it is likely that many other α KG hydroxylases will be similarly stabilized by α KG binding.

Screening Inhibitors by a Thermal Shift Assay.

FIH is the subject of inhibition studies as it regulates key physiological processes related to proliferation. These studies have led to many successes, as reviewed previously;¹⁸ however, a challenge with identifying new inhibitors for FIH is that the activity assays are not easily amenable to high-throughput screening methods.¹⁸ A general thermal shift assay was described in 2004 for ligand binding to proteins, based on the change in fluorescence from a dye that is sensitive to protein denaturation.⁴⁴ Binding of a ligand to protein will stabilize the protein and lead to a shift in the melting temperature. In this assay, the thermocycler function on an RT-PCR instrument was used to vary the melting temperature while SYPRO Orange dye provided the fluorescent signal, suggesting that such an assay might work to detect ligand binding to FIH.

FIH was screened against *N*-oxalylglycine (NOG), a ligand that is known to bind to the enzyme at the α KG site.¹¹ In view of the cooperative binding of M^{2+} along with α KG, we tested different combinations of the ligand and metal for their ability to shift the melting temperature (T_M) of FIH. The addition of Mn^{2+} or α KG to FIH did not increase T_M ($T_M = 52.9$ – 53.0), consistent with the results from intrinsic fluorescence quenching (Figure 2) and amide HDX (Figure 4), showing that Mn^{2+} did not bind to FIH. In contrast, the T_M increased significantly when Mn^{2+} was added in the presence of $100\ \mu M$ α KG (55.3) or NOG (57.1), indicating that these ligands bound cooperatively with Mn^{2+} to stabilize FIH from unfolding (Figure 10).

The thermal shift results lead to two important outcomes. First, a fluorometric thermal stability assay can be used to detect ligand binding to FIH. This assay should be amenable to high-throughput screens of compound libraries for FIH inhibitors, provided users observe the synergy between metal and α KG binding sites. Second, in concert with the amide HDX and proteolysis results, ($Mn + \alpha$ KG) binding leads to a decrease in protein dynamics, stabilizing FIH from denaturation.

Implications for Catalysis.

Binding ($M + \alpha$ KG) causes FIH to adopt a less flexible structure, providing a structural basis for the sequentially ordered mechanism of FIH. Upon binding ($M + \alpha$ KG), the CTAD binding site shifts from low affinity ($K_D > 1\ mM$) to moderate affinity ($K_D = 80\ \mu M$), leading to a rigorously sequential binding order.²⁴ This has a practical effect, as there is but a narrow solvent accessible channel to the active site in the FIH/CTAD adduct,¹⁴ which would prevent α KG from entering the active site were CTAD present. Otherwise, one could envision that CTAD binding to apoFIH might inhibit turnover by blocking α KG from reaching the active site.

The contrast between binding of HIF-1 α to FIH and PHD2 is striking, despite the fact that these enzymes bind peptides from the respective domains of HIF-1 α in constrained orientations.^{11,45} Although both enzymes become less dynamic once bound to ($M + \alpha$ KG), the $\beta 2$ – $\beta 3$ loop of PHD remains dynamic even when the oxygen-dependent degradation

domain (ODDD) is bound.³⁸ While this work shows that FIH requires (M+ α KG) to bind to the HIF-CTAD domain, PHD2 binds HIF-ODDD in the absence of added (M+ α KG).^{46,47} This difference may arise due to the more buried active site in PHD2, which is closed from the solvent once the β 2– β 3 loop closes onto the ODDD domain, in contrast to the more open active site of FIH, which is closed from the solvent only by the CTAD domain itself. In both examples, prime substrate binding limits the access of the solvent to the cofactor, which sets the stage for the chemical steps leading to hydroxylation.

This has important ramifications for catalysis by FIH and other α KG-dependent hydroxylases. The stabilizing effect of (M+ α KG) binding on FIH both reduces protein flexibility and ensures sequential substrate binding, which demonstrates that global protein flexibility is an effective strategy for enforcing ordered chemistry. When we discuss enzyme/substrate binding, it is common to think in terms of concepts related to small substrates binding within large active sites, such as the lock-and-key or the induced fit models that can destabilize the substrate for subsequent reaction. These concepts are inexact for FIH/CTAD, due to the large protein/peptide interface that would involve a large decrease in entropy upon binding from conformational entropy, solvent entropy, and translational entropy. The decreased dynamics of the (M+ α KG)FIH enzyme form would help to alleviate some of this entropic cost associated with binding CTAD. In this sense, while FIH/CTAD can be viewed as an induced fit binding, this is largely a matter of overcoming the entropic penalty inherent to organizing three reactants (α KG, CTAD, and O₂) in the correct orientation and proximity for reaction.

The flexibility of the CTAD binding site also suggests that FIH inhibition may require targeting of the α KG binding site rather than the CTAD binding site, or at least ensuring that α KG be filled. As the buried α KG site is more attractive for small molecule inhibitors than a protein/peptide interface, the general approach for FIH inhibitors has been to target the α KG initially.¹⁸ However, targeting the α KG binding site leads to challenges due to the potential for bidentate chelators to bind metals in solution leading to metal sequestration, as well as due to the wide array of enzymes using α KG as a substrate. These challenges are similar to those faced in targeting other metalloproteins⁴⁸ and are not insurmountable. For example, generic inhibitor templates for several α KG-dependent hydroxylases have been identified,¹⁸ leading to a degree of selectivity for several of the enzymes. In the case of FIH, selective inhibition may be best achieved by targeting both the α KG and CTAD binding site simultaneously,¹⁸ as reported for a family of *N*-oxalyl amino acids selective for FIH over PHD2.⁴⁹

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

The authors thank Prof. Richard Vachet for the use of the PTI Quantamaster-4 SE fluorimeter and Prof. Michelle Farkas for the use of the Bio-Rad CFX Connect Real-Time PCR Detection System. Mass spectral data were obtained at the University of Massachusetts Mass Spectrometry Core Facility.

Funding

This research was supported by National Institutes of Health Grant 1R01-GM077413 to M.J.K.

ABBREVIATIONS

αKG	α -ketoglutarate
CTAD	C-terminal transactivation domain
EDTA	ethylenediaminetetraacetic acid
ESI-TOF MS	electrospray ionization/time-of-flight mass spectrometry
FIH	factor-inhibiting HIF-1
GST	glutathione <i>S</i> -transferase
HDX-MS	hydrogen/deuterium exchange mass spectrometry
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF	hypoxia-inducible factor 1 α
MALDI-TOF MS	matrix-assisted laser desorption ionization/time-of-flight mass spectrometry
MW	molecular weight
NOG	<i>N</i> -oxalylglycine
SDS-PAGE	sodium dodecyl sulfate–polyacrylamide gel electrophoresis
TFA	trifluoroacetic acid

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Figure 1. Polar contacts between FIH (wheat) and CTAD (cyan) found in Protein Data Bank entry 1H2K¹¹ imaged using PyMOL.³⁰ Residue numbers are included for select residues, NOG (*N*-oxalylglycine).

Figure 2.

Binding of HIF-1 α /CTAD substrate to FIH in the presence of αKG (●) and in the absence of αKG (○; apoFIH, ▲). Samples contained one, two, or all of the following components: 2 μM FIH, 50 μM MnSO_4 , and 500 μM αKG in 50 mM HEPES (pH 7.00). The experiment was performed in duplicate at an excitation λ of 295 nm, and the intrinsic fluorescence quenching was monitored between 300 and 375 nm. Nonlinear regression analysis of the plot for the αKG -bound enzyme using eq 1 gave a K_D of $92 \pm 9 \mu\text{M}$ (Figure 2).

Figure 3.

Thermal shift curves showing the effect of α KG on the thermal stability of FIH. The inset is the derivative plot to facilitate apparent melting point determination. The thermal shift of the assay mix containing one, two, or all of the components [5 μ M FIH, 50 μ M MnSO₄, and 500 μ M α KG in 50 mM HEPES (pH 7.0)] was monitored using SYPRO Orange dye using a thermocycler.

Figure 4.

Global deuterium uptake plots showing the effect of α KG on the backbone amide solvent accessibility of FIH. H/D exchange was initiated by diluting samples in 10 mM ammonium acetate buffer (pD 7.4) to give final concentrations of 20 μ M FIH, 50 μ M MnSO_4 , and 500 μ M α KG.

Figure 5.

Limited proteolysis of FIH in the absence and presence of α KG as analyzed via SDS-PAGE. N indicates the negative control, (Mn+ α KG)FIH without trypsin incubated for 4 h (240 min) at 37 °C; M is the molecular weight marker. Samples were analyzed in a 12% polyacrylamide gel. (A) Protein samples [150 μ g of FIH, 100 μ M MnSO₄, and 500 μ M α KG in 50 mM Tris (pH 7.5)] were incubated with 0.5 mg/mL trypsin in a 1:300 protease:substrate ratio for the indicated time points at 37 °C and then quenched in 1 mM AEBSF. The sample loading volume was 1 μ L per well corresponding to ~2 μ g of protein. (B) A second replicate sample was performed as in panel A, but the sample loading volume used this time was 3 μ L to make band 2 more visible. (C) A third replicate sample was prepared as in panel A, but the incubation time was extended to 24 h. The arrow indicates the ~34 kDa fragment assigned as band 4.

Figure 6. Analysis of the limited proteolysis data of FIH by MALDI-TOF mass spectrometry in linear mode. The same quenched samples [150 μ g of FIH, 100 μ M MnSO₄, and 500 μ M α KG in 50 mM Tris (pH 7.5)] used in SDS-PAGE analysis were mixed with 10 mg/mL sinapinic acid in 70% CH₃CN/0.03% TFA in a 1:5 sample:matrix ratio.

1 MAATAAEAVA SGSGEP**REEA** GALGPAWDES **QLRSYSFPTR** PIP**RLS**QSDP **RAEELIENEE** 60
 61 PVVLTDNLV YPAL**KWD**LEY LQENIGNGDF SVYSAST**HKF** LYYDE**KK**MAN FQNF**KPR**SNR 120
 121 EEM**KF**HEFVE **KLQDIQQRGG** EERLYLQOTL NDTVGR**KIVM** DFLGFNWNWI **NKQQGKR**GWG 180
 181 QLTSNLLIG MEGNVTPAHY DEQONFFAQI **KGYKRCILFP** PDQFECLYPY PVHHPCDR**QS** 240
 241 QVDFDNP**DYE** **RFPNFQNVVG** YETVVGP**GDV** LYIPMYWWHH IESLLNGG**IT** ITVNF**WYKGA** 300
 301 PTP**KRIEYPL** **KAHQKVAIMR** NIEKMLGEAL GNPQEVGP**LL** NTMI**KGRYN** 360

Figure 7.

Primary structure of FIH showing all of the possible trypsin cleavage sites colored red.

Highlighted in gray are the amino acids involved in the FIH dimer interface. The expressed protein used has three additional residues (GSH-) at the N-terminus, which are not included in the figure.

Figure 8.

Top-down sequence verification of the ~34 kDa tryptic fragment (band 4) of FIH using ISD coupled to MALDI-TOF MS. The sample quenched after protease incubation for 4 h was used for the analysis. It was mixed with 10 mg/mL sinapinic acid in 30% CH₃CN/0.1% TFA in a 1:1 ratio. (A) Spectrum showing the c , $z_n + 2$, and a few y ions identified for the m/z range of <2000. Also shown are the verified N- and C-terminal sequences of FIH. (B) Spectrum for the m/z range of >2000.

Figure 9. Biological dimer of FIH (PDB entry 1H2K), showing CTAD (cyan) and the trypsin fragments of FIH: N-terminus–Arg17 (red), Glu18–Arg44 (green), Leu45–Arg51 (blue), and Ala52–Asn349 (wheat).

Figure 10.

Thermal shift assay for ligand binding to FIH. Assays were conducted using 5 μ M WT FIH, 5 μ M MnSO_4 , 200 μ M NOG or 100 μ M α KG, and 5 $\times$ SYPRO Orange dye in 50 mM HEPES (pH 7.00).

Table 1.

Summary of Global HDX Data

	apoFIH^a	(Mn)FIH^a	(Mn+αKG)FIH^a
N_0	186 ± 1	199 ± 5	157 ± 2
A_{slow}	47 ± 2	60 ± 8	55 ± 4
B_{medium}	55 ± 3	50 ± 11	51 ± 6
C_{fast}	84 ± 3	89 ± 10	51 ± 6

^aThe corresponding average rate constants were fixed accordingly during the fitting: k_1 (0.05 min⁻¹), k_2 (1 min⁻¹), and k_3 (20 min⁻¹).³⁶

The initial parameters obtained were relative values; the number of exchangeable amides reported above was calculated by multiplying the relative values by N ($N = 324$). The HDX experiments were performed in duplicate.

Table 2.

Summary of the Average Observed Masses for the Four Major Tryptic Fragments Determined after MALDI-TOF MS Analysis

protein species	observed MW ^a	calculated MW ^b	fragment ^c
band 1 (intact FIH)	41186 ± 635	40567	–3 to 349
band 2	39601 ± 489	38728	18–349
band 3	36654 ± 585	35714	45–349
band 4	34904 ± 609	34931	52–349

^aThe observed MW and uncertainties represent the full width at half-maximum values generated after fitting the peaks to a Gaussian function.

^bThe calculated MW is the average mass of FIH fragments most closely corresponding to the observed fragments.

^cThe processed FIH as used contains a leading sequence Gly-Ser-His-.

Table 3.

Summary of the Observed *c* and *z* Ions for the 34 kDa Fragment (band 4) Generated during ISD-MALDI-TOF MS^a

N-terminus: AEELIENEETPVVLTDTNLVYPALKWDLEYLQE

C-terminus: MRNIEKMLGEALGNPQEVGPLLNTMIKGRYN

sequence (N-terminus)	observed (<i>c</i> ions)	calculated (<i>c</i> ions)	sequence (C-terminus)	observed (<i>z</i> ions)	calculated (<i>z</i> ions)
V	1270	1271	G	2028	2028
V	1369	1370	N	1915	1915
L	1484	1483	P	1819	1818
T	–	1584	Q	1689	1689
D	–	1699	E	–	1560
T	1799	1800	V	1461	1461
N	1915	1914	G	–	1404
L	2028	2027	P	1308	1307
K	2699	2698	N	3186	3185
W	2885	2884	I	3070	3071
D	3001	3000	E	2959	2958
L	–	3113	K	–	2828
E	3242	3242	M	2701	2700

^aThe regions of the N- and C-termini identified from sequence analysis are highlighted in gray.