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Substrate promotes productive gas binding in the α KG-dependent oxygenase FIH

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Abstract

The $\text{Fe}^{2+}/\alpha\text{KG}$ -dependent oxygenases use molecular oxygen to carry out a wide variety of reactions with important biological implications, such as DNA base-excision repair, histone demethylation, and the cellular hypoxia response. These enzymes follow a sequential mechanism in which O_2 binds and reacts after the primary substrate binds, making those structural factors that promote productive O_2 binding central to their chemistry. A large challenge in this field is to identify strategies that engender productive turnover. Factor Inhibiting HIF (FIH), is a $\text{Fe}^{2+}/\alpha\text{KG}$ -dependent oxygenase that forms part of the O_2 sensing machinery in human cells by hydroxylating the c-terminal transactivation domain (CTAD) found within the HIF-1 α protein. The structure of FIH was solved with the O_2 analogue NO bound to Fe, offering the first direct insight into the gas binding geometry in this enzyme. Through a combination of DFT calculations, $\{\text{FeNO}\}^7$ EPR spectroscopy, and UV-Vis absorption spectroscopy we demonstrate that CTAD binding stimulates O_2 reactivity by altering the orientation of the bound gas molecule. Although FIH binds NO with moderate affinity, the bound gas can adopt either of two orientations with similar stability; upon CTAD binding, NO adopts a single preferred orientation that is appropriate to support oxidative decarboxylation. Combined with other studies on related enzymes, our data suggests that substrate induced reorientation of bound O_2 is the mechanism utilized by the αKG oxygenases to tightly couple O_2 activation to substrate hydroxylation.

Introduction

The $\text{Fe}^{2+}/\alpha\text{KG}$ -dependent oxygenases constitute the largest family of mononuclear nonheme oxygenases.¹ These enzymes activate molecular oxygen (O_2) and couple the oxidative decarboxylation of the αKG co-substrate to many important biological transformations, including repair of alkylated DNA and RNA, antibiotic synthesis, and protein modifications.^{2–5} Although these enzymes utilize a sequential mechanism in which primary substrate binding precedes O_2 activation, thereby coupling O_2 consumption to substrate hydroxylation, the mechanical linkage between these two steps remains elusive despite a number of studies testing the role of changes in Fe coordination number, 2° sphere contacts,

and O₂ affinity.^{5–9} The strategy by which coupled turnover is achieved is central to the normal function of these αKG-dependent oxygenases.

The Factor Inhibiting HIF (FIH) is an Fe²⁺/αKG-dependent enzyme that hydroxylates the HIF transcriptional activator specifically at Asn⁸⁰³ within the c-terminal transactivation domain (CTAD), regulating O₂ homeostasis in human cells.^{10,11} A key feature of FIH function is that O₂ activation is stimulated by the binding of the CTAD domain of the HIF transcription factor,¹² which ensures that the rate of CTAD hydroxylation is directly proportional to the concentration of O₂ (Scheme 1).^{13–15} Recently, it was shown that CTAD binding leads to a mixture of 6-coordinate (6C) and 5-coordinate (5C) Fe²⁺ in FIH/CTAD,⁷ suggesting that greater O₂ access to the Fe²⁺ may only partially explain the increased O₂ reactivity upon CTAD binding. The rate-limiting step for O₂ activation by FIH occurs before early in turnover, making FIH an excellent enzyme to interrogate the link between O₂ activation and substrate hydroxylation in the broad class of αKG oxygenases.

In order to gain insight into the structural factors that influence productive gas binding in the αKG oxygenases, we used NO as an O₂ mimic to study the changes in the gas binding site of FIH induced by substrate binding. Nitric oxide (NO) is commonly used as an O₂ surrogate to learn about intermediates and the chemistry of non heme O₂ activating enzymes as bound NO adopts a similar geometry to that of O₂.^{16–18} NO is capable of reversibly binding to Fe²⁺ within enzyme active sites, altering the electronic properties to allow characterization by conventional EPR and electronic absorption spectroscopy.^{19–21} Metal-nitrosyl complexes are generally described as {MNO}ⁿ, where n represents the sum of the metal d and NO π* electrons; Fe²⁺ bound NO is {FeNO}^{7,22}

Although gas binding is crucial to defining the chemistry in non heme enzymes, little geometrical data exists. We are aware of only one structure of NO bound to an Fe²⁺/αKG-dependent oxygenase, clavaminic synthase (CAS).¹⁸ The structure of CAS with NO bound revealed a pseudo octahedral metal center with NO oriented above αKG in the presence of substrate.¹⁸ DFT calculations in a related enzyme, taurine dioxygenase (TauD), indicate that NO preferentially adopts two conformations, one in which the O-atom is directed towards αKG and another pointing over the active site carboxylate moiety.¹⁷ As only the conformation in which NO is oriented above αKG is expected to be reactive based on the consensus mechanism, factors governing the switch in NO orientation may be central to coupled turnover in αKG oxygenases.

Here we report our use of crystallography, electronic spectroscopy, and DFT calculations to test the role of substrate sterics on productive gas binding in FIH. CTAD binding, specifically the target residue (Asn⁸⁰³), was tested for its role in forming the proper gas binding geometry which leads to substrate hydroxylation. This is particularly important as evidence suggests that the gas binding geometry may be the fundamental process controlling turnover in the αKG-dependent oxygenases.

Materials and Methods

Protein Purification

Unless noted otherwise, all reagents were purchased and used as received from commercial vendors. FIH expression and purification was carried out as previously described.^{12,15} Briefly, the N-terminal His₆ tagged protein was purified using a Ni-NTA column followed by removal of the tag via thrombin digestion. Post thrombin cleavage, three non-native residues (Gly, Ser, His) remain at the N-terminus of FIH. Exogenous metals were removed with EDTA treatment. FIH was further purified using size-exclusion chromatography to afford the active dimer. Protein concentrations were calculated by absorption spectroscopy ($\epsilon_{280} = 48800 \text{ M}^{-1} \text{ cm}^{-1}$).¹²

CTAD Purification

A 39 amino acid peptide substrate corresponding to the c-terminal transactivation domain of HIF α ^{788–826} (CTAD) containing a Cys⁸⁰⁰ → Ala substitution with sequence DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN (Asn⁸⁰³ in bold) was ordered from EZBiolabs as a desalted synthetic peptide with free N and C termini. Another 39 residue peptide also with a Cys⁸⁰⁰ → Ala point mutation containing the target residue Asn⁸⁰³ → Ala point mutation with sequence DESGLPQLTSYDAEVAAPIQGSRNLLQGEELLRALDQVN (Ala⁸⁰³ in bold) was also ordered from EZBiolabs as a desalted peptide with free N and C termini. The peptides were dissolved in 25% acetonitrile and purified by reverse-phase HPLC as previously reported.¹⁵ After two rounds of purification, the samples were >95% pure as determined using matrix assisted laser desorption ionization mass spectrometry (MALDI-MS). The concentrations were established by measuring the UV-absorption at 293 nm in 0.1 M NaOH ($\epsilon = 2400 \text{ M}^{-1} \text{ cm}^{-1}$).¹²

Crystallography and Data Refinement

Crystals of (Fe+NOG+NO)FIH were grown anaerobically using conditions similar to those previously reported in the literature.²³ Crystals were grown in an anaerobic glovebox by the hanging drop vapor diffusion method by mixing 3 μL of a 20 mg/ml protein solution containing 0.55 mM FeSO₄, and 2mM αKG in 50 mM Hepes pH 7.00 with 1 μL of the reservoir buffer containing 0.1 M Hepes, 1.2 M (NH₄)₂SO₄, and 3 % polyethylene glycol (PEG) 400, pH 7.50. After setting up trays, the nitric oxide donor, Diethylammonium (Z)-1-(N,N-diethylamino)diazen-1-ium-1,2-diolate (DEANO) was added only to the reservoir solution to facilitate NO release into the headspace during crystallization. 100 μL of a 0.35 M stock of DEANO was added to the reservoir solutions (total volume = 500 μL), such that 70 mM DEANO was present, and the coverslips were quickly resealed. Crystals were allowed to grow in the glovebox at 23°C with NO filling the headspace in the wells. Yellow crystals grew within 4 days (Figure S2), at which time they were harvested on the bench top within 1 minute of the coverslip being flipped to minimize gas diffusion and flash frozen in reservoir solution containing 24% glycerol. Clear, colorless crystals of (Fe+ αKG)FIH were grown anaerobically in the same fashion without the addition of DEANO to the reservoir solution.

Data was collected at the Advanced Photon Source facility in Chicago at 100 K using radiation of $\lambda = 0.979 \text{ \AA}$. 150 frames were collected at a crystal to detector distance of 345 mm with 1.5° oscillation and 1 second exposure per image for (Fe+NOG+NO)FIH. For (Fe+ α KG)FIH, 150 frames were captured with 2° oscillations at a crystal to detector distance of 380 nm and 1 second exposure per image. The data sets were processed using iMOSFILM.²⁴ Coordinates from PDB 3D8C²³ were employed for molecular replacement using Phaser²⁵ and the model building and refinement done using COOT²⁶ and Refmac5²⁷ in the CCP4²⁸ software package. During refinement, 5% of the data was withheld and used to obtain an R_{free} value. Data collection and crystallographic analysis statistics are reported in the supplementary material (Table S1). Anaerobic crystals of (Fe+ α KG+NO)FIH, (Fe+ α KG+NO)FIH/CTAD and (Fe+ α KG+NOG+NO)FIH/CTAD were of poor quality and most dissolved during harvesting. Unfortunately the few that were successfully harvested failed to diffract well and we were unable to collect high resolution data.

Electronic Absorption Spectroscopy

For anaerobic experiments, stocks of FIH, α KG, NOG, and Hepes buffer were sealed with rubber septa and degassed with argon gas. FeSO_4 was brought into the glovebox as a solid and prepared using degassed H_2O while DEANO stocks were prepared in degassed 10 mM NaOH and the concentration verified by its published extinction coefficient and characteristic UV absorbance at 250 nm ($\epsilon_{250} = 6500 \text{ M}^{-1}\text{cm}^{-1}$).^{29,30} DEANO decomposes to form 1.5 equivalents³¹ of NO with a rate constant of $3.75 \times 10^{-3} \pm 8 \times 10^{-5} \text{ s}^{-1}$ under our conditions (Figure S1). Anaerobic UV-Vis samples contained FIH (0.25 mM), FeSO_4 (0.25 mM), α KG (0.5 mM), CTAD (0.5 mM) and DEANO (0.13 – 2 mM) in 50mM Hepes pH 7.00. Samples were incubated in the glovebox at 23°C for 20 minutes to permit NO release and binding. The samples were capped and then UV-Vis absorption spectra were collected immediately after removal from the glovebox on an HP 8453 UV-Visible spectrophotometer from 300nm to 900nm in 1 cm path length quartz cuvettes. Data used to construct the binding isotherm is reported in the supplementary material (Figure S3). As FIH has comparable affinities for CTAD ($K_D = 80 \text{ }\mu\text{M}$) and N803A ($K_D = 180 \text{ }\mu\text{M}$),⁸ FIH was ~84% bound with CTAD and ~70% bound with the N803A substrate.³²

EPR Spectroscopy

Anaerobic EPR samples containing FIH (0.10 mM), FeSO_4 (0.10 mM), α KG (0.50mM), CTAD (0.50 mM), Asn⁸⁰³ $\rightarrow$ Ala peptide (0.50 mM) and DEANO (0.50 mM) in 50 mM Hepes pH 7.00 were prepared and aged anaerobically at 23°C for 20 minutes. The samples were capped then flash frozen in liquid nitrogen (LN_2) immediately upon removal from the glovebox. X-Band EPR data was collected using a Bruker Elexsys E-500 EPR equipped with a DM4116 cavity and a Bruker ER 4118CF-O LHe/ LN_2 cryostat at 9.624 GHz frequency, 2.0mW power, 10G modulation amplitude, 100 GHz modulation frequency, 163 ms time constant at 4K.

The EPR data was analyzed by means of the standard spin Hamiltonian ($H_{\text{Fe}} = \beta \cdot \mathbf{B} \cdot \mathbf{g} \cdot \mathbf{S} + \mathbf{S} \cdot \mathbf{D} \cdot \mathbf{S}$) where β is the Bohr magneton, $\mathbf{B}$ is the applied field, $\mathbf{g}$ is the electron Zeeman term, $\mathbf{S}$ is the electron spin operator, and $\mathbf{D}$ is the zero field splitting tensor. Simulations of the EPR spectra were calculated using the XSophe software package version 1.1.14.³³ As the S

$= 3/2 \{FeNO\}^7$ spin is in the weak field limit ($D \gg h\nu$), D was fixed at 10 cm^{-1} , corresponding to the measured and calculated values of D for $\{FeNO\}^7$ adducts of related enzymes 4-hydroxyphenylpyruvate dioxygenase (HPPD) ($\sim 8 \text{ cm}^{-1}$) and TauD ($\sim 12 \text{ cm}^{-1}$).^{16,17}

DFT Calculations

Geometry optimizations were carried out on a truncated active site starting from the geometry of the crystal structure of FIH (PDB 1H2L).³⁴ Methyl imidazoles were used for His¹⁹⁹ and His²⁷⁹, propionic acid for Asp²⁰¹, 2 oxopropionate for α KG, and Asn⁸⁰³ was truncated and contained a methyl group from the adjacent amino acid (Figure 6). The $\{FeNO\}^7 S = 3/2$ spin state was generated using the spin-coupling scheme of a high spin Fe^{3+} ($S_{Fe} = 5/2$) antiferromagnetically coupled to two electrons from the $NO^- \pi^*$ orbitals ($S_{NO} = 1$) as this represents the proper electronic description of ferrous NO complexes.^{16,20} Using the geometry optimized structures, a rigid potential energy surface scan (B3LYP/6-311+g(2d,p)) was performed to investigate the energy barrier for rotating the bent NO ligand in the axial coordination position of FIH. The DFT calculations were completed with and without the target Asn⁸⁰³ residue in order to study the influence of the target residue on NO rotational barriers.

Results

Crystal Structure of FIH in Complex with Fe and NO

To gain insight into the O_2 binding site of FIH, we attempted to grow FIH bound to Fe^{2+} , α KG, NO, and CTAD. Anaerobic crystals of $(Fe+\alpha KG+NO)FIH$, $(Fe+\alpha KG+NO)FIH/CTAD$ and $(Fe+\alpha KG+NOG+NO)FIH/CTAD$ were of poor quality and most dissolved during harvesting. Unfortunately the few that were successfully harvested failed to diffract well and we were unable to collect high-resolution data. Good quality crystals grew for $(Fe+\alpha KG)FIH$ and $(Fe+NOG+NO)FIH$, which were harvested and sent for diffraction at Advanced Photon Source at Argonne National Laboratories. The structure of $(Fe+\alpha KG)FIH$ was refined to $2.4 \text{ \AA}$ with $R_{\text{factor}} = 20.0\%$ and $R_{\text{free}} = 25\%$ while the structure of the $(Fe+NOG+NO)FIH$ complex was refined to $2.1 \text{ \AA}$ resolution ($R_{\text{factor}} = 19.0\%$ and $R_{\text{free}} = 24\%$). For both $(Fe+\alpha KG)FIH$ and $(Fe+NOG+NO)FIH$, the active site metal retained pseudo octahedral geometry and Fe^{2+} was coordinated by His¹⁹⁹, His²⁷⁹, Asp²⁰¹ and α KG/NOG with the C1 carboxylate of α KG/NOG bound *trans* to His¹⁹⁹ as previously reported for structures of $(Fe+\alpha KG)FIH$ ³⁵ and $(Fe+NOG)FIH$.³⁴ The backbone of residues 9-349 were essentially unchanged upon binding NO. (rmsd = $0.153 \text{ \AA}$) (Figure S4).³⁶

For $(Fe+\alpha KG)FIH$, the $2F_o - F_c$ map displayed electron density at the coordination position *trans* to His²⁷⁹, indicating that the active site Fe of FIH was 6-coordinate as previously suggested by spectroscopic studies on FIH⁷ and other $Fe^{2+}/\alpha KG$ oxygenases.^{9,37} The density was too small to accommodate a diatomic molecule such as O_2 or two aquo ligands and refined best with one water molecule in this position (Figure 1A). Previously reported structures of $(Fe+\alpha KG)FIH$ at resolutions of $2.84 \text{ \AA}$ and $2.4 \text{ \AA}$ contained unresolved density above the metal centers which is likely to be due to water.^{34,35} Although the resolution of our structure was also modest ($2.5 \text{ \AA}$), the aquo ligand was reasonably well ordered with a

refined B -factor of $46.0 \text{ \AA}^2$ and a reasonable Fe-OH₂ bond length ($2.00 \text{ \AA}$). For comparison, the B -factors for the other atoms bonded to Fe were in the $36 - 40 \text{ \AA}^2$ range, with the exception of the very well ordered Asp²⁰¹ O atom ($26 \text{ \AA}^2$).

For (Fe+NOG+NO)FIH, the $2F_o - F_c$ map displayed electron density at the diffusible ligand site above the metal center which was approximately twice the density observed in the (Fe+ α KG)FIH structure. This density was consistent with the presence of a diatomic molecule as a single water molecule did not adequately refine into the density. Additionally, the $F_o - F_c$ difference Fourier map for the structure without NO subtracted from the structure with NO also displayed electron density at this site, the size and shape of which was consistent with a diatomic molecule (Figure 1C). We refined this as NO due to the size of the electron density, the spectroscopic data indicative of {FeNO}⁷ adduct formation in solution, and the yellow crystals that were used for X-ray diffraction.

The active site metal was pseudo octahedral with NO bound at the axial ligand site *trans* to His²⁷⁹, displacing water (Figure 1B). Although it cannot be interpreted from the density that NO ligated with the nitrogen atom, chemically, this is observed in model complexes and calculations.^{16,17,38} The Fe-N bond length ($1.83 \text{ \AA}$) and the Fe-N-O angle (133°) (Table 2), were within range of calculations and experimental data on Fe-N bond lengths ($\sim 1.70 \text{ \AA} - 1.88 \text{ \AA}$) and Fe-N-O angles ($\sim 120^\circ - 169^\circ$) for related enzymes and model complexes.^{16-18,38-40} The B -factors for NO were $50 \text{ \AA}^2$ for the N atom and $67 \text{ \AA}^2$ for the O atom, indicating that the N atom was reasonably well ordered while the O atom has greater disorder, as compared to the well-ordered N atoms on His¹⁹⁹ ($38 \text{ \AA}^2$) and His²⁷⁹ ($37 \text{ \AA}^2$) in the (Fe+NOG+NO)FIH structure, further supporting our refinement of this density as an NO ligand. A partially resolved PEG molecule was bound above the NO ligand; as the active site of FIH is solvent exposed, the presence of PEG may speak to an evolutionary preference for peptide like constructs to be bound above the metal center. The structures of (Fe+ α KG)FIH (PDB: 4Z2W) and (Fe+NOG+NO)FIH (PDB: 4Z1V) were deposited to the PDB and based on the PDB metrics (Clashscore, Sidechain outliers, Ramachandran outliers, etc.), they are amongst the most reliable structures of FIH that exist.

Electronic Absorption Spectroscopy

Formation of an {FeNO}⁷ center in the active site of a nonheme enzyme leads to moderately intense ($\epsilon < 1000 \text{ M}^{-1} \text{ cm}^{-1}$) absorption bands near 440 nm with a broad shoulder between $500 - 700 \text{ nm}$, arising from NO π^* donor interaction into the Fe t_{2g} orbitals.^{20,21,41} Addition of DEANO to (Fe+ α KG)FIH led to a bright yellow solution with the expected electronic absorption bands (Figure 2) for an {FeNO}⁷ center.^{16,19,21,41,42} Upon addition of CTAD, the spectra increased in intensity, suggesting either tighter gas binding as proposed by the consensus mechanism or a geometric change resulting in stronger orbital overlap.

A binding isotherm was constructed following the individual addition of aged DEANO solutions to premixed samples of (Fe+ α KG)FIH (Figure S3) in order to test the effect of CTAD binding on gas-binding affinity of FIH. The dissociation constant (K_D) for NO was obtained by fitting A_{440} vs. [NO] to a saturation curve (equation 1) similar to previously reported for related extradiol dioxygenases⁴² and phenylalanine hydroxylase²¹ in which A_{max} represents the A_{440} at saturation.

$$\Delta A = \frac{A_{max} * [NO]}{K_D + [NO]} \quad (1)$$

The samples were aged for 20 minutes (~7 half-lives) to allow NO release and binding, by which point the absorbance had ceased increasing. The NO affinity of (Fe+αKG)FIH was moderate ($K_{D(NO)} = 330 \pm 25 \mu\text{M}$) and increased slightly upon binding CTAD ($K_{D(NO)} = 200 \pm 15 \mu\text{M}$) (Table 1), indicating that CTAD did not promote gas binding significantly. In other αKG dependent oxygenases, a much more pronounced increase in NO affinity was observed in the presence of substrate, ranging from 5 – 100 fold.^{21,42}

To test the effect of the steric and polar contacts created by the Asn⁸⁰³ target residue within CTAD on gas binding, we measured the NO affinity using a variant substrate containing the Asn⁸⁰³ → Ala point mutation (N803A). For the (Fe+αKG+NO)FIH/N803A complex, the $K_{D(NO)} = 300 \pm 30 \mu\text{M}$ (Table 1), similar to the affinity for NO in the absence of CTAD (Figure 3). This indicated that the steric imposition of the Asn⁸⁰³ target residue did not overly affect the gas affinity in FIH.

Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy measurements were made after the addition of NO to samples of FIH in order to gain further insight into the geometry and electronic environment of the metal center. The electronic fine structure of {FeNO}⁷ is a sensitive reporter of geometric changes in the {FeNO}⁷ moiety, such as changes in the FeNO bond angle or orientation.^{16,20,42–45} The {FeNO}⁷ is an S = 3/2 spin system which is typically characterized by a large axial zero field splitting (E/D ~ 0) such that the M_s = ± 3/2 and ± 1/2 Kramers doublets are well separated from each other. Due to the large zero field splitting (D ≫ hv) the EPR resonance arises solely from the lower doublet with effective g values at g_x ≈ g_y ≈ 4 and g_z = 2.^{20,42} As the zero-field splitting becomes more rhombic (E/D > 0), the g_{eff} = 4 feature will split into two separate g_{eff} features.

Despite the modest increase in NO affinity in the presence of CTAD, we tested the impact of CTAD binding on the geometry of the {FeNO}⁷ moiety in FIH. Addition of NO to the FIH samples produced the expected yellow color and complex g_{eff} ~ 4 EPR line-shape (Figure 4), along with an S = 5/2 feature due to oxidation of Fe²⁺ to Fe³⁺ as seen in related nonheme {FeNO}⁷ sites.^{16,20,21,41,42}

The spectrum of (Fe+αKG+NO)FIH displayed a slightly rhombic S = 3/2 line-shape with g_{eff} = 4.11, 3.93 (E/D = 0.016) (Figure 4). Addition of NO to samples containing the native CTAD led to formation of (Fe+αKG+NO)FIH/CTAD which exhibited a highly axial line-shape with g_{eff} = 4.06, 4.00 (E/D = 0.005) (Figure 4), along with the slightly rhombic S = 3/2 species observed in the CTAD free complex. With the addition of NO to samples containing the N803A variant substrate, the (Fe+αKG+NO)FIH/N803A sample displayed a slightly rhombic electronic fine structure (E/D = 0.014, g_{eff} = 4.10, 3.93) as seen for the CTAD-free sample (Figure 4). This new axial line-shape corresponds to the active enzyme complex as CTAD contained the proper target residue, suggesting that the Fe-NO geometry changed upon binding the Asn⁸⁰³ target residue. The change in line-shape is indicative of a

change in gas binding geometry, likely due to a rotational change as Solomon et al showed experimentally and computationally that the Fe-NO bond length and angle do not change to any appreciable degree in the presence of substrate for related enzyme HPPD.¹⁶

We simulated the three different species present in the spectra of the (Fe+NO+αKG)FIH/CTAD complex (Figure 4B) using XSophe.³³ A summary of the simulation parameters and experimental g_{eff} values is shown in Table 1. The sum of the simulations overlaid well with the experimental spectra (Figure 4A), and showed that the spectra of (Fe+αKG+NO)FIH/CTAD consisted of two $S = 3/2$ species and one $S = 5/2$ species (Figure 4B). By comparing the experimental and simulated spectra, we observed that the highly axial $S = 3/2$ signal with $E/D = 0.005$ is unique to CTAD bound FIH in which the proper target residue is present as the N803A substrate did not exhibit this line-shape.

DFT Calculations of FIH- $\{\text{FeNO}\}^7$

In view of the $\{\text{FeNO}\}^7$ EPR line-shape changes induced by CTAD binding, we turned to geometry optimization calculations to test the effect of the Asn⁸⁰³ target residue on the gas binding site in FIH. A truncated ligand set was used in the geometry optimization for structures corresponding to (Fe+NO+αKG)FIH and (Fe+NO+αKG)FIH/CTAD. The starting coordinates were taken from a crystal structure of FIH/CTAD,³⁴ in which methyl terminated amino acid sidechains were used along with pyruvate in the place of αKG. Final geometry optimization employed an antiferromagnetic spin coupling between a high spin Fe^{3+} ($S = 5/2$) and NO^- ($S = 1$) to obtain an $S = 3/2$ state, as this was shown to be the lowest energy electronic structure by prior workers.^{16,17,20} Following geometry optimization for each structure, the NO was rotated as a rigid body about the O1-Fe-N-O dihedral angle and the energy of each orientation was calculated.

A single minimum was found for (Fe+αKG+NO)FIH at a dihedral angle 226° (Figure 5), however the entire energy surface was relatively independent of dihedral angle with all orientations within 5 kJ/mol of this minimum. In the minimum energy structure, NO was pointed between the π -acceptor groups of His¹⁹⁹ and the C2 keto of αKG (Figure 6), similar to the orientation observed for the TauD- $\{\text{FeNO}\}^7$ complex¹⁷ and very close to that observed in our crystallographic refinement of the (Fe+NOG+NO)FIH. Our calculated Fe-N bond length was 1.83 Å and the Fe-N-O angle was 156° (Table 2). These calculated bond metrics were in good agreement with those reported for the (Fe+NO+αKG)TauD based on calculations¹⁷ and ESEEM data,^{46,47} as is the low rotational barrier about the Fe-N axis.¹⁷

In the energy minimized structure corresponding to FIH/CTAD, a single energy minimum was found in which the O1-Fe-N-O dihedral angle was 3° (Figure 5), similar to that found in the crystal structure of NO bound to CAS.¹⁸ This placed the bound NO directly above the O1. The energetic barrier for NO rotation about the Fe-N bond vector was quite large (147 kJ/mol), indicating that the sterics of the Asn⁸⁰³ sidechain greatly outweighed electronic factors in orienting the bound gas molecule. As O_2 is isosteric with NO, it is very likely that sterics will place the bound O_2 into a similar orientation en route to oxidative decarboxylation.

The presence of Asn⁸⁰³ led to nearly unchanged {FeNO}⁷ bonding metrics between the geometry optimized structures, with the exception of the O1-Fe-NO dihedral angle. As a consequence, the distance between the C_{keto} and the distal O of NO decreased in the structure corresponding to FIH/CTAD (3.48 Å) relative to the structure corresponding to FIH (4.18 Å). This suggests that the steric environment created by the Asn⁸⁰³ target residue will play a significant role in directing O₂ to react at the keto position during normal turnover.

Discussion

A number of mechanistic and spectroscopic studies have been undertaken to study the substrate stimulated O₂ activation by αKG-dependent oxygenase,^{13,17,48–54} leading to the view that substrate binding primes the enzyme to react with O₂ to form the ferryl intermediate. In light of the fact that primary substrate does not bind directly to Fe²⁺, and the observation that contacts throughout the active site greatly impact reactivity,^{15,55} it appears that substrate binding alters the steric and non-covalent contacts near the gas binding site to stimulate O₂ activation. Understanding factors governing O₂ binding are key to understanding why the ferryl forms preferentially after the primary substrate is in position for subsequent hydroxylation. The present study integrated spectroscopic, computational, and crystallographic analysis to test the linkage between CTAD binding and NO binding in FIH as a mimic for O₂ binding. Our crystallographic refinement of the (Fe+NOG+NO)FIH structure offers new insight into O₂ binding, as this is only the second structure reported for an enzyme from the Fe/αKG oxygenase superfamily bound to NO. Our data suggests that the target residue (CTAD-Asn⁸⁰³) plays a pivotal role in directing O₂ reactivity by providing steric constraints that orient O₂ for oxidative decarboxylation, resulting in coupled turnover in FIH.

The current dominant model to explain O₂ activation and coupled turnover in the αKG-dependent oxygenases is centered on the idea that O₂ reacts at a vacant coordination site that is present only when both αKG and substrate are present.³ O₂ is thought to become reactive as a superoxo radical anion (O₂^{•−}) upon binding the 5-coordinate Fe center, but no direct evidence of this has been established for the Fe/αKG oxygenase family. Electronic spectroscopy of Fe/αKG oxygenases and related His₂Asp facial triad-containing enzymes reveal that the Fe²⁺ center typically converts from a 6-coordinate to a 5-coordinate geometry upon binding primary substrate,^{3,37,56} implying that creation of an open coordination site is the origin of the increased NO/O₂ affinity for these enzymes. In contrast, CTAD binding to FIH creates an Fe²⁺ center that is a mixture of 5- and 6-coordinate geometries,⁷ suggesting that an open coordination site only partially explains the increased reactivity.

In order for oxidative decarboxylation to occur, the consensus mechanism for Fe/αKG oxygenases relies on the ferric superoxide attacking the C2-keto position of αKG. Although the Fe³⁺(O₂^{•−}) intermediate has never been observed for any αKG oxygenase, its existence in this class of enzyme is supported by computational predictions,^{16,38,57,58} the observation of {FeNO}⁷ adducts,¹⁷ and the observation of such an intermediate in homoprotechatechuate 2,3-dioxygenase, an extradiol dioxygenase.⁵⁹ Our EPR and UV-Vis spectroscopic data showed that NO bound to the Fe²⁺ center of FIH, forming a typical non-heme {FeNO}⁷,

while the bonding metrics found in the crystallographic structure of the {FeNO}⁷ center of FIH were similar to those reported for TauD, CAS, and the oxo-acid oxygenase HPPD. This indicated that the gas binding center of FIH was comparable to these other oxygenases, despite the O₂-sensing role of FIH.

The backbone and sidechains of FIH were nearly superimposable for the (Fe+αKG)FIH and (Fe+NOG+NO)FIH structures, indicating that only minor alterations in contacts were needed for NO binding. Similarly, slight shifts in nearby residues accommodated substrate and NO binding in the oxygen binding cavity in the extradiol dioxygenase BphC.³⁹ In contrast to the orientation of NO found in CAS, where NO pointed toward αKG,¹⁸ the (Fe+NO+NOG)FIH structure showed NO oriented away from the active site carboxylate towards the most accessible site near His¹⁹⁹. There was a water molecule above NO that appeared to play a stabilizing role for the bound gas orientation. The energetic penalty for reorientation would be approximately equal to the bond dissociation energy to break a typical hydrogen bond, roughly 25 kJ/mol.⁶⁰ The previously published structure of FIH with bound CTAD³⁴ showed that the CTAD-Asn⁸⁰³ target residue occupied the same region of the active site that was occupied by PEG and the water molecule in our (Fe+NO+NOG)FIH structure, suggesting that CTAD displaces any stabilizing contacts to the bound gas. In addition, the most accessible site in the gas binding pocket after CTAD binding rests directly over αKG, suggesting that steric occlusion of bound gas due to the presence of Asn⁸⁰³ plays a role in directing nucleophilic attack on the C2-keto position of αKG.

Remarkably, the impact of binding primary substrate on gas affinity was very small for FIH. Nonheme Fe²⁺ oxygenases typically bind NO with a moderate affinity ($K_D \sim 200 \mu\text{M}$)^{21,42} which then becomes significantly higher after binding primary substrate. For example, upon substrate binding phenylalanine hydroxylase exhibited nearly a 5 fold increase in gas binding affinity ($K_{D(\text{NO})} = 31 \mu\text{M}$ with substrate)²¹ while protocatechuate 4,5-dioxygenase exhibited a 100-fold increase in NO affinity ($K_{D(\text{NO})} = 3 \mu\text{M}$ with substrate).⁴² In contrast, upon substrate binding FIH/CTAD has an unusually small increase in NO affinity ($K_{D(\text{NO})} = 200 \mu\text{M}$ with substrate) (Table 1), suggesting that gas access to the coordination site on Fe is nearly independent of CTAD binding. The weak affinity of FIH/CTAD for NO is mirrored by its relatively low Michaelis constant toward O₂,^{8,61} both of which indicate that CTAD binding does not stimulate diatomic gas binding. This in turn strongly suggests that oxygenation by FIH is not stimulated by a change in gas affinity, raising the potential for factors other than access to an open Fe coordination site to govern coupling between oxidative decarboxylation and substrate hydroxylation.

The spectroscopically characterized Fe³⁺(O₂⁻) intermediate in homoprotocatechuate 2,3-dioxygenase provides intriguing clues as to the effect of altered local contacts on reactivity in αKG oxygenases. In WT homoprotocatechuate 2,3-dioxygenase, the initially formed Fe³⁺(O₂⁻) rapidly undergoes electron transfer with the coordinated catechol substrate to form the reactive Fe²⁺(O₂⁻) + semiquinone intermediate en route to forming product.^{59,62} However, a single point mutation and the use of an electron withdrawing 4-nitrocatechol substrate permits accumulation of the Fe³⁺(O₂⁻) on the seconds timescale,⁵⁹ underscoring how subtle changes in active site contacts can impact oxygenase reactivity.

The active site of FIH changes upon binding CTAD, leading to a number of altered contacts near the gas binding site. We observed changes in the $\{\text{FeNO}\}^7$ EPR lineshape of (Fe+ α KG+NO)FIH upon binding CTAD, which we attribute to changes in the orientation of the bound NO based on the geometry optimization calculations. Although the EPR lineshape is complex, the dominant $S=3/2$ species changes from a slightly rhombic species ($E/D = 0.016$) to a highly axial species ($E/D = 0.005$) upon binding CTAD. As the E/D ratio reflects overlap between the NO π_{ip}^* and π_{op}^* orbitals and the Fe t_{2g} orbitals, rotation about the Fe-NO bond vector will alter the EPR line-shape due to changes in the overlap of these π -symmetry orbitals. Our geometry optimization calculations predict a large barrier to rotation about the O1-Fe-NO dihedral angle in FIH/CTAD, which agrees with the observation that the highly axial $\{\text{FeNO}\}^7$ EPR line-shape was not induced by the less bulky Asn⁸⁰³ $\rightarrow$ Ala variant CTAD. Consequently, we attribute the more axial ($E/D = 0.005$) EPR line-shape in FIH/CTAD to a CTAD-induced change in the gas orientation.

Similar variations in the $\{\text{FeNO}\}^7$ EPR line-shape have been attributed to conformational changes in other nonheme Fe²⁺ enzymes. DFT calculations showed that NO adopted at least 2 conformations in TauD,¹⁷ implying that O₂ reactivity in TauD could vary in response to the distribution between multiple conformers. The EPR spectra for protocatechuate 4,5-dioxygenase and homoprotocatechuate 2,3-dioxygenase showed the presence of multiple $S = 3/2$ species, attributed to pH-dependent protein conformational changes.⁴² Although it is not obvious how the altered electronic structure of $\{\text{FeNO}\}^7$ translates into the reactivity of the $\{\text{FeO}_2\}^8$ intermediate formed during normal turnover, the oxidative decarboxylation step is likely to require proximity between the distal O-atom of bound superoxide and the keto position of α KG.

We propose that CTAD binding induces oxidative decarboxylation in FIH by restricting the conformational freedom of bound O₂ such that it is lined up for reaction with α KG. This follows from our calculations showing that in the presence of Asn⁸⁰³, NO adopted a conformation in which it was positioned near the keto group of α KG (Figure 6), consistent with the observed orientation of NO in substrate bound CAS,¹⁸ suggesting that this orientation may depict the preferred gas orientation in the α KG-dependent oxygenases following substrate binding.

Conclusion

The key feature of the consensus mechanism for α KG oxygenases is the coupling of O₂ reactivity to the presence of primary substrate. Although this is commonly attributed to the creation of an open coordination site on the Fe(II), our results for FIH indicate that such a view is incomplete. In particular, the affinity of FIH for NO, an excellent mimic of O₂, is essentially unchanged by CTAD binding. Taken together, the spectroscopic, structural, and computational data support reorientation of bound gas as a mechanistic strategy employed by FIH to couple O₂ reactivity to primary substrate binding. This raises the intriguing possibility that sterics within the active site directs O₂ activation in FIH, and may play a similar role in other α KG oxygenases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviated Terms

CAS	Clavamate synthase
CTAD	C-terminal transactivation domain, of HIF-1 α
DEANO	Diethylammonium (Z)-1-(N,N-diethylamino)diazene-1,1,2,2-tetrolate
FIH	Factor Inhibiting HIF
HIF	Hypoxia Inducible Factor
HPPD	4-Hydroxyphenylpyruvate dioxygenase
NOG	N-oxalylglycine
TauD	Taurine dioxygenase
αKG	α -ketoglutarate

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Figure 1.

Structure of (Fe+αKG)FIH and (Fe+NOG+NO)FIH. Iron is shown in magenta, water in red. (A) View of the (Fe+αKG)FIH active site with the 2F_o - F_c map contoured to 1σ. (B) (Fe+NOG+NO)FIH active site and the 2F_o - F_c map contoured to 1σ. (C) (Fe+NOG+NO)FIH active site with the F_o(Fe+NOG+NO)FIH - F_o(Fe+αKG)FIH map contoured to 1σ. (D) (Fe+NOG+NO)FIH active site measurements.

Figure 2.

Electronic absorption spectra of FIH (250 μ M), FeSO_4 (250 μ M), α KG (500 μ M), DEANO (2 mM) and CTAD or CTAD_{N803A} (500 μ M) at 23°C in anaerobic Hepes (50 mM pH 7.00). Difference spectra were created by subtracting the spectrum of (Fe)FIH from each experimental spectrum.

Figure 3.

Binding isotherm of (Fe+NO+αKG)FIH, (Fe+NO+αKG)FIH/N803A and (Fe+NO+αKG)FIH/CTAD. FIH (0.25 mM), FeSO₄ (0.24 mM), αKG (0.5 mM), CTAD (0.5 mM), N803A (0.5 mM) and DEANO (.013 – 2 mM).

Figure 4.

Experimental and simulated X-Band EPR spectra of $\{\text{FeNO}\}^7$ FIH complexes: (A) Spectra of $(\text{Fe}+\alpha\text{KG}+\text{NO})\text{FIH}/\text{CTAD}$, $(\text{Fe}+\alpha\text{KG}+\text{NO})\text{FIH}/\text{N803A}$, $(\text{Fe}+\alpha\text{KG}+\text{NO})\text{FIH}$, and $(\text{Fe}+\text{NO})\text{FIH}$ samples (B) Simulation of $\{\text{FeNO}\}^7$ species. FIH (0.10mM), FeSO_4 (0.10mM), αKG (0.50mM), CTAD (0.50mM), N803A (0.50mM), DEANO (0.50 mM), 9.624 GHz frequency, 2.0mW power, 10G modulation amplitude, 100 GHz modulation frequency, 163 ms time constant, 4K. (*) = Fe^{3+} with $g_{\text{eff}} = 4.30, 4.25$, present for all spectra.

Figure 5. Rigid scanned potential energy surface for rotation about the O1-Fe-NO axis in the FIH- $\{\text{FeNO}\}^7$ complex.

Figure 6.
Optimized geometries of A and B used in DFT calculations as models for FIH-{FeNO}⁷

Scheme 1.
Proposed mechanism for FIH.

Table 1

Summary of EPR Data and NO Dissociation Constants

sample	experimental		simulation	E/D
	$K_{D(NO)}$ (μM)	g_{eff}	g tensors ($g_{\perp}$, $g_{\parallel}$)	
(Fe+NO+ α KG)FIH/CTAD	200 ± 15	4.06, 4.00	2.015, 2.00	0.005
		4.13, 3.91	2.015, 2.00	0.017
(Fe+NO+ α KG)FIH/N803A	300 ± 30	4.10, 3.93	2.010, 2.00	0.014
(Fe+NO+ α KG)FIH	330 ± 25	4.11, 3.92	2.010, 2.00	0.016
(Fe+NO) FIH	ND	4.10, 3.93	2.010, 2.00	0.014

Table 2
Comparison of Calculated and Experimental Geometric Parameters for {FeNO}⁷ centers in FIH and related enzymes

	FIH ^a	Δ^b	B ^c	CAS ^d	HPPD ^e	TauD ^f
E _(ionization) (kJ/mol)		147	5.4			9.4 ^f
Fe – N (Å)	1.83	1.85	1.83	1.79	1.76	1.88 ^f (1.98) ^g
N – O (Å)	1.19	1.17	1.16	1.16	1.17	1.17 ^f (1.19) ^g
Fe – N – O (deg)	133	143	156	146	149	144 ^f (122) ^g
O1 _(αKG) –Fe – N – O (deg)	290	3	226	30		
Fe – O _(Cl1-O αKG) (Å)	2.02	2.01	2.03	2.01		2.06 ^f
Fe – O _(C2=O αKG) (Å)	2.15	2.35	2.32	2.27		2.37 ^f
Fe – N _(His199) (Å)	2.03	2.23	2.19	2.10		
Fe – N _(His279) (Å)	2.01	2.24	2.34	2.23		
Fe – O _(Asp201) (Å)	2.04	1.98	1.97	2.06		
O _(NO) – C2 _(αKG) (Å)	4.0	3.48	4.18	2.85		
N _(NO) – Fe – N _(His279) (deg)	159	174	169	171		

^a Measured from the (Fe+NOG+NO)FIH structure.

^b FIH- {FeNO}⁷ orientation A

^c FIH- {FeNO}⁷ orientation B.

^d Measured from PDB: 1GVG (reference 18). Values given correspond to residues in the same position as those for FIH.

^e Taken from reference 16.

^f Taken from reference 17.

^g Taken from reference 46 based on ESEEM data.