Evren Saban

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CONTROLLED OXYGEN ACTIVATION IN HUMAN OXYGEN SENSOR FIH

A Dissertation Presented

by

EVREN SABAN

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

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September 2011

Department of Chemistry
CONTROLLED OXYGEN ACTIVATION IN HUMAN OXYGEN SENSOR FIH

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Stephen J. Eyles, Member

Craig T. Martin, Department Head
Department of Chemistry
DEDICATION

This thesis is dedicated to my parents who have raised me and supported me. If I am here at this point that is because of my parents.

This thesis is also dedicated to Sinem who motivated me and showed her support by being with me.
I would like to give my special thanks to my advisor Professor Knapp. I am grateful for all his contributions of ideas and knowledge to this research.

Thanks to Committee members
Prof. Eyles, thank you for helping me on mass spectroscopy.
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John and Cornelius, thank you for contributions of your time and effort on this thesis.
ABSTRACT

CONTROLLED OXYGEN ACTIVATION IN HUMAN OXYGEN SENSOR FIH

SEPTEMBER 2011

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Directed by: Professor Michael J. Knapp

One of the primary oxygen sensors in human cells, which controls gene expression by hydroxylating the hypoxia inducible transcription factor (HIFα) is the factor inhibiting HIF (FIH). As FIH is an alpha-ketoglutarate dependent non-heme iron dioxygenase, oxygen activation is thought to precede substrate hydroxylation. The coupling between oxygen activation and substrate hydroxylation was hypothesized to be very tight, in order for FIH to fulfill its function as a regulatory enzyme. Coupling was investigated by looking for reactive oxygen species production during turnover. Alkylsulfatase (AtsK), a metabolic bacterial enzyme with a related mechanism and similar turnover frequency, was used for comparison, and both FIH and AtsK were tested for \( \text{H}_2\text{O}_2 \), \( \text{O}_2^- \) and \( \text{OH}^- \) formation under steady and substrate-depleted conditions. Coupling ratios were determined by comparing the ratio of substrate consumed to product formed. AtsK reacted with \( \text{O}_2 \) on the seconds timescale in the absence of prime substrate, and uncoupled during turnover to produce \( \text{H}_2\text{O}_2 \); neither \( \text{O}_2^- \) nor \( \text{OH}^- \) were detected. In contrast, FIH was unreactive toward \( \text{O}_2 \) on the minutes timescale in the absence of prime substrate, and tightly coupled during steady-state turnover; any reactive oxygen species produced by FIH was not available for detection. Inactivation mechanisms of these enzymes were also investigated. AtsK likely inactivated due to deoligomerization,
whereas FIH inactivated by slow autohydroxylation. Autohydroxylated FIH could not be reactivated by dithiothreitol (DTT) nor is ascorbate, suggesting that autohydroxylation likely to be irreversible under physiological conditions.

Iron in the FIH active site is coordinated by a (His$_2$Asp) facial triad, $\alpha$KG, and H$_2$O. Hydrogen bonding between the facial triad, the HIF-Asn$^{803}$ sidechain, and various second-sphere residues suggests a functional role for the second coordination sphere in tuning the chemistry of the Fe(II) center. Point mutants of FIH were prepared to test the functional role of the $\alpha$KG-centered (Asn$^{205}$, Asn$^{294}$) or HIF-Asn$^{803}$ centered (Arg$^{238}$, Gln$^{239}$) second-sphere residues. The second sphere was tested for local effects on priming Fe(II) to react with O$_2$, oxidative decarboxylation, and substrate positioning. Steady-state kinetics were used to test for overall catalytic effects, autohydroxylation rates were used to test for priming and positioning, and electronic spectroscopy was used to assess the primary coordination sphere and the electrophilicity of $\alpha$KG. Asn$^{205}\rightarrow$Ala and Asn$^{294}\rightarrow$Ala exhibited diminished rates of steady-state turnover, while minimally affecting autohydroxylation, consistent with impaired oxidative decarboxylation. Blue shifted MLCT transitions for (Fe+$\alpha$KG)FIH indicated that these point mutations destabilized the $\pi^*$ orbitals of $\alpha$KG, further supporting a slowed rate of oxidative decarboxylation. The Arg$^{238}\rightarrow$Met mutant exhibited steady-state rates too low to measure and diminished product yields, suggesting impaired substrate positioning or priming; Arg$^{238}\rightarrow$Met was capable of O$_2$-activation for the autohydroxylation reaction. The Gln$^{239}\rightarrow$Asn mutant exhibited significantly slowed steady-state kinetics and diminished product yields, suggesting impaired substrate positioning or priming. As HIF binding to
Gln$^{239}$ → Asn stimulated autohydroxylation, it is more likely that this point mutant simply mis-positions the HIF-Asn$^{803}$ sidechain. By combining kinetics and spectroscopy, it was shown that these second sphere hydrogen bonds play roles in promoting oxidative decarboxylation, priming Fe(II) to bind O$_2$, and positioning HIF-Asn$^{803}$. 
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CHAPTER 1

KEY PLAYERS OF OXYGEN SENSING

1.1 Introduction

Molecular oxygen has a prime function in aerobes. It is the final electron acceptor in cellular aerobic respiration system to produce energy in the form of ATP. Energy production starts with glycolysis in cytoplasm and continues with oxidative decarboxylation of pyruvate, citric acid cycle and oxidative phosphorylation steps in mitochondria. Most of the ATP synthesis occurs in the last step in which oxygen is the terminal electron acceptor in electron transport chain. During glycolysis 2 ATP molecules are produced whereas 36 ATP molecules are formed with the help of molecular oxygen (1). Due to its high energy potential, oxygen is crucial for our life. Oxygen does not only play a key role in energy metabolism, it is also used as a substrate for the production of key cellular components in signaling. So this makes molecular oxygen even more important in our life.

A Constant source of energy is necessary to maintain our lives, but oxygen concentrations may vary due to environmental conditions or due to the type of tissues in which O₂ is distributed throughout the human body (2, 3). Lack of oxygen for brief periods of time can be tolerated, but a continuous lack of oxygen requires more complicated responses (4). An adaptive response to the lack of oxygen in an effort to maintain energy requirements is called oxygen homeostasis (2). At high altitudes oxygen levels can decrease up to 30% due to air density and our bodies respond to this new
environment by triggering more red blood cell production so that more oxygen can be
delivered to the cells. Low oxygen concentrations might also occur locally in our body as
in the case of tumorous cells. They need oxygen to grow, so the adaptive response to lack
of oxygen is to trigger angiogenesis.

1.2 Hypoxia Inducible Factor

When dioxygen levels change in a cellular environment, adaptation to a new environment
is achieved by the transcriptional factor HIF. Under hypoxia (when dioxygen levels are
low), HIF-1 induces the expression of over 100 genes associated with angiogenesis in
cancer, immune response, erythropoiesis, energy metabolism, nutrient transport and cell
migration (5-12). HIF is a heterodimeric transcription factor consisting of a constitutively
expressed HIF-β subunit and oxygen regulated HIF-α subunit (13-15). There are three
isoforms of HIF which are HIF-1, HIF-2 and HIF-3 in which HIF-1 is the most important
and abundant and they are accepted as the master regulators in oxygen homeostasis (16-
19). HIF-1α/β proteins contain basic-helix-loop-helix and Per-ARNT-SIM (bHLH-PAS)
domains for DNA binding and heterodimerization (9). Studies showed that both bHLH
and PAS domains are required for complex formation and DNA binding. Truncation of
one of the domains revealed that bHLH can dimerize alone but the PAS domain is also
required for DNA binding (20). HIF-1α also has oxygen dependent degradation (ODDD)
and transcriptional activation domains (NTAD and CTAD). These domains are required
for the regulation of HIF activity in the presence of normal oxygen levels (Figure 1.1)
(14, 21, 22).
Under hypoxic conditions HIF-1α levels rise and HIF-1α dimerizes with HIF-1β and initiates transcriptional activation. To activate gene transcription, the heterodimeric HIF-1α/β complex requires coactivators including p300, CBP, SRC-1 and TIF2. Once the active transcription complex forms, it binds to a core DNA motif (G/ACGTG) in hypoxia response elements (HREs) that are associated by HIF target genes. This results in the induction of over 100 genes including angiogenesis, erythropoiesis, vascular remodeling, inflammation and other processes to achieve oxygen homeostasis (23).

1.3 HIF Hydroxylases

At normal oxygen levels HIF activity is regulated either by proteasomal degradation or prevention of coactivator binding. The key players in hypoxic response are the HIF hydroxylases, which regulate the activity level of HIF by dioxygen dependent modification under conditions of normal or elevated oxygen (7, 24-26). The regulation of HIF is shown in Figure 1.2.

1.3.1 Prolyl Hydroxylase

Two types of modification have been identified both of which inhibit HIF. In the first type of modification, the ODD domain of HIF-1α is hydroxylated at proline residues.
(Pro-402 and Pro-564 in HIF-1α), which are mediated by PHD (Prolyl hydroxylase). The ODD domain possesses two sub-domains, NODD and CODD (N- and C- terminal ODD) each of which can be independently hydroxylated. This hydroxylation enables the recognition of modified HIF-1α ODD domain by von Hippel-Lindau tumor suppressor protein (pVHL) by increasing the affinity between these proteins. The hydroxylated proline residue excludes a water molecule and forms two hydrogen bonds with Ser-111 and His-115 residues of pVHL which makes this complex more stable. Ubiquitin ligase binds this complex and ubiquitinylates and targets HIF-1α for hydrolysis via ubiquitin-proteasome pathway (27, 28).

1.3.2 Factor Inhibiting HIF

In the second type of modification, the C-terminal transactivation domain (CTAD) of HIF-1α is hydroxylated at the β-Carbon of asparagine residue (Asn-803) by FIH (factor inhibiting HIF) in a process which is independent of ODD modification (29-32). Hydroxylation at this asparagine residue prevents interaction of HIF-1α with the CH-1 (Cysteine/Histidine rich) domain of the transcriptional co-activator p300. NMR structures of unhydroxylated CTAD bound to CH-1 of p300 showed that Asn-803 residue is buried at the complex interface, and this suggests that hydroxylation of β-Carbon of Asn-803 may decrease binding affinity either by disrupting the interaction between pVHL and HIF-1α and/or disrupting the α-helix structure of CTAD formed at the binding interface (30, 33, 34).
Subtle changes in the oxygen concentrations in our body are sensed by these HIF hydroxylases. There are three isoforms of PHD enzyme (PHD1, PHD2 & PHD3) and with FIH; all four enzymes exist at different concentrations and in different compartments of cells. The activity of these enzymes depends on the available oxygen concentration and they are accepted as the primary oxygen sensors in our body. Generally $K_m$ of oxygen for these enzymes are higher than the ambient oxygen levels, so small changes in the oxygen concentration have large impacts on the activity of these enzymes. $K_m(O_2)$ of FIH was determined to be less than for PHD enzymes. This suggests that HIF-1α which survived from the degradation pathway can continue to be regulated by FIH when PHD activity has already been suppressed by lowered $O_2$ levels (35-37). The HIF hydroxylases have different tissue distributions in our body at RNA level. PHD1 is abundant in testis, PHD2 and FIH are ubiquitous and PHD3 exist mostly in heart and
smooth muscles. Within the cell, PHD1 is nuclear, PHD2 and FIH are mainly in cytoplasm, and PHD3 is present both in nucleus and cytoplasm (38-42). Our body has a different number of copies of these enzymes, PHD2 is the most abundant and active among PHDs; together with FIH, they keep the system under check at all times by sensing small changes in available oxygen levels (4, 43).

1.3.3 Proposed Reaction Mechanism of FIH

The HIF-hydroxylases (FIH and PHD) belong to the Fe(II) and α-ketoglutarate (αKG) dependent dioxygenase superfamily. These enzymes are characterized by their jelly-roll motif in which eight anti-parallel β-strands form two β-sheets and Fe(II) is coordinated by the His$_2$(Asp/Glu) residues between β-sheets. Unlike heme-containing enzymes, Fe(II) forms a facial triad with His$_2$(Asp/Glu); leaving the other three coordination sites for reaction chemistry. They consume molecular oxygen and α-ketoglutarate to produce CO$_2$ and succinate while hydroxylating the primary substrate (R-H) to give product (R-OH) with a simplified reaction shown below (32, 44-53).

\[ \text{αKG} + \text{O}_2 + \text{RH} \rightarrow \text{succinate} + \text{CO}_2 + \text{ROH} \]

A consensus mechanism, which is shown in Scheme 1.1, has been proposed for FIH based on other αKG dependent enzymes. In the resting enzyme, Fe(II) is six coordinate which has 2-His-1-carboxylate facial triad and three water molecules bound. Initially the substrate α-ketoglutarate binds to the iron, releasing the two water molecules. Upon
primary substrate binding, the last water molecule leaves, making Fe(II) five-coordinated and creating an open coordination site for molecular oxygen.

Scheme 1.1 Consensus reaction mechanism of FIH

Fe(II) is relatively unreactive toward molecular oxygen unless the coordination of metal center changes from six- to five-coordinate and when this happens the quaternary complex composed of Fe(II), α-ketoglutarate and substrate bound to the enzyme active site reacts with dioxygen. Because six coordinate Fe(II) is not reactive towards $O_2$, 

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substrate binding is accepted as the switch which activates Fe(II) for oxygen activation. First, Fe(II) activates oxygen by one-electron reduction to form a superoxide radical, which attacks C2 of α-ketoglutarate. The decarboxylation of α-ketoglutarate yields succinate and CO₂ with the concomitant formation of a highly reactive Fe(IV)=O intermediate (54-58). This reactive intermediate then abstracts a hydrogen atom from the primary substrate forming a substrate-centered radical. Finally, this radical binds the oxygen on the iron center and generates the final hydroxylated product and the enzyme returns to its Fe(II) active state. This final step is called a hydrogen abstraction - rebound mechanism (59-64).

### 1.4 α-Ketoglutarate Dependent Enzymes

The members of the α-ketoglutarate dependent dioxygenase family exist both in prokaryotes and eukaryotes and catalyze a wide range of reactions in which hydroxylation chemistry is the main reaction. These enzymes are accepted as dioxygenases but individual atoms of dioxygen are distributed to different products; one oxygen atom is incorporated into product while the other oxygen atom is incorporated into succinate that is derived from α-ketoglutarate. While the typical reaction is the hydroxylation, other oxidative processes like ring closure, ring opening, desaturation, elimination and epoxidation reactions are also common in this class of enzymes. In some of these reactions it is possible that one atom of dioxygen is incorporated into succinate, and the other oxygen atom is liberated as a water molecule. These enzymes are a part of important biological processes including post-translational modification of proteins, DNA repair, antibiotic biosynthesis and fatty acid metabolism, and they all posses α-
ketoglutarate dependent non-heme Fe(II) active site with His$_2$Asp/Glu facial triad (44, 47, 65, 66).

α-Ketoglutarate dependent enzymes are only a subfamily of the non-heme iron enzyme superfamily, which possesses His$_2$Asp/Glu facial triad and catalyzes many diverse reactions. The diversity of these catalyzed reactions is broader than the reactions associated with heme-containing enzymes. The porphyrin of heme occupies four coordination sites of Fe and one proximal residue binds the fifth coordination site; leaving only one available site for binding which is for molecular oxygen. However in non-heme enzymes Fe is coordinated by three endogenous protein ligands forming a facial triad, and leaving the other three coordination sites for exogenous ligands such as oxygen, substrate and/or cofactor. This facial triad combined with the protein flexibility tunes the reactivity of active center and makes these enzymes versatile (67). This superfamily can be categorized into five families based on the reaction they catalyze and/or cofactors they use. These families are the extradiol cleaving catechol dioxygenases, Rieske dioxygenases, α-ketoglutarate dependent dioxygenases, tetrahydropterin dependent hydroxylases and biosynthetic oxidases (45, 46, 61, 62, 68).

1.5 Extradiol Cleaving Catechol Dioxygenases

Extradiol cleaving catechol dioxygenases catalyze the ring cleavage reactions of aromatic compounds (48-50). Aromatic ring cleavage of catechols occurs at C-C bond adjacent to enediol group and both atoms of molecular oxygen are incorporated into the product via four-electron oxidation. The best characterized enzyme in this class is
homoprotocatechuic 2,3-dioxygenase (HPCD). To better characterize this enzyme 4-nitrocatechol was used as an alternative substrate instead of homoprotocatechnic acid (HPCA). Crystal structure studies revealed three different intermediates during the catalytic cycle. The first one is Fe(II)-O₂ complex which showed that Fe-O bond length is long (2.4 Å) and the 4-nitrocatechol ring is not planar. This suggests that iron remains in the Fe(II) state and substrate gives an electron to activate oxygen. The second intermediate is an Fe(II)-peroxo-substrate complex in which oxygen attacks to C2 of 4-nitrocatechol forming a peroxo bridge. The third and final intermediate is the Fe(II)-product complex (Scheme 1.2). During the catalytic turnover, the reaction is driven by not only close coupling of the iron center and the substrate, but also the second sphere residues.

Scheme 1.2 Extradiol catechol dioxygenase catalytic cycle
There are two important second sphere residues (Tyr257 and His200), which form hydrogen bonds to stabilize the deprotonated substrate and promote heterolytic dioxygen bond cleavage, respectively (69-75).

1.6 Rieske Dioxygenases

Rieske dioxygenases catalyze the cis-dihydroxylation of aromatic compounds, which in turn become catechols during the downstream processes (76). The active site of these enzymes differs slightly from other non-heme enzymes in a way that Asp in the facial triad binds in a bidentate manner. The two other coordination sites are generally occupied with solvent molecules (77, 78). Near the active site, there is a Rieske type cluster (Fe2-S2), which acts as an external electron source. For the cis-dihydroxylation reaction two external electrons are needed, and these are supplied by NADH. A good example for this class of enzymes is naphthalene dioxygenase (NDO). Single turnover and crystallographic studies of NDO showed that at the end of one turnover both metal centers are fully oxidized. For another turnover these centers must be reduced by NADH (79, 80). The activated oxygen is proposed to be Fe(III)-hydroperoxo, in which O-O bond cleavage occurs and the hydroxylating reagent Fe(V)-oxo-hydroxo is formed, or it can directly react with the aryl substrate via a radical mechanism. The proposed reaction mechanism is shown Scheme 1.3. The yield of NDO catalytic cycle in the presence of alternate substrate benzene is about 50%. Oxygen consumption and benzene hydroxylation are not tightly coupled, and hydrogen peroxide is released during the turnover. Studies showed that hydrogen peroxide released acts as an inactivator, and it is
proposed that this occurs via a Fenton-type reaction forming strong reactive oxygen species like hydroxyl radicals (OH\(^-\)) (81-84).

Scheme 1.3 Proposed Reaction mechanism of Naphthalene 1,2-dioxygenase

1.7 Tetrahydropterin Dependent Hydroxylases

Tetrahydropterin dependent hydroxylases catalyze the hydroxylation of aromatic amino acid residues using tetrahydropterin as a two-electron donor cofactor like \(\alpha\)-ketoglutarate dependent enzymes. Tryptophan (TrpH), tyrosine (TryH) and phenylalanine (PheH) hydroxylases are responsible for the formation of the neurotransmitters serotonin and 3,4-dihydroxyphenylalanine, and the amino acid tyrosine, respectively (85, 86). Binding of substrate and cofactor near the iron active site leaves an open coordination site for oxygen (Scheme 1.4). Once dioxygen binds, it forms a peroxo bridge between Fe(II) center and the cofactor. Heterolytic O-O bond cleavage of this peroxo bridge yields 4a-
hydroxypterin and an Fe(IV)=O species (87, 88). So two electrons come from cofactor and the other two electrons are supplied by Fe(II). Once the Fe(IV)=O intermediate forms, it can abstract a hydrogen from the near substrate. The active hydroxyl group then rebounds to the radical substrate like in the α-ketoglutarate dependent enzymes (89). When the substrate is not present near the active site, the enzyme chooses another pathway to protect itself from reactive oxygen species. In the absence of a hydroxylation reaction, two tetrahydropterin molecules are oxidized to dihydropterins per molecule of dioxygen consumed. The four-electron reduction of dioxygen to water prevents reactive oxygen species formation like hydrogen peroxide (90, 91).

Scheme 1.4 Reaction mechanism of Aromatic aminoacid hydroxylases
1.8 Biosynthetic Oxidases

Biosynthetic oxidases are a family of enzymes that play key roles in the synthesis of biologically important molecules. In this family, substrates bind to the iron center like in extradiol dioxygenases, and using electrons from the substrate and/or a cofactor, oxygen is reduced to two water molecules in a four-electron reduction with the concomitant substrate catalysis. The best known example of this family is isopenicillin-N synthase (IPNS). IPNS catalyzes the synthesis of isopenicillin-N from δ-(L-α-amino adipoyl)-L-cysteinyld-valine (ACV), which is a key step in biosynthesis of penicillin and cephalosporin antibiotics (92).

Scheme 1.5 Catalytic cycle of IPNS
When substrate (ACV) and oxygen bind to iron active site, it is proposed that Fe(III)-superoxo forms and electron transfer from substrate is promoted which causes the β-lactam ring closure, formation of the first water molecule and the Fe(IV)=O reactive intermediate. The Fe(IV)=O then causes the second ring closure and formation of the second water molecule (Scheme 1.5).

Kinetic studies showed that IPNS has no detectable uncoupling between oxygen depletion and ACV consumption, but the enzyme inactivates itself slowly with time. It is proposed that IPNS is vulnerable to oxidative damage, which is irreversible and cannot be rescued by reducing agents. Catalase protects IPNS from damage and reduces the inactivation to some extent, which suggests H₂O₂ formation. Since catalase does not rescue the enzyme at 100%, it is concluded that internal ROS formation also gives rise to oxidative damage near active site (93, 94).

Other examples of biosynthetic oxidases family are aminocyclopropane carboxylate oxidase (ACCO) and fosfomycin oxidase (FOS), which catalyze synthesis of the plant hormone ethylene and the fosfomycin antibiotic respectively. Unlike IPNS, these enzymes use two electrons from from substrate and two electrons from cofactors (ACCO uses ascorbic acid and FOS uses NADH) (67).

1.9 Activated Oxygen

As seen from the previously mentioned examples, mononuclear non-heme iron enzymes all possess His₂Asp/Glu facial triad and catalyze very diverse reactions by utilizing O₂.
Compared to heme proteins, the non-heme iron active site can accommodate more than one ligand. The facial triad occupies generally three coordination sites in these enzymes leaving the other three coordination sites for binding of O$_2$ and, in some cases, substrate or cofactor. All these enzymes activate dioxygen in different ways. The observed and proposed iron-oxo species are as the following: Fe(II)-superoxo, Fe(III)-hydroperoxo, Fe(III)-superperoxo and Fe(II)-alkylperoxo intermediates are converted to high valent Fe(IV)=O and Fe(V)=O-H after O-O bond cleavage (67). Activated oxygen and reactive intermediates are summarized in Figure 1.3 and Figure 1.4.

Figure 1.3 activated dioxygen in nonheme iron enzymes
It would be interesting to understand how these enzymes control reactive iron-oxo intermediates when dioxygen is activated. If the oxygen activation is not coupled with the substrate catalysis, side reactions can occur. Reactive iron-oxo intermediates can attack internal amino acid residues causing self-hydroxylation and self-inhibition or they can produce reactive oxygen species (ROS) like H$_2$O$_2$, O$_2^-$, and ´OH and give oxidative damage to their environment.

In α-ketoglutarate dependent enzymes the proposed and, in some cases, observed intermediates are Fe(III)-peroxo and Fe(IV)=O intermediates. If the catalytic cycle is not
tightly coupled, $\text{H}_2\text{O}_2$ and $\text{O}_2^-$ can be formed, likely from an Fe(III)-peroxo intermediate. As the catalytic turnover proceeds, $\cdot\text{OH}$ can also be formed after Fe(IV)=O step.

### 1.10 Coupling of Turnover

The active site structure including the second coordination sphere plays a key role in the reaction turnover. The protein flexibility, substrate binding, electron donating cofactor, second sphere residues and facial triad are all important players during the catalytic cycle. Any oxygen leakage can be avoided by coupling the dioxygen activation to the hydroxylation step so that the reaction is driven to the product without byproducts. However, uncoupling of oxygen activation to substrate hydroxylation can occur. The ideal stoichiometric ratio between oxygen consumption and product catalysis would be 1:1. Any oxygen leakage would cause more oxygen consumption and the stoichiometry would be higher than 1 in which reactive oxygen species can be generated or the enzyme can hydroxylate itself. Self hydroxylation generally occurs when the substrate is not present or not in the close proximity of iron active site. In the case of FIH, the rate of self-hydroxylation is diminished when the substrate CTAD is present. If the regulatory role of FIH in oxygen sensing is considered, uncoupling between oxygen activation and product catalysis must be low such that no excess reactive oxygen should give oxidative damage. The release of reactive oxygen species should also be minimal in order to prevent oxidative stress in the cells. Determining the extent of uncoupling and the propensity to produce ROS in FIH will help us to understand the link between oxygen activation and product hydroxylation.
1.11 Second Coordination Sphere

In order to determine how FIH controls activated oxygen, second coordination sphere residues should also be investigated. These residues play important roles in hydroxylation chemistry in which they tune the active site by making hydrogen bonds. The hydrogen bonding network with the second coordination sphere can also be found in other enzymes. In the case of FIH, there are four residues present in the second sphere which control the hydroxylation by either facilitating the decarboxylation of α-ketoglutarate or positioning the primary substrate. When activated oxygen attacks αKG, a buildup of negative charge can be pulled away by hydrogen bond donors in the second sphere so that decarboxylation can be facilitated. Subsequent to decarboxylation, hydroxylation of the primary substrate occurs via the hydrogen abstraction/rebound mechanism involving a putative Fe(IV)=O intermediate. It is crucial that the substrate is oriented in the correct position for hydroxylation. Hydrogen bonding networks in the second sphere provide stabilization for optimal orientation of the substrate.

Because of the simpler substrate requirements of AtsK, this enzyme will also be studied and compared with FIH. AtsK is a metabolic αKG-dependent dioxygenase involved in bacterial sulfur metabolism. Under sulfur starvation conditions, AtsK catalyzes the hydroxylation of alkylsulfate and releases inorganic sulfate to the medium. Hexylsulfate is hydroxylated to form 1-hydroxylhexylsulfate which decomposes spontaneously to hexanal and inorganic sulfate (95).
How FIH controls activated O$_2$, and the role of the second sphere residues on hydroxylation, will be the focus of this research. The steps between O$_2$ binding and decarboxylation are very important because the enzyme turnover rate is determined by these steps and it is related to the O$_2$ sensing function of FIH. So it is crucial to understand how oxygen activation is controlled by FIH, which performs its unique oxygen sensing role in our body.

1.12 Appendix

1.12.1 Abbreviations

HIF, Hypoxia Inducible Factor;
ODDD, Oxygen Dependent Degradation Domain;
N- and C- terminal TAD, Transcriptional activation domain of HIF-1$\alpha$
p300, Transcriptional coactivator;
CBP, CREB-binding protein;
SRC-1, Steroid receptor coactivator 1;
TIF2, Transcriptional intermediary factor 2;
HRE, Hypoxia responsive elements;
pVHL, Von Hippel-Lindau tumor suppressor;
PHD, Prolyl hydroxylase;
FIH, Factor inhibiting HIF;
HPCD, Homoprotocatechuate 2,3-dioxygenase;
HPCA, Homoprotocatechnic acid;
NDO, Naphthalene dioxygenase;
TrpH, Tryptophan hydroxylase;
TryH, Tyrosine hydroxylase;
PheH, Phenylalanine hydroxylase;
IPNS, Isopenicillin-N synthase;
ACV, δ-(L-α-aminoadipoyl)-L-cysteinyl-D-valine;
ACCO, aminocyclopropane carboxylate oxidase;
FOS, Fosfomycin oxidase;
AtsK, alkylsulfatase;

1.13 Bibliography


CHAPTER 2

UNCOPLED O$_2$-ACTIVATION IN THE HUMAN HIF-ASPARAGINYL HYDROXYLASE, FIH, DOES NOT PRODUCE REACTIVE OXYGEN SPECIES

2.1 Introduction

Cellular oxygen-sensing in metazoans is directly controlled by enzymes which hydroxylate the alpha-subunit of the hypoxia inducible factor (HIF$\alpha$ or HIF-1$\alpha$) (1-3). As the HIF-hydroxylases are key regulators of angiogenesis and basal metabolism, they are potential targets for treating diseases such as cancer and stroke (4-6). The two types of HIF-hydroxylase are the factor inhibiting HIF-1 (FIH) and prolyl hydroxylase (PHD) (7-9), both of which are Fe(II), $\alpha$-ketoglutarate-dependent hydroxylases. The best characterized of these enzymes is the human enzyme FIH, which hydroxylates the Asn$^{803}$ residue within the C-terminal transactivation domain (CTAD) of HIF$\alpha$ (10), thereby preventing transcriptional machinery from binding to HIF$\alpha$.

$\alpha$KG hydroxylases catalyze two half-reactions resulting in the transfer of oxidizing equivalents from O$_2$ to both $\alpha$KG and the primary substrate (Scheme 2.1) (4, 11, 12). The first half-reaction is O$_2$-activation, in which the $\alpha$KG/[Fe]$^{2+}$ is oxidatively decarboxylated to form succinate/[FeO]$^{2+}$ and CO$_2$ (13, 14). The second half-reaction is transfer of the oxidant from [FeO]$^{2+}$ to the prime substrate (15, 16), which may include reactions such as desaturation, demethylations, ring-closure, and hydroxylation (11, 12, 17); in the case of FIH, this reaction is the hydroxylation of the $\beta$-carbon of Asn$^{803}$ (10). Tight coupling between these two half-reactions is challenging, and many of the $\alpha$KG-
dependent hydroxylases exhibit reactions with O$_2$ that are uncoupled (18-26). In some cases this leads to auto-hydroxylation of residues within enzyme active sites (18, 21, 22, 25), however, more commonly, it leads to metal oxidation that can be rescued by ascorbate (12, 23, 26).

![Scheme 2.1 Consensus chemical mechanism of αKG-dependent hydroxylases. The (Fe+αKG)Enz binds the primary substrate to form the ES complex.](image)

The prevailing model for how αKG hydroxylases may achieve coupled turnover focuses on changes in the coordination number about the Fe(II) upon substrate binding. In this model, the Fe(II) is six-coordinate prior to prime substrate binding, coordinated by a His$_2$(Asp/Glu) facial triad, a bidentate αKG, and a single H$_2$O ligand. Once the prime substrate binds, the H$_2$O ligand (17) is released to form a five-coordinate Fe(II) center which is ready to react with O$_2$. Crystal structures of several αKG hydroxylases support this model (12, 27-29), as does mechanistic data indicating that poor substrates stimulate uncoupling (20, 24, 26, 30). Perhaps the strongest evidence comes from advanced spectroscopic studies that clearly show this coordination change occurs upon substrate binding (31-33). Tight coupling would be highly beneficial to an O$_2$-sensing enzyme, such as FIH.
There are three criteria for effective O₂-sensing by HIF hydroxylases. First, the $K_{M(O2)}$ should lie above the physiological $pO_2$, so that the rate of HIF hydroxylation is proportional to the $pO_2$. As the reported $K_{M(O2)}$ for FIH is much higher than the cellular $pO_2$ under physiological conditions (34, 35), it appears that FIH is well suited for its regulatory role by this first criterion. Second, uncoupling between the two half-reactions must be low, ideally such that no molecule of O₂ reacts without HIF hydroxylation, due to the potential for oxidative damage. Although αKG hydroxylases are mechanistically predisposed to some uncoupling between O₂-activation and substrate hydroxylation (12, 36), and FIH can autohydroxylate in the absence of prime substrate (18), the extent of uncoupling during steady-state turnover by FIH is unknown. Third, release of reactive oxygen species (ROS) from the active site should be minimal in order to avoid oxidative damage to the cell. The propensity of FIH to produce ROS has never been tested.

Here we use chemical methods to test the uncoupling and ROS production from human FIH in comparison to AtsK, a metabolic αKG hydroxylase involved in bacterial sulfur metabolism (37). As metabolic enzymes are expected to favor fast catalysis over tight coupling, we felt that AtsK would be an instructive contrast to FIH. We observed that FIH does not uncouple during turnover conditions, nor does it release ROS under any tested conditions. FIH does, however, autohydroxylate in a slow reaction in the absence of substrate, forming an inactive form FIH. In contrast, AtsK uncouples under turnover conditions and releases H₂O₂.
2.2 Experimental Procedures

2.2.1 Materials

The prime substrate for FIH was a 39-residue peptide corresponding to HIFα788-826 with a Cys800→Ala point mutation, which corresponds to the C-terminal transactivation domain of HIF-1α (CTAD). The CTAD sequence used (Asn803 underlined) was DESGL-PQLTS-YDAEV-NAPIQ-GSRNL-LQGEE-LLRAL-DQVN, which was unmodified at the peptide termini. CTAD peptide was purchased from EZBiolabs as a desalted product, and was further purified by reverse-phase HPLC. Hexylsulfate (HexSO₄) and NADH were obtained from Acros Organics. The enzymes used in coupled assays were from commercial sources: alcohol dehydrogenase and Cu/Zn superoxide dismutase were from MP Biomedicals, horseradish peroxidase was from Fluka. A succinate detection kit was purchased from R-Biopharm.

2.2.2 Protein Expression, Purification and Activity

AtsK and FIH were expressed and purified as previously described (19, 37). AtsK activity was measured continuously with both oxygen sensor using a Clark-type electrode and coupling the enzyme reaction with NADH/alcohol dehydrogenase at 25°C. In O₂ consumption assay, 200 µM ascorbate, 1 mM αKG, 0-1 mM HexSO₄ and 100 µM FeSO₄ were premixed in 1 mL volume with 10 mM HEPES pH 7.00, and the reaction was initiated by adding 1 µM AtsK, and oxygen consumption was monitored. AtsK activity was also monitored by coupling the enzyme reaction with 160 µM NADH and 5 unit/mL alcohol dehydrogenase in 100 µL volume by monitoring the absorbance change of NADH at 340 nm in UV-Visible(UV-Vis) Spectrometer.
FIH activity was monitored by a quenched time point assay and analyzed by LC-MS. In a 50 µL reaction volume, 2 mM ascorbate, 0.1 mM DTT, 5 unit/mL catalase, 500 μM αKG, 25 μM FeSO₄, 0-600 μM CTAD were preincubated at 37°C and 0.5-5 μM of FIH was added to initiate the reaction. At certain time points 5 µL aliquots were taken and the reaction was quenched with 45 µL 0.1% formic acid. For each reaction 5-10 samples were prepared on a 3-10 min time scale and analyzed by LC-MS using a C₈ column. The +3 charge-state of parental and hydroxylated CTAD peaks were observed at m/z of 1419.1 and 1424.4 respectively, as expected for the anticipated mass gain of 16 amu in the product. The ratio of hydroxylated CTAD peak intensity to overall peak intensity was calculated for each sample and the rate was calculated from a linear fit of these time points.

2.2.3 Uncoupling

2.2.3.1 Absence of prime substrate

Small volumes of an AtsK stock (0.38 mM) were injected into a 1.00 mL solution containing 100 μM FeSO₄, 200 μM ascorbate, 1 mM αKG and were mixed in 10 mM HEPES buffer pH 7.00, 25 °C. The FIH assay was performed with 50 μM FeSO₄, 500 μM αKG, and 11.7 μM FIH in 50 mM HEPES, pH 7.50, 37°C. O₂ consumption was monitored by a Clark-type electrode (YSI Incorporated).

2.2.3.2 Presence of prime substrate

The coupling ratio of each enzyme was determined in the presence of varied concentrations of primary substrate. For AtsK, the amount of consumed oxygen was
compared to the amount of product formed by oxygen consumption and NADH coupled assays, respectively. The coupling ratio of FIH was obtained by comparing the amount of succinate formed to the amount of hydroxylated peptide. Succinate was measured by UV-Vis spectroscopy using a succinate detection kit and hydroxylated CTAD was analyzed by LC-MS.

2.2.4 ROS Production

2.2.4.1 H$_2$O$_2$ / O$_2^-$ assays

Hydrogen peroxide production was detected by coupling H$_2$O$_2$ oxidation of 50 µM 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfoic acid) (ABTS) with 1 Unit/mL horseradish peroxidase (HRP). Hydrogen peroxide production was continuously monitored at 405 nm. Ascorbic acid was excluded from the reaction mixtures to prevent reduction of ABTS$. Superoxide detection was accomplished in a same manner of peroxide detection, by the use of 100 Unit/mL Cu/Zn superoxide dismutase (Cu/Zn SOD) to convert O$_2^-$ into H$_2$O$_2$. The reaction conditions for H$_2$O$_2$ and O$_2^-$ detection included the coupling reagents with the respective enzyme assays; for AtsK: 11.4 µM AtsK, 1 mM αKG, 0-100 µM HexSO$_4$, 100 µM FeSO$_4$; for FIH: 5 µM FIH, 500 µM αKG, 50 µM FeSO$_4$, 0-100 µM CTAD.

2.2.4.2 OH$^-$ radical assay

Hydroxyl radical detection of AtsK and FIH was achieved by mixing enzyme activity assay solutions with 15 mM 2-deoxyribose (38). Primary substrate concentrations were varied for AtsK, 0-600 µM HexSO$_4$; and for FIH, 0-120 µM CTAD. The reactions were
initiated by adding enzyme then incubating at 37 °C for 1 hour. Reactions were quenched with 100 µl 1% thiobarbituric acid in 50 mM NaOH and 100 µL 2.8 % trichloroacetic acid in water. The solution was heated at 100 °C for 20 minutes. After cooling down to room temperature, the absorbance at 532 nm was measured. The zero substrate sample was treated as a reference.

2.2.5 Inactivation and Rescue of Inactivated Enzyme

2.2.5.1 Inactivation of AtsK

Inactivation of AtsK was tested by changing the concentration of AtsK in a reaction from 1 to 10 µM, in the NADH-coupled assay detected at 340 nm. 1 mM αKG, 1mM HexSO₄, 100 µM Fe(II), 0-2 mM ascorbic acid, 1-10 µM AtsK, 30 µM NADH and 30 units of alcohol dehydrogenase were used in a 100 µL reaction volume.

2.2.5.2 Inactivation and rescue of autohydroxylated FIH

Autohydroxylated FIH was prepared to test for re-activation conditions. Under anaerobic conditions, 50 mM HEPES (pH 7.50) αKG (100 µM), FIH (49 µM) and FeSO₄ (50 µM) were added to a septum-sealed UV cuvette. An initial UV-Vis absorption spectrum was collected; the septum was then removed to introduce air and initiate the reaction. The characteristic absorption peak of autohydroxylated FIH (λ<sub>max</sub> = 583 nm) grew over several hours (18). Autohydroxylated FIH was tested for activity in an assay mixture containing αKG (500 µM), FeSO₄ (25 µM), CTAD (70 µM), autohydroxylated FIH (5 µM), and a mild reductant. The mild reductants ascorbate (0 - 2 mM) and DTT (0 - 0.1 mM) were tested for their ability to re-activate auto-hydroxylated FIH. Samples were analyzed for hydroxylated CTAD by LC-ESI-MS.
2.3 Results

Uncoupling for FIH and AtsK was evaluated by comparing the steady-state kinetic signatures of the two half reactions. As O$_2$ reacts with αKG to form succinate and CO$_2$ in the first half-reaction, the stoichiometry of O$_2$-activation was measured by O$_2$ consumption or succinate formation. This assumes that O$_2$ is only activated by oxidative decarboxylation of αKG. The rate of prime substrate hydroxylation was monitored by mass spectrometry for FIH or by a UV-Vis assay for AtsK. A fully coupled reaction would exhibit an [O$_2$]:[hydroxylated product] ratio of unity; deviations from this ratio would indicate that O$_2$ was being activated in the absence of substrate hydroxylation.

Uncoupling for AtsK and FIH was measured with variable concentrations of substrate. The solution conditions for AtsK included 0 – 1000 µM HexSO$_4$, with up to 3.0 µM AtsK. AtsK was tightly coupled under steady-state conditions, as the number of enzyme turnovers was equivalent by both Clark electrode and by the UV-Vis assay. These assays indicated a coupling ratio of 1.04 ± 0.07 in the steady-state for AtsK (Figure 2.1A). As the $K_M$(HexSO$_4$) is 15 µM for AtsK, the measured steady-state coupling ratio for AtsK reflected that of an ES complex, indicating that once AtsK bound HexSO$_4$ and entered its catalytic cycle it completed the hydroxylation with relatively high fidelity (ca. 96%). This indicated that AtsK turnover was much faster than uncoupling from the ES complex.

Similarly, FIH was tightly coupled as shown by the stoichiometry of succinate to hydroxylated peptide present in quenched reactions. FIH (2 µM) was incubated with CTAD (200 µM) and the reaction quenched at several time points for analysis. These
assays indicated a coupling ratio of 1.03 ± 0.17 in the steady-state for FIH (Figure 2.1B). FIH distributed between predominantly an (Fe²⁺+αKG)FIH form at low [CTAD], and an ES complex at high [CTAD], as we measured the $K_{M(CTAD)}$ as 77 µM (39). Nevertheless, FIH exhibited a tightly coupled reaction under steady-state conditions, indicating that $O_2$-activation by FIH is tightly controlled. It should be noted that the succinate assay used to measure $O_2$-activation by FIH was less precise than the simpler UV-Vis assay used for AtsK, due to the many reaction components and manipulations needed.

The consumption of $O_2$ by AtsK and FIH in the absence of prime substrate was directly monitored by use of a Clark electrode (Figure 2.2). Small volumes of enzyme (1-3 µL) were injected into thermostated 1.00 mL reaction buffer containing FeSO₄ (100 µM), ascorbate (200 µM), and αKG (1mM). Despite the tight coupling ratios observed during steady-state turnover, $O_2$ consumption was not absolutely tied to substrate hydroxylation for either enzyme. In the case of AtsK, $O_2$ was consumed within 30 seconds at a molar stoichiometry of 2.5 (+/- 0.2) $O_2$ per AtsK active site (Figure 2.3). Such a rapid reaction with $O_2$ is reminiscent of the fast inactivation observed for several other αKG hydroxylases, such as TauD and TfdA (25, 26).
Figure 2.1 Coupling of O₂ to prime substrate for AtsK and FIH. A) O₂ consumption vs. HexSO₄ hydroxylation for AtsK. AtsK (0.38 – 3.0 µM), ascorbate (200 µM), αKG (1 mM), FeSO₄ (100 µM), HexSO₄ (1 mM), NADH (160 µM), alcohol dehydrogenase (5 Unit/mL) in 10 mM HEPES (10 mM, pH 7.00). B) Succinate production vs. CTAD hydroxylation for FIH. FIH (2.0 µM), ascorbate (2000 µM), DTT (100 µM), αKG (500 µM), FeSO₄ (50 µM), CTAD (240 µM) in HEPES (50 mM, pH 7.50).

In contrast, injections of FIH at a final concentration of 11.7 µM consumed no measurable O₂ on the minutes timescale (Figure 2.2A). Although we were unable to measure any uncoupled O₂-consumption for FIH on the minutes timescale, we did reproduce the autohydroxylation reaction (18). Autohydroxylation required O₂, and formed a hydroxylated Trp²⁹⁶ residue on FIH (18), which is formed via hydroxylase activity in the absence of CTAD.
Although the coupling ratios for both FIH and AtsK were near unity during the steady-state, the consumption of O₂ by AtsK in the absence of prime substrate suggested that ROS may be produced by the resting form of AtsK, (Fe⁺ αKG)AtsK. As an enzyme will partition amongst various forms depending on substrate concentrations, ROS formation was measured while varying prime substrate concentrations for both FIH and AtsK. The tested ROS species were superoxide (O₂⁻), hydrogen peroxide (H₂O₂), and hydroxyl radical (OH'), which may form depending on the number of electrons transferred to O₂.

Figure 2.2 Oxygen consumption of FIH and AtsK measured with O₂ sensor; A) FIH (11.7 µM) mixed with FeSO₄ (50 µM), αKG (500 µM) in 50 mM HEPES pH 7.5 B) AtsK (1.14 µM) mixed with ascorbate (200 µM), FeSO₄ (100µM), αKG (1 mM) in 10 mM HEPES pH 7.00.

Both H₂O₂ and O₂⁻ were assayed by a peroxidase/ABTS assay in which H₂O₂ was indicated by a characteristic absorption at 405 nm due to the formation of ABTS+. In the
absence of HexSO₄, AtsK (11.4 µM) produced 0.12 µM H₂O₂; this was much less than the anticipated 2.5 equivalents of O₂ consumed by AtsK. Under steady-state conditions, AtsK produced up to 0.47 µM H₂O₂, with [HexSO₄] ranging from 0 – 800 µM. Notably, H₂O₂ production was a saturable function of [HexSO₄], with an apparent half-maximal value of 10 µM HexSO₄ (Figure 2.4). As this is similar to the reported Kₘ(HexSO₄) (37), it suggested that H₂O₂ was also released from the ES complex of AtsK, (Fe²⁺+αKG+HexSO₄)AtsK.

![Figure 2.3 Oxygen consumption of AtsK in the absence of HexSO4. AtsK (0.2-1.2 µM) mixed with ascorbate (200 µM), FeSO₄ (100 µM) and αKG (1 mM) in 10 mM HEPES pH 7.00.](image)

FIH (5 µM) failed to produce measurable levels of H₂O₂ under any tested condition. FIH concentrations were varied between 5 and 50 µM in the absence of CTAD to test for H₂O₂ production by (Fe²⁺+αKG)FIH, however H₂O₂ was not detected even at 50 µM FIH. FIH was also tested under steady-state conditions with 50 µM CTAD; however,
H₂O₂ was not detected suggesting that the ES complex, (Fe²⁺αKG+CTAD)FIH, did not produce H₂O₂. This was consistent with the near unity coupling ratio for FIH (Figure 2.4 inset).

Figure 2.4 H₂O₂ produced by AtsK (11.4 µM) during steady state turnover. Inset) Timecourse for H₂O₂ production by AtsK (upper line) and FIH (lower line). AtsK (11.4 µM) was added into a reaction mixture containing FeSO₄ (100 µM), αKG (1 mM), HexSO₄ (100 µM), ABTS (50 µM) and HRP (1 unit/mL) in 10 mM HEPES pH 7.00. FIH (5 µM) was added into a solution which has FeSO₄ (25 µM), αKG (500 µM), CTAD (50 µM), ABTS (50 µM) and HRP (1 unit/mL) in 50 mM HEPES pH 7.50.

Superoxide was measured by the use of Cu/Zn SOD to convert two equivalents of O₂⁻ into one equivalent of H₂O₂ for the peroxidase/ABTS assay. Neither AtsK nor FIH produced detectable O₂⁻ by this coupled assay in the absence of prime substrate; experiments in the presence of added prime substrate similarly yielded no detectable O₂⁻.
Hydroxyl radicals were tested by the deoxyribose method, a qualitative colorimetric assay that compares OH• production to a baseline condition by normalized absorptivities (A/A₀); an increase in A/A₀ would indicate that more OH• radical were produced. AtsK (1.14 µM) was tested at varied concentrations of HexSO₄ (0-600 µM). This allowed a comparison of different enzyme forms, with free enzyme prevalent at sub-saturating concentrations of HexSO₄, and an ES complex prevalent at saturation. A plot of the normalized data indicated a slight decrease in OH• production in the presence of HexSO₄, however this was independent of [HexSO₄] (A/A₀ = 0.91 +/- 0.05) (Figure 2.5A). This showed that OH• radical production was not a function of enzyme form, suggesting that neither the free enzyme nor the ES complex produced diffusible OH•. Similarly, FIH (0.5 µM) was assayed for OH• production at varied CTAD concentrations. The normalized data was a line of zero slope with respect to [CTAD] (A/A₀ = 1.03 +/- 0.03) (Figure 2.5B). This indicated that OH• radical production was not a function of enzyme form. As with AtsK, this suggested that FIH did not produce diffusible OH• radicals.

During steady-state kinetics assays, we noted a biphasic time course for AtsK, indicating enzyme inactivation. Inactivation could result from the oxidation of the iron pool into Fe(III), or from specific changes to AtsK. Oxidation of iron was excluded as the reason for inactivation by serial injections of AtsK (0.5-1 µM, 1.25-2.5 µL) into a 1.0 mL reaction solution containing HexSO₄ (1 mM) and FeSO₄ (100 µM); ascorbate was omitted to simplify data interpretation. Each injection of AtsK consumed O₂ for approximately 60 seconds, followed by a return to baseline; subsequent AtsK injections were similarly active (Figure 2.6A). This showed that the Fe(II) and HexSO₄ in solution
were sufficient for turnover, and that diluted AtsK (1 μM) itself became inactive within one minute.

Figure 2.5 Hydroxyl radical assays of AtsK and FIH. A) AtsK (1.14 μM), ascorbate (200 μM), αKG (1 mM), FeSO₄ (100 μM), HexSO₄ (0-600 μM), 2-deoxyribose (15 mM) in 10 mM HEPES pH 7.0 B) FIH (0.5 μM), ascorbate (2 mM), αKG (500 μM), FeSO₄ (25 μM), CTAD (0-120 μM), 2-deoxyribose (15 mM) in 50 mM HEPES pH 7.50.

As AtsK is a tetramer (29), we tested de-oligomerization as the cause of the rapid inactivation. Timecourses for varied concentrations of AtsK (0.5 – 10 μM) were monitored by either the UV-Vis assay or the O₂-consumption assay. High concentrations of AtsK (5 – 10 μM) were active for hundreds of seconds, whereas low concentrations
(0.5 – 1 µM) inactivated within 50 seconds (Figure 2.6 A, B), suggesting that de-oligomerization was the root cause of the rapid inactivation.

Figure 2.6 Inactivation of AtsK measured by O₂-electrode. A) Sequential injection of AtsK (1 µM, 1 µM, 0.5 µM) into a mixture of FeSO₄ (100 µM), ascorbate (200 µM), αKG (1 mM), HexSO₄ (1 mM) in 10 mM HEPES pH 7.00. B) Injection of high concentration of AtsK (7 µM) into same reaction conditions as in A.

FIH exhibited linear progress curves for more than 4 minutes in steady-state assays that included ascorbate, indicating that enzyme inactivation was not a rapid process. However, we previously observed that FIH would autohydroxylate over several hours to form an Fe(III) form of the enzyme called ‘purple FIH’ in which Fe(III) was coordinated by the hydroxy-group of hydroxylated Trp. As Saari and Hausinger noted that inactivated TfdA was partially rescued by ascorbate (26), we tested two common reductants for their ability to rescue auto-hydroxylated FIH. First, we tested auto-hydroxylated FIH for activity; purple FIH (5 µM) exhibited no activity following
incubation with CTAD (70 µM), FeSO₄ (25 µM) and αKG (500 µM), indicating that this enzyme form was inactive. Next, we tested ascorbate (2 mM) and/or DTT (100 µM) for their ability to re-activate purple FIH by including these reductants in purple FIH assay mixtures, however no activity was observed with these mild reductants (Table 2.1).

<table>
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<tr>
<th>FIH (µM)</th>
<th>Ascorbate (mM)</th>
<th>DTT (mM)</th>
<th>v (µM/min)</th>
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<tr>
<td>5 (purple)</td>
<td>0</td>
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<td>0.05±0.04</td>
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<td>5 (purple)</td>
<td>2</td>
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<td>0.06±0.05</td>
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<td>5 (purple)</td>
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<tr>
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<td>0</td>
<td>0</td>
<td>4.4±0.4</td>
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<tr>
<td>5 (fresh)</td>
<td>2</td>
<td>0.1</td>
<td>7.3±0.5</td>
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2.4 Discussion

Although the αKG hydroxylases are mechanistically predisposed to uncouple the oxidative and reductive parts of their catalytic cycle, the rate and products of uncoupling depend greatly on the identity of the enzyme and on reaction conditions. The reported coupling ratio during turnover was within error of unity for all reported examples (20, 26, 40, 41), indicating that uncoupling occurs on less than ~ 5% of turnovers in the presence of prime substrate. However, reactivity toward O₂ in the absence of prime substrate is variable, as some αKG oxygenases release ROS whereas some autohydroxylate, suggesting that structural differences may control both the rates and products of uncoupling. Neither FIH nor AtsK were significantly uncoupled during turnover;
however they differed in both their reactivity toward O$_2$ in the absence of prime substrate, as well as in their uncoupling products.

Coupling between O$_2$-activation and substrate hydroxylation likely relies on three structural factors. The first being local structures which link the binding of prime substrate to coordination changes at Fe, such as proposed for hydrogen bonding between the facial triad and the Fe-bound H$_2$O (31). In a highly organized active site, the (Fe$^{2+}+\alpha$KG) form of enzyme would be unable to react with O$_2$ until prime substrate bound, and would tightly couple O$_2$ activation to substrate oxidation. The second factor is a closed active site to prevent solvent access, such as by loop closure or the binding of a large substrate. The (Fe$^{2+}+\alpha$KG+Substrate) form of enzyme, the ES complex, would be tightly coupled if solvent were unable to reach reactive intermediates, such as (FeO)$^{2+}$. The third factor is the presence of oxidizable residues near the active site which may react with (FeO)$^{2+}$ or scavenge any generated ROS. Any ROS formed by O$_2$-activation in the absence of prime substrate could then damage the protein rather than diffusing away, and has been proposed as a general protective strategy for $\alpha$KG-dependent hydroxylases (36).

The free enzyme forms of AtsK and FIH were quite distinct in their propensity to react with O$_2$, suggesting differences in their structures. As described below, the primary factor is likely to be hydrogen bonding between the facial triad and the coordinated H$_2$O. In the context of the inner-sphere O$_2$-activation model, the Fe(II) in the free enzyme (eg: (Fe+$\alpha$KG)FIH) should be 6-coordinate due to a H$_2$O ligand which is hydrogen bonded to
the Asp/Glu ligand. In order for free enzyme to react with O$_2$, this H$_2$O ligand must be released in the absence of prime substrate. As (Fe+$\alpha$KG)FIH was unreactive toward O$_2$ on the minutes timescale (Figure 2.2), the H$_2$O-ligand is likely to be tightly bound in FIH. In contrast, (Fe$^{2+}$$\alpha$KG)AtsK consumed 2.5 equivalents of O$_2$ (Figure 2.3), suggesting weaker hydrogen bonding from the facial triad in AtsK.

While the reactivity data presented herein do not directly probe the strength of this hydrogen bond, the structural data for FIH and AtsK are consistent with weakened hydrogen bonding in AtsK. In particular, while Asp$^{201}$ of the facial triad in FIH forms a hydrogen bond to the Fe-bound H$_2$O (27), the orientation of AtsK Asp$^{110}$ precludes any hydrogen-bond from the facial triad of AtsK (29, 31). The confluence of structural data with our reactivity data suggest that hydrogen bonding from the facial triad is a significant factor in deactivating FIH towards uncoupled O$_2$-activation.

Differential solvent access to the active sites of FIH and AtsK is unlikely to be the origin of the very different uncoupling behavior in the absence of prime substrate, as both active sites are highly solvent exposed. The crystal structures of AtsK suggested that the active site would be very solvent accessible, as a ‘lid’ formed by residues 80 – 102 was so disordered in the crystal structure that it could not be refined (29). This is also consistent with the broad substrate tolerance of AtsK, and suggests that solvent access to the active site should be facile. In a similar vein, FIH has no structural obstruction to solvent access to the active site in the (Fe+ $\alpha$KG)FIH enzyme form, as the active site only becomes closed upon binding of the prime substrate, CTAD (27). As a consequence, any
uncoupled O2-activation in the absence of prime substrate should lead to a highly solvent exposed (FeO)2+ center in both AtsK and FIH. That AtsK released ROS, but FIH did not, indicated that some combination of slow initial reactivity and internal scavenging (see below) is most likely operative in FIH.

The only ROS produced by (Fe+αKG)AtsK was H2O2, suggesting that simple hydrolysis of the (FeO)2+ intermediate formed H2O2 and regenerated the Fe2+ cofactor. ROS have been implicated during uncoupling of αKG oxygenases, but seldom observed. Two negative examples are enzymes structurally related to AtsK, which also inactivated during turnover: TFdA and TauD. The irreversible inactivation of TFdA was suggested to involve oxidative damage from hydroxyl radicals, however diffusible OH• was not observed (26); neither H2O2 nor O2− were observed during uncoupling by TauD (41). The rare positive example is for CS2, which releases H2O2 in the absence of substrate as does AtsK (40). A crucial difference between AtsK and CS2 is that unlike CS2, AtsK also released H2O2 during normal turnover. This may relate to the size of the active site pocket, as the AtsK active site is highly disordered (29) and may therefore be more prone to hydrolytic attack at the FeO2+ intermediate than CS2.

The presence of a sacrificial site in FIH may explain the absence of diffusible ROS from this enzyme. FIH autohydroxylation led to irreversibly inactivated enzyme on the timescale of hours. The autohydroxylated form of FIH, in which the Fe(III) is coordinated by the hydroxylated ring of Trp296 (18, 19), could not be rescued by the addition of common reducing agents nor by excess αKG (Table 2.1). Irreversible
inactivation by ‘sacrificial’ reactivity near the active site has precedent in the autohydroxylation reactions of TauD, AlkB and TfdA, where new chromophores arise from Fe(III) coordination of the newly formed hydroxylated residue, and for which no reactivation pathway has been identified (21, 22, 25). We propose that FIH is deactivated from aberrant O₂ reactivity by strong hydrogen bonding from the facial triad, and further insured against ROS release by the internal reaction with Trp²⁹⁶.

2.5 Conclusions

FIH is a proximate O₂-sensor for human cells, controlling vital processes such as basal metabolism and angiogenesis. Understanding the link between O₂-activation and substrate hydroxylation is crucial to developing therapies targeting FIH function. Our findings show that FIH minimizes uncoupled O₂ reactivity at two levels: through an inherently low reactivity in the absence of prime substrate; and through presentation of a sacrificial acceptor (Trp²⁹⁶) near the active site. The tight control over O₂-reactivity for FIH is in contrast to the facile production of H₂O₂ by the bacterial enzyme AtsK, both in the presence and absence of the prime substrate for AtsK. We propose that this low reactivity for FIH may be due to strong hydrogen bonding between the coordinated water and the facial triad. Such tight control over O₂-activation by FIH is compatible with the significance of O₂ and O₂-derived species to both gene expression and cellular toxicity.
2.6 Appendix

2.6.1 Supplemental

Figure 2.7 AtsK (1.14 µM), ascorbate (200 µM), αKG (1 mM), FeSO₄ (0-100 µM), HexSO₄ (1 mM) in 10 mM Hepes pH 7.00. Reaction was initiated by adding the enzyme and oxygen consumption was monitored.

Figure 2.8 AtsK (1.14 µM), ascorbate (200 µM), αKG (0-100 µM), FeSO₄ (100 µM), HexSO₄ (1 mM) in 10 mM Hepes pH 7.00. Reaction was initiated by adding the enzyme and oxygen consumption was monitored.
Figure 2.9 AtsK (1.14 µM), ascorbate (200 µM), αKG (1 mM), FeSO₄ (100 µM), HexSO₄ (0-200 µM) in 10 mM Hepes pH 7.00. Reaction was initiated by adding the enzyme and oxygen consumption was monitored.

Figure 2.10 AtsK (1.14 µM), ascorbate (200 µM), αKG (1 mM), FeSO₄ (0-100 µM), HexSO₄ (1 mM), O₂ (0-100%) in 10 mM Hepes pH 7.00. Reaction was initiated by adding the enzyme and oxygen consumption was monitored.
Table 2.2 Summary of Michaelis-Menten kinetics of AtsK

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<th>$K_d \text{ (µM)}$</th>
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<td>Fe(II)</td>
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<td>αKG</td>
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<td>5.9 ± 1.2</td>
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<tr>
<td>HexSO$_4$</td>
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<td></td>
</tr>
<tr>
<td>O$_2$</td>
<td>0.47 ± 0.03</td>
<td>40 ± 9</td>
<td></td>
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2.6.2 Abbreviations

ABTS, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid);

αKG, alpha-ketoglutarate;

AtsK, oxygenative alkylsulfatase;

CS2, clavaminate synthase-2;

CTAD, C-terminal transactivation domain of HIFα;

ESI-MS, electrospray ionization mass spectrometry;

HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;

FIH-1, the factor inhibiting HIF;

HexSO$_4$, hexylsulfate;

HIF, Hypoxia Inducible Factor;

HRP, horseradish peroxidase;

SOD, superoxide dismutase;

TauD, taurine dioxygenase;

TfdA, (2, 4-dichlorophenoxy)acetate dioxygenase.
2.7 Bibliography


CHAPTER 3
THE SECOND COORDINATION SPHERE OF FIH CONTROLS HYDROXYLATION

3.1 Introduction

A small number of Fe(II), α-ketoglutarate (αKG)-dependent dioxygenases directly control cellular oxygen sensing in humans (1-3). These enzymes hydroxylate specific residues within the α-subunit of the hypoxia inducible factor (HIF), thereby affecting the transcriptional level of hundreds of genes (4). The best characterized of these enzymes is ‘factor inhibiting HIF-1’ (FIH), which hydroxylates the β-carbon of HIF-Asn⁸⁰³ (5); HIF-Asn⁸⁰³ lies within the C-terminal transactivation domain (CTAD) of HIFα. Hydroxylating HIF-Asn⁸⁰³ turns off HIF-dependent gene expression and is a key step in down-regulating angiogenesis. Understanding the chemical basis of how FIH reacts with HIFα and O₂ is crucial to understanding tissue O₂ homeostasis, and holds potential for treating disease states such as cancer or stroke (6-8). According to the consensus chemical mechanism (Scheme 3.1), O₂ binds to (Fe²⁺+αKG)FIH only after HIFα binds (9, 10). In this mechanism, the active site Fe(II) of FIH is proposed to change its coordination geometry upon binding HIFα, thereby creating a binding site for O₂. A close correlation between pO₂ and HIF hydroxylation would then result, as the rate would be proportional to pO₂. X-ray crystallography of FIH in various substrate-bound states revealed a structural linkage between HIF-binding and O₂ activation through changes in multiple hydrogen-bonding interactions (11-13). Controlling oxygenation chemistry through varied hydrogen bonding to atoms coordinated to the metal has been found in
other enzymes and models (14-16), suggesting that the structural changes in FIH might also have functional significance.

Scheme 3.1 Consensus chemical mechanism

The chemistry within the FIH active site occurs at the Fe(II) cofactor, making the extended coordination environment of this metal the focus of our investigation. FIH provides a primary coordination sphere of His^{199}, Asp^{201}, and His^{279}, forming a facial triad (17), with other coordination sites occupied by H₂O or αKG (Figure 3.1). The secondary coordination sphere is comprised of those residues that form hydrogen bonds to the ligands. Spectroscopic and structural data from FIH and related enzymes suggest
that coordination changes at Fe are crucial to turnover (9, 18, 19). Based on evidence from related enzymes, these key coordination geometries are proposed to be a six-coordinate (6C) center for the (Fe$^{II}$+αKG)FIH, and a five-coordinate (5C) center for (Fe$^{II}$+αKG+CTAD)FIH.

![Figure 3.1 Hydrogen bonding network in the active site of wild type FIH (PDB 1H2K). FIH (gray) and CTAD (cyan) shown as strands; hydrogen bond distances (Å), FIH residues, and HIF-Asn$^{803}$ are noted.]

Hydrogen bonds between surrounding residues, substrates, and iron ligands may comprise a functionally significant second-coordination sphere for FIH, both for priming as well as for oxidative decarboxylation. X-ray crystal structures of FIH imply that the single H$_2$O-ligand is lost from the 6C (Fe$^{II}$+αKG)FIH upon binding CTAD, which would prime the Fe(II) to react with O$_2$ (11-13). Concomitant alterations in nearby hydrogen
bonds between Arg$^{238}$, Asp$^{201}$, and the H$_2$O ligand suggest that these second-sphere interactions may be necessary for priming. We prepared and characterized the isosteric mutant Arg$^{238}$$\rightarrow$Met in order to determine how loss of this hydrogen bond linkage affected priming of the Fe(II) to react with O$_2$.

Decarboxylating α-ketoacids generally requires stabilization of negative charge within the transition state (18-20). Notably, a Lys or Arg is positioned as a hydrogen bond donor to the C1-carboxylate of αKG in most structures of the αKG dioxygenase superfamily (9). In the case of FIH, Asn$^{205}$ and Asn$^{294}$ appear to fulfill this role via hydrogen-bond donation to the C-1 carboxylate. We prepared and characterized the single-point Asn $\rightarrow$ Ala mutants to test how loss of each hydrogen bond affected O$_2$-activation in normal turnover as well as autohydroxylation.

Subsequent to O$_2$-activation, CTAD hydroxylation likely proceeds via a hydrogen transfer/rebound steps involving a putative [FeO]$^{2+}$ oxidant observed in related enzymes (21, 22). Precise substrate positioning is necessary to ensure that the putative [FeO]$^{2+}$ oxidant attacks the proper C-H bond. The side chain of the HIFα-Asn$^{803}$ residue is located directly above the open coordination site of the Fe(II) in FIH, and forms a hydrogen bond pair to FIH-Gln$^{239}$. This interaction may be crucial for substrate positioning, such that the [FeO]$^{2+}$ oxidant reacts selectively with the β-carbon of HIF-Asn$^{803}$. Although Gln$^{239}$ is not strictly a second-sphere residue in FIH, the Gln239$\rightarrow$Asn mutant was included in the present study as a way to investigate the role of CTAD positioning on catalysis.
Testing the functional role of the second-sphere is crucial to understand the intricate relationship between the overall structure and function of FIH. This manuscript reports the activity, metal binding, and catalytic precision of second-sphere point mutants of FIH. Our results indicate that several second-sphere residues are key to tuning the \( \text{O}_2 \)-reactivity of FIH.

3.2 Materials and Methods

3.2.1 Protein expression and purification

FIH and its mutants were expressed from \textit{E. coli} with an N-terminal His\(_6\) tag and purified as previously described (23). Following purification via Ni-NTA column chromatography, the His\(_6\) tag was removed by thrombin digestion. Exogenous metal was removed by prolonged incubation with EDTA, and then FIH was further purified by size-exclusion chromatography to yield the FIH dimer.

3.2.2 Differential Scanning Calorimetry

DSC experiments were performed using a MicroCal VP-DSC microcalorimeter (24). 50 mM Hepes buffer pH 7.50 was used and 50 \( \mu \)M samples were heated in the calorimeter over a 25-75 °C range at a scan rate of 60°C/hour. A buffer scan was subtracted from each dataset to correct for base-line drift. Data analysis was performed using Origin Microcal software,(25) the results of which are provided in the supplementary material.

3.2.3 Metal Binding

The experimental protocol was modified from Marletta et al. (26). Citrate was used as a chelator to buffer the concentration of free Co(II) ion, \([\text{Co(H}_2\text{O)}_6]\)^{2+}, for which stability
constants are reported (27). Co(II) binding to FIH was monitored by fluorescence quenching in a deoxygenated solution at room temperature. FIH was present in a fluorescence cuvette as a 200 μL solution of 1.00 mM sodium citrate, 20 μM FIH, 100 μM α-ketoglutarate, and 50 mM HEPES pH 7.50. Small volumes of CoCl₂ were added from a solution of 1.00 mM CoCl₂ and 1.00 mM sodium citrate, 50 mM HEPES pH 7.50. Following addition of Co(II), the cuvette was gently rocked by hand and the fluorescence measured over several minutes until a steady reading was obtained. Samples were excited at 280 nm and the fluorescence intensity was measured at 340 nm. Data fitting and results are provided in the supplementary material.

3.2.4 EPR

X-band EPR spectra were recorded on a Bruker Elexsys E-500 ESR Spectrometer equipped with a Bruker ER 4118CF-O LHe/LN₂ cryostat. EPR samples were prepared by reconstituting enzymes with CuSO₄ in a FIH:Cu²⁺ ratio of 1:0.9; the CuSO₄ solution was slowly added to the enzyme solution to prevent precipitation. Similarly, FIH(Cu+αKG) was prepared by adding αKG slowly as the final step. Samples totaling 300 μL of 1 mM enzyme, 0.9 mM CuSO₄ and 1 mM αKG in 50 mM Hepes, pH 7.50, were frozen in quartz tubes with liquid nitrogen. The spectra were obtained by averaging 4 scans at 9.438 GHz frequency, 20 mW power, 20 G modulation amplitude, 100 GHz modulation frequency and a 327 ms time constant. The microwave power was varied to ensure the samples were not saturated under reported conditions.
3.2.5 UV-Vis Spectroscopy

Enzyme stocks, \(\text{FeSO}_4\), and \(\alpha\text{KG}\) were made anaerobic under an Argon flush. 50 mM HEPES pH 7.50 was also made anaerobic by a repeating cycle of vacuum and nitrogen flush. \((\text{Fe}^{II}+\alpha\text{KG})\text{FIH}\) spectra were obtained on anaerobic samples containing FIH (250 µM), \(\text{FeSO}_4\) (230 µM) and \(\alpha\text{KG}\) (500 µM) in buffer. Apo FIH spectra were recorded for similar samples by omitting \(\alpha\text{KG}\) and \(\text{FeSO}_4\), and used as background spectra.

3.2.6 Activity Assays

Initial rate measurements were performed in 50 mM HEPES pH 7.50 and incubated at 37 °C in 50 µL reaction volume. Reaction buffer included 2.00 mM ascorbate, 100 µM DTT, 5 unit/mL catalase, 600 µM \(\alpha\text{KG}\), 25 µM \(\text{FeSO}_4\), and 0-600 µM CTAD. The reaction was initiated by adding the enzyme (0.5-5 µM), and at certain time points 5 µL aliquots were taken and quenched in 45 µL 0.1% formic acid. For each reaction 10 time points were collected and quenched, then analyzed by LC-ESI-MS. Samples were first loaded onto a C8 column for desalting, and CTAD and hydroxylated CTAD (CTAD\(^{\text{OH}}\)) were detected by ESI-MS to determine the mole fraction of peptide that had been converted to product, \(\chi_{\text{CTAD-OH}}\). Product concentrations were calculated as \([\text{CTAD}\^{\text{OH}}] = \chi_{(\text{CTAD-OH})} \times [\text{CTAD}]_0\), and used to determined initial rates, which were then used for Michaelis-Menten fits.

3.2.7 Autohydroxylation

Autohydroxylation was measured as described previously, with minor changes (28). FIH (100 µM), \(\text{FeSO}_4\) (500 µM), and \(\alpha\text{KG}\) (500 µM) were anaerobically incubated in 50 mM HEPES pH 7.50 for 20 min. Autohydroxylation was initiated by adding an equal volume
of buffer that had been equilibrated under air, and the reaction monitored at 583 nm. Competitive autohydroxylation assays included 50 µM CTAD that was pre-incubated with the FIH.

3.2.8 Coupling

A Hamilton PRP-X300 anion exclusion column and UV detection at 210 nm was used to separate and detect succinate yield from quenched steady-state reactions of FIH. The concentration of hydroxylated CTAD, CTAD$_{OH}$, was determined as per the activity assays. The coupling ratio was defined as $C = [\text{succinate}]/[\text{CTAD}^{\text{OH}}]$.

3.3 Results

Inspection of the X-ray crystal structure of FIH revealed hydrogen-bonding networks surrounding the Fe, which were centered on either αKG or CTAD. Asn$^{205}$ and Asn$^{294}$ residues donate hydrogen-bonds to the C-1 carboxylate of αKG, suggesting that these second-sphere residues may stabilize charge buildup during decarboxylation. Arg$^{238}$ and Gln$^{239}$ make hydrogen bonds with CTAD, suggesting that these residues may position the HIF-Asn$^{803}$, or may play a role in priming the Fe for oxygenation chemistry (Figure 3.1). The role of these hydrogen bonds was tested by functional assays and electronic spectroscopy of point mutants.
Table 3.1 Kinetic parameters for WT-FIH and its mutants for αKG and the synthetic peptide CTAD (39 residues).\textsuperscript{a,b}

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<th>$K_{M(CTAD)}$ (μM)\textsuperscript{a}</th>
<th>$k_{\text{cat}}/K_{M(CTAD)}$ (μM(^{-1}) min(^{-1}))\textsuperscript{a}</th>
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<td>0.24 ± 0.07</td>
</tr>
<tr>
<td>N294A</td>
<td>2.2 ± 0.1</td>
<td>14 ± 2</td>
<td>137 ± 26</td>
<td>0.016 ± 0.004</td>
</tr>
<tr>
<td>Q239N</td>
<td>0.123 ± 0.002</td>
<td>24 ± 4</td>
<td>38 ± 2</td>
<td>0.0032 ± 0.0002</td>
</tr>
<tr>
<td>R238M\textsuperscript{c}</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Assays in which CTAD was the varied substrate were performed with FIH and mutants (0.5-5 μM), ascorbate (2 mM), DTT (100 μM), catalase (5 unit/mL), αKG (500 μM), FeSO\(_4\) (25-50 μM), CTAD (0-600 μM) in 50 mM HEPES pH 7.50 at 37 °C. CTAD corresponds to a Cys→Ala point mutant of HIF-1α\textsuperscript{788-826}

\textsuperscript{b} Assays in which αKG was the varied substrate were as above, with these exceptions: CTAD (200 μM), and αKG varied (3 – 160 μM).

\textsuperscript{c} R238M was too slow to assay initial rates.

The effect of hydrogen bonding network on turnover was investigated by steady-state kinetics (Table 3.1). Saturating conditions were determined for ascorbate, αKG and Fe(II) and then initial rates of WT-FIH and its mutants were acquired by varying the CTAD concentration. DTT and catalase were used in the steady-state assays to prevent ferroxidase chemistry by Fe(II) in solution. WT-FIH showed $k_{\text{cat}}$ of 31 min\(^{-1}\) with a $k_{\text{cat}}/K_{M(CTAD)}$ of 0.41 μM\(^{-1}\) min\(^{-1}\), similar to previously reported values (Figure 3.2) (29-31). Each of the mutants had a modest effect on $K_{M(CTAD)}$, as both $k_{\text{cat}}$ and $k_{\text{cat}}/K_{M}$ were diminished by a similar factor for each mutant: N205A (3-fold), N294A (15-fold), and
Q239N (150-fold) (Figure 3.2 and Figure 3.3). R238M exhibited very low activity, which we could only detect through endpoint assays.

Figure 3.2 Steady state kinetics of WT-FIH and N205A. WT-FIH (0.5 µM), N205A (1 µM), ascorbate (2 mM), DTT (100 µM), catalase (5 unit/mL), αKG (500 µM), FeSO₄ (25 µM), CTAD (0-250 µM) in 50 mM HEPES pH 7.50.

Figure 3.3 Steady-state kinetics of N294A and Q239N. N294A (5 µM), Q239N (5 µM), ascorbate (2 mM), DTT (100 µM), catalase (5 unit/mL), αKG (500 µM), FeSO₄ (50 µM), CTAD (0-600 µM) in 50 mM HEPES pH 7.50.
Endpoint assays showed that CTAD-centered mutants were impaired in terms of product yield, relative to WT-FIH and the αKG-centered mutants (Table 3.2). WT-FIH hydroxylated 94% of CTAD during prolonged incubations; the αKG-centered mutants, N205A and N294A, were nearly as thorough as WT-FIH, converting over 79% of CTAD to product. In contrast, both CTAD-centered mutants were significantly compromised in their ability to hydroxylate CTAD, as Q239N and R238M converted less than 25% of CTAD to hydroxylated product.

The ratio of succinate production to CTAD hydroxylation was measured, to check for coupling between O$_2$-activation and hydroxylation (Table 3.2). As O$_2$-activation produces succinate, but might not lead to hydroxylated CTAD, uncoupling between these two enzymatic steps would lead to elevated coupling ratios, $C = [\text{succinate}]/[\text{CTAD}^{\text{OH}}]$. WT-FIH was tightly coupled (43), with $C$ equal to unity within experimental uncertainty ($C = 0.98 \pm 0.03$). The Q205A and Q294A point mutants exhibited slightly elevated $C$ values, however Q239N exhibited a coupling ratio was appreciably greater than unity ($C = 3.1 \pm 0.4$).
Table 3.2 Coupling and yield of hydroxylated CTAD by FIH variants.

<table>
<thead>
<tr>
<th></th>
<th>C&lt;sup&gt;a&lt;/sup&gt;</th>
<th>% CTAD&lt;sup&gt;OH&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-FIH</td>
<td>0.98 ± 0.03</td>
<td>94%</td>
</tr>
<tr>
<td>N205A</td>
<td>1.08 ± 0.04</td>
<td>90</td>
</tr>
<tr>
<td>N294A</td>
<td>1.3 ± 0.1</td>
<td>79</td>
</tr>
<tr>
<td>Q239N</td>
<td>3.1 ± 0.4</td>
<td>23</td>
</tr>
<tr>
<td>R238M</td>
<td>n.d.</td>
<td>5</td>
</tr>
</tbody>
</table>

<sup>a</sup> C = [succinate]/[CTAD<sup>OH</sup>] as determined under steady-state turnover conditions; see text for details. Not determined (n.d.).

<sup>b</sup> Qualitative endpoints: FIH (100μM) FeSO₄ (500 μM), αKG (500μM), CTAD (50 μM) 50 mM HEPES, pH 7.50.

FIH will slowly activate O₂ in the absence of CTAD, autohydroxylating Trp<sub>296</sub> to form an Fe(III)-O-Trp<sub>296</sub> chromophore with λ<sub>max</sub> = 583 nm (23, 28). While the rate-limiting step in autohydroxylation is not known, priming of the (Fe<sup>II</sup> + αKG)FIH is a likely requirement to permit O₂-activation. WT-FIH and the point mutants were tested for autohydroxylation rates in the absence of CTAD in order to determine how the priming of Fe(II) changed upon mutation. The autohydroxylation rates for the point mutants were only moderately altered from WT-FIH (Table 3.3), suggesting that the second coordination sphere had minor effects on steps directly involved in autohydroxylation. It is notable that both αKG-centered mutants exhibited 2-fold increases in the autohydroxylation rates relative to that for WT-FIH.
Table 3.3 Autohydroxylation rates for FIH variants.

<table>
<thead>
<tr>
<th></th>
<th>auto-hydroxylation initial rates (μM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 CTAD</td>
</tr>
<tr>
<td>WT FIH</td>
<td>0.0442 ± 0.0007</td>
</tr>
<tr>
<td>N205A</td>
<td>0.100 ± 0.002</td>
</tr>
<tr>
<td>N294A</td>
<td>0.0816 ± 0.0008</td>
</tr>
<tr>
<td>Q239N</td>
<td>0.034 ± 0.001</td>
</tr>
<tr>
<td>R238M</td>
<td>0.0262 ± 0.0008</td>
</tr>
</tbody>
</table>

100 μM FIH, 500 μM FeSO₄, 500μM αKG, 0 or 50 μM CTAD, 50 mM HEPES, pH 7.50.

We felt that a competition assay, in which autohydroxylation was monitored in the presence of CTAD, would be an interesting way to further test the effect of second-sphere mutations on positioning. In the competition assay, HIF-Asn⁸⁰³ and Trp²⁹⁶ were both present as hydroxylation targets (Table 3.3). The rate of autohydroxylation for WT-FIH, and most of the point mutants, decreased in the presence of CTAD. The lone exception to this was the CTAD-centered mutant Q239N, which underwent autohydroxylation 60% faster in the presence of CTAD than in its absence. This increased autohydroxylation rate for Q239N strongly suggested that CTAD binding primed Fe(II) to react with O₂, but that HIF-Asn⁸⁰³ was improperly oriented to receive the oxidant in this point mutant.
The ability of WT-FIH and its mutants to stabilize negative charge on C-1 of αKG was analyzed spectroscopically by the energy of the metal to ligand charge transfer (MLCT) transition. UV-Vis absorption spectra of (Fe²⁺+αKG)FIH form of each enzyme was measured, with the spectrum of the apo enzyme subtracted as a background. Each of the (Fe²⁺+αKG)FIH samples exhibited an MLCT peak near 500 nm (Figure 3.4). This absorption band has been attributed to three overlapping transitions between populated Fe $T_{2g}(\pi)$ orbitals and empty $\pi^*$ orbitals delocalized over the C1 carboxylate and C2 keto group of αKG (18). WT-FIH displayed an MLCT maximum at 500 nm, similar to that of other αKG hydroxylases such as TauD (530 nm) (32) and CS2 (476 nm) (33). In αKG-centered mutants this MLCT was blue shifted (Table 3.4), with the MLCT peaks for N205A and N294A appearing at 485 and 490 nm respectively. This blue shift indicated that the αKG $\pi^*$ orbitals in these point mutants were destabilized from WT-FIH, consistent with the hydrogen-bonds donated by Asn²⁰⁵ and Asn²⁹⁴ pulling electron density away from the keto-group of αKG.
Figure 3.4 UV-Vis spectra of (Fe$^{II}$+$\alpha$KG)FIH under anaerobic conditions after subtraction of (Fe$^{II}$)FIH spectra. FIH & mutants (250 µM), FeSO$_4$ (230 µM), αKG (250 µM) in 50 mM HEPES pH 7.50.

<table>
<thead>
<tr>
<th></th>
<th>$\lambda_{\text{max}}$ (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FIH</td>
<td>500</td>
</tr>
<tr>
<td>N205A</td>
<td>485</td>
</tr>
<tr>
<td>N294A</td>
<td>490</td>
</tr>
<tr>
<td>Q239N</td>
<td>495</td>
</tr>
<tr>
<td>R238M</td>
<td>488</td>
</tr>
</tbody>
</table>

FIH (250 µM), FeSO$_4$ (230 µM), αKG (250 µM) in 50 mM HEPES pH 7.50.
Substrate centered mutations also showed blue-shifted MLCT bands (Table 3.4). Charge transfer complexes of Q239N and R238M appeared at 495 and 488 nm respectively. The slight shift in the MLCT energy for Q239N is consistent with the short distance between residue 239 and αKG, suggesting only minor structural perturbations. The larger shift observed for R238M is similar in magnitude to that observed for N294A, and may be tentatively attributed to polarization effects from the loss of the positive charge at residue 238.

Cu(II) was used as a spectroscopic probe for the electronic fine structure in FIH. EPR spectra of both (Cu)FIH and (Cu+αKG)FIH were measured for WT-FIH and each point mutant (Table 3.5). The spectra of the point mutants were similar to that of WT-FIH, with some heterogeneity evident in the (Cu)FIH samples that diminished significantly in the (Cu+αKG)FIH samples. As FIH only provided three protein derived ligands, heterogeneity in the (Cu)FIH samples likely arose due to the presence of two or three solvent-derived ligands with variable bond lengths to the Cu(II). The WT (Cu)FIH sample exhibited an axial spectrum with apparent \( g_{\text{eff}} \) and \( A_{||} \) values (\( g_{\perp} = 2.06; g_{||} = 2.3; A_{||} = 148.1 \) G) appropriate for a “Type 2” Cu(II) site with mixed N and O-donor ligands (Figure 3.5) (34). Spectra of the WT (Cu+αKG)FIH sample exhibited increased anisotropy in \( g_{\text{eff}} \) (\( g_{\perp} = 2.06, g_{||} = 2.35 \)) and reduced hyperfine coupling (\( A_{||} = 136.4 \) G) as compared to (Cu)FIH (Figure 3.6). The small \( A_{||} \) is similar to that reported for other αKG hydroxylases (35-37), where it was attributed to an O-rich anionic ligand set and distorted planarity (37). The spectra for the (Cu+αKG)FIH point mutants were nearly identical to
those of WT-FIH, showing that the point mutations did not alter the metal coordination geometry in the (Cu+αKG)FIH enzyme form.

Table 3.5 EPR spectral parameters for (Cu)FIH and (Cu+αKG)FIH.

<table>
<thead>
<tr>
<th>EPR parameters</th>
<th>g∥</th>
<th>g⊥</th>
<th>A∥ (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Cu)FIH</td>
<td>2.30</td>
<td>2.06</td>
<td>146.2</td>
</tr>
<tr>
<td>(Cu)N205A</td>
<td>2.31</td>
<td>2.06</td>
<td>131.0</td>
</tr>
<tr>
<td>(Cu)N294A</td>
<td>2.31</td>
<td>2.06</td>
<td>134.6</td>
</tr>
<tr>
<td>(Cu)Q239N</td>
<td>2.29</td>
<td>2.06</td>
<td>135.0</td>
</tr>
<tr>
<td>(Cu)R238M</td>
<td>2.31</td>
<td>2.06</td>
<td>144.8</td>
</tr>
<tr>
<td>(Cu+αKG)FIH</td>
<td>2.35</td>
<td>2.06</td>
<td>136.4</td>
</tr>
<tr>
<td>(Cu+αKG)N205A</td>
<td>2.35</td>
<td>2.06</td>
<td>136.4</td>
</tr>
<tr>
<td>(Cu+αKG)N294A</td>
<td>2.35</td>
<td>2.07</td>
<td>136.4</td>
</tr>
<tr>
<td>(Cu+αKG)Q239N</td>
<td>2.35</td>
<td>2.07</td>
<td>136.8</td>
</tr>
<tr>
<td>(Cu+αKG)R238M</td>
<td>2.35</td>
<td>2.07</td>
<td>136.1</td>
</tr>
</tbody>
</table>

Parameters observed using Spincount(44)
Global enzyme stability was not altered by point mutation, as shown by the irreversible melting temperatures ($T_{M(app)}$) determined by DSC (Table 3.). The $T_{M(app)}$ of wild type FIH was 54.5 °C, whereas the $T_{M(app)}$ for the mutants were, in fact, slightly higher. Substrate centered mutants R238M and Q239N had slightly higher $T_{M(app)}$ than wild type, 56.2 and 56.7 °C respectively. The $T_{M(app)}$ of αKG centered mutants N205A and N294A were 58.6 and 58.9 °C respectively, again indicating that the second sphere mutations did not alter overall protein stability.

Figure 3.5 X-band EPR spectra of (Cu$^{II}$)FIH variants. FIH and point mutants (1 mM), CuSO$_4$ (0.9 mM) in 50 mM HEPES pH 7.50. 9.438 GHz, 20 mW power, 20 G modulation amplitude, 100 GHz modulation frequency, 327 ms time constant, 77 K.
The metal binding of FIH was slightly affected by point mutation, as shown by the Co(II) titrations (Table 3.). The equilibrium dissociation constant for \((\text{Co}^{2+}+\alpha\text{KG})\text{FIH}\) was \(1.38(6) \times 10^{-7}\), indicating a relatively strong affinity for Co\(^{2+}\). Each of the point mutants exhibited a similar dissociation constant, with \(K_D\) ranging from \(1.0 - 1.9 \times 10^{-7}\). This indicated that the primary coordination sphere of the active site was not altered by mutagenesis in the secondary sphere.

![Figure 3.6 X-band EPR spectra of (Cu\(^{1+}\)+\alpha\text{KG})FIH variants. FIH and mutants (1 mM), CuSO\(_4\) (0.9 mM), \alpha\text{KG} (1 mM) in 50 mM HEPES pH 7.50. 9.438 GHz frequency, 20 mW power, 20G modulation amplitude, 100 GHz modulation frequency, 327 ms time constant at 77 K.](image)
3.4 Discussion

Hydrogen bonding networks have been shown to be crucial to the function of enzymes reacting with O$_2$-derived species, such as lipoxygenase and SOD (15, 16), as well as for O$_2$-activating models (14). In the case of FIH, hydrogen bonding was observed between the facial triad ligands of Fe(II), the αKG, and various second-sphere residues, suggesting a functional role in tuning the chemistry of the Fe(II) center. In addition Gln$^{239}$ hydrogen bonds to the CTAD substrate, and its role in substrate positioning has been included. The present work combines kinetics and spectroscopy to show that these second sphere hydrogen bonds play roles in promoting oxidative decarboxylation, priming Fe(II) to bind O$_2$, and positioning HIF-Asn$^{803}$.

The steady-state rate constants, $k_{cat}$ and $k_{cat}/K_{M(CTAD)}$, were diminished for each point mutant of FIH (Table 3.1), indicating a reduction in the rate of one or more steps during turnover. These rate constants can be interpreted within the context of an ordered sequential mechanism, which is the consensus chemical mechanism for αKG hydroxylases (9, 10). A minimal kinetic model (Scheme 3.2) for FIH in the presence of saturating αKG involves separate microscopic steps for binding $S = CTAD$ ($k_1$) and O$_2$ ($k_2$), with an irreversible chemical step ($k_3$) followed by product release ($k_4$).

![Scheme 3.2 Minimal kinetic model for FIH](image)

The steady-state rate constants are composites of the above microscopic steps. All steps after substrate binding contribute to $k_{cat}$, while $k_{cat}/K_{M(CTAD)}$ is a function of all steps up through the first irreversible step. The reduced values to both $k_{cat}$ and $k_{cat}/K_{M(CTAD)}$ for
each point mutant suggests that the second coordination sphere plays a significant role in determining \( k_3 \), the common microscopic step. This first irreversible step may be oxidative decarboxylation of \( \alpha KG \) to form the \([FeO]^{2+}\) intermediate, as seen for TauD (38, 39), or a another step forming an Fe(II) center, as implied for PHD (40). In the absence of additional mechanistic probes such as kinetic isotope effects, we turned to the use of electronic spectroscopy and inactivation kinetics to test the effect of the second sphere residues.

Point mutations to second sphere residues were non-perturbing to the protein structure and primary coordination of the metal center in mutated FIH, as shown by the thermodynamic stability and electronic spectroscopy of each mutant. EPR spectra of the point mutants showed that the metal center in (Cu+\( \alpha KG \))FIH retained a virtually identical ground-state electronic environment to WT-FIH (Figure 3.6). The MLCT transitions for (Fe\( ^{II} \)+\( \alpha KG \))FIH variants revealed that the \( \alpha KG \) was a bidentate ligand to Fe(II), while also providing a view of the changing Lewis acidity of the C2-position of \( \alpha KG \) (Figure 3.4). As nucleophilic attack of superoxide at C2 is thought to be a key step in \( O_2 \)-activation by \( \alpha KG \)-dependent oxygenases (41), the Lewis acidity of the keto group is essential for oxidative decarboxylation. The blue shifts in the MLCT energies for point mutants indicated that the \( \alpha KG \) \( \pi^* \) orbitals were destabilized in the N294A and N205A mutants, suggesting that the hydrogen bonds found in WT-FIH serve to pull electron density away from the C2 position of \( \alpha KG \).
Oxidative decarboxylation is promoted by stabilizing negative charge buildup in the transition state, as shown for enzymes (20) and model complexes (41). Structural analysis and the MLCT shifts in FIH indicate that both Asn$^{205}$ and Asn$^{294}$ pull electron density away from αKG, although the Fe(II) likely plays the predominant role in activating αKG for decarboxylation. Asn$^{294}$ plays a more significant role than Asn$^{205}$ in stabilizing charge, as shown by the relative values for $k_{cat}$: WT > N205A > N294A (Table 3.1). This may be due to Asn$^{294}$ being the sole residue pulling electron density away from the distal O of the C1 carboxylate of αKG, while both Asn$^{205}$ and Fe(II) pull electron density from the proximal O of the C1 carboxylate (Figure 3.1).

The significant decrease in the steady-state rate constants for CTAD-centered mutants may arise from changes in Fe(II) priming or CTAD positioning. The consensus chemical mechanism suggests that mutations affecting priming should principally reduce $k_{cat}/K_{M(CTAD)}$, as steps up through O$_2$-activation determine this rate constant. Conversely, mutations affecting positioning should reduce $k_{cat}$, as steps following O$_2$-binding determine this rate constant. Although the structure of FIH with CTAD bound suggests that there may be hydrogen bonds with specific roles, such as those between Gln$^{239}$ and HIF-Asn$^{803}$ (positioning), and Arg$^{238}$ and Asp$^{201}$ (priming), the kinetics data is less clear as each point mutation reduced $k_{cat}$ as well as $k_{cat}/K_{M(CTAD)}$ when compared to WT FIH. This may arise from an effect on a chemical step common to both rate constants, such as O$_2$-activation, or from the extended hydrogen bond network linking Asp$^{201}$ to HIF-Asn$^{803}$ thorough Arg$^{238}$ and Gln$^{239}$ (Figure 3.1). It appears that positioning the sidechain of HIF-
Asn\textsuperscript{803} is integral to the priming of Fe(II), and may even be a design feature to ensure tight coupling between O\textsubscript{2}-activation and substrate hydroxylation.

While priming could not be separated from substrate positioning by steady-state kinetics, the two roles could be distinguished by the coupling ratio, and by analysis of hydroxylation products, as shown for TauD (42). A functional view of priming is the ability of FIH to activate O\textsubscript{2} only once CTAD binds, leading to close coupling between the production of succinate and CTAD\textsuperscript{OH}. In the case of WT-FIH, breakdown in priming leads solely to autohydroxylation as FIH does not release reactive oxygen species (23, 28, 43). This was supported by our observation of a coupling value of unity ($C = 0.98 \pm 0.03$) for WT-FIH, and a near-unity coupling ratio for N205A and N294A.

Positioning HIF-Asn\textsuperscript{803} in the proper registry within the active site is crucial to ensure that the [FeO]\textsuperscript{2+} oxidant attacks the correct residue and forms the CTAD\textsuperscript{OH} product. The Q239N mutant exhibited notable effects on the coupling ratio, indicating that Gln\textsuperscript{239} was crucial for local positioning (}
Table 3.2). Q239N was the only mutant in which the coupling ratio differed significantly from unity (3.1 ± 0.4), and the only mutant that autohydroxylated faster in the presence of CTAD. These observations indicate that, while Q239N was able to activate O₂, the HIF-Asn\textsuperscript{803} sidechain was improperly positioned to form normal product.

FIH serves a crucial role in sensing hypoxia within human cells. As an O₂-sensing enzyme, it must maintain tight coupling between O₂-activation and substrate hydroxylation, which it achieves by hydrogen bonding between ligands to the Fe and the second coordination sphere. These second sphere hydrogen bonds play roles in promoting O₂-activation as well as maintaining the structural registry between CTAD and Fe. Hydrogen bonds from Asn\textsuperscript{205} and Asn\textsuperscript{294} pulled electron density away from αKG, as shown by the LMCT transitions; removal of either hydrogen bond in the Asn→Ala point mutants slightly accelerated autohydroxylation, but made normal turnover much slower in the case of Asn\textsuperscript{294}→Ala. It is likely that autohydroxylation rates reflect the intrinsic reactivity of αKG, whereas the rate of normal turnover is more dependent on precise positioning of CTAD. For this reason, the rate constants for normal turnover are greatly reduced in Gln\textsuperscript{239}→Asn and Arg\textsuperscript{238}→Met, both CTAD-centered mutants. By virtue of their respective locations relative to Asp\textsuperscript{201} and HIF-Asn\textsuperscript{803}, it is likely that Arg\textsuperscript{238} is necessary for priming of the Fe(II) in response to CTAD binding, whereas Gln\textsuperscript{239} is more involved in positioning HIF-Asn\textsuperscript{803}. The second sphere interactions play a significant role in O₂-sensing function of FIH by tuning the chemistry at the Fe(II) center.
3.5 Appendix

3.5.1 Supplemental

3.5.1.1 Thermal stability of FIH variants

The thermal stability of FIH variants was measured by differential scanning calorimetry, as described in the text.

<table>
<thead>
<tr>
<th></th>
<th>( T_{M(app)} ) (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WtFIH</td>
<td>54.5</td>
</tr>
<tr>
<td>N205A</td>
<td>58.6</td>
</tr>
<tr>
<td>N294A</td>
<td>58.9</td>
</tr>
<tr>
<td>R238M</td>
<td>56.7</td>
</tr>
<tr>
<td>Q239N</td>
<td>56.2</td>
</tr>
</tbody>
</table>

FIH (50 μM) in 50 mM HEPES pH 7.50, temperature range: 25-75 °C, scan rate: 60 °C/hour.

3.5.1.2 Co\(^{II}\) Binding thermodynamics to FIH variants

3.5.1.2.1 Metal titration data fitting

The binding affinity of (Co\(^{2+}\)+αKG)FIH for Co\(^{2+}\) was obtained by competitive titration in 50 mM HEPES, pH 7.50 at 23°C. A 200 μL solution of apo FIH (20 μM), citrate (1.00 mM), and αKG (100 μM) was temperature equilibrated in a fluorescence cuvette, while a separate solution of CoCl\(_2\) (1.00 mM) with citrate (1.00 mM) in buffer was loaded into a titrating syringe. As FIH-1 utilizes αKG as a co-substrate as well as a bidentate ligand for metal, αKG was included to complete the relevant ligand set of (Co\(^{2+}\)+αKG)FIH.
This experiment used citrate to buffer the concentration of free Co\textsuperscript{2+}, or Co(H\textsubscript{2}O)\textsubscript{6}\textsuperscript{2+}, as the log \(\beta\) values for Co(II) binding to citrate are well defined (1). Upon addition of small volumes of CoCl\textsubscript{2}, apo FIH bound the available \(\alpha\)KG and Co\textsuperscript{2+}. The fluorescence intensity of FIH-1 at 340 nm (F340) was plotted against \(\log[\text{Co}\textsuperscript{2+}]\text{free}\), exhibiting the sigmoidal shape characteristic of a binding equilibrium. The binding curve was fitted to a simple 1:1 binding equilibrium in which the Co\textsuperscript{2+} dissociation constant of (Co\textsuperscript{2+}+\(\alpha\)KG)FIH (\(K_D\)) is with respect to Co(H\textsubscript{2}O)\textsubscript{6}\textsuperscript{2+} (Eq. S1). The fluorescence intensity (F340) was fitted to Eq. S2 (2) which yielded \(\log K_D\) (x) relative to the \(\log[\text{Co}\textsuperscript{2+}]\text{free}\) (c), using a cooperativity parameter (B) which accounted for both chemical cooperativity involved with dimeric FIH-1 as well as the spectroscopic cooperativity involved with fluorescence signal from the eight Trp residues of FIH. The results are in Table 3.

\[
(Co^{2+}+\alpha \text{KG})FIH \leftrightarrow Co(H_2O)_6^{2+} + apoFIH \quad (S1)
\]

\[
F_{340} = F_{\text{min}} + (F_{\text{max}} - F_{\text{min}})/(1+10^{(c-x)B}) \quad (S2)
\]

Table 3.6 Co\textsuperscript{II} binding affinity of FIH-1 mutants in the presence of \(\alpha\)KG

<table>
<thead>
<tr>
<th></th>
<th>(K_D) (M)</th>
<th>B</th>
</tr>
</thead>
<tbody>
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<td>WTIH</td>
<td>1.38(6)x10\textsuperscript{-7}</td>
<td>2.2 ± 0.2</td>
</tr>
<tr>
<td>N205A</td>
<td>1.63(3) x10\textsuperscript{-7}</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>N294A</td>
<td>1.01(4) x10\textsuperscript{-7}</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td>R238M</td>
<td>1.45(8) x10\textsuperscript{-7}</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Q239N</td>
<td>1.9(2) x10\textsuperscript{-7}</td>
<td>1.3 ± 0.1</td>
</tr>
</tbody>
</table>

CoCl\textsubscript{2} (1 mM) / citrate (1 mM) was titrated into FIH (20 \(\mu\)M), \(\alpha\)KG (100 \(\mu\)M) in 50 mM HEPES pH 7.50.
3.5.1.2.2 References:


3.5.2 Abbreviations

αKG, alpha-ketoglutarate;

CTAD, C-terminal transactivation domain of HIFα;

DSC, differential scanning calorimetry;

EPR, electron paramagnetic resonance;

FIH, factor inhibiting HIF-1;

HIF, hypoxia inducible factor;

LC-ESI-MS, liquid chromatography – electrospray ionization mass spectrometry;

MLCT, metal-to-ligand charge transfer;

NTA, nitrilotriacetate;

SOD, superoxide dismutase
3.6 Bibliography


44. Hendrich, M. P. Spincount software, Carnegie Mellon Univ.
4.1 Introduction

Solvent isotope effects (SIE) can be seen when enzymatic rates are measured in H₂O, D₂O or mixture of these solvents. Solvent isotope effects are more global when compared to substrate isotope effects which use one or few isotopic positions. Because the physical properties of D₂O are different, replacing H₂O with D₂O might affect the enzyme stability, conformational change, substrate binding and kinetic & equilibrium constants associated with enzymatic reactions. Overall change is termed as solvent isotope effects which is expressed as ratios of the rate constants in two solvents (1, 2). When the kinetic parameters \( k_{H}/k_{D} \), \( V_{H}/V_{D} \) or \( (V/K)_{H}/(V/K)_{D} \) are larger than unity (larger in H₂O) a normal solvent isotope effect is observed. When the parameters are less than unity (larger in D₂O), it is called an inverse solvent isotope effect.

Acid dissociation constant for H₂O (1x10⁻¹⁴) is about ten times larger than that of D₂O (1x10⁻¹⁵). While the pH of H₂O is 7.00, pD of D₂O is 7.41 which is a significant isotope effect and might have important consequences on pH and pD sensitive rate constants. Generally pD values are corrected by a factor of 0.4 (pD=pH+0.4) and thus accurate measurements of pH and pD are required for sensitive solvent isotope effects (3-6).

If the enzyme structure/function is maintained by hydrogen bonding network, conformation and stability can change in heavy water. Because the intermolecular hydrogen bond length is longer in D₂O, conformation and stability can slightly alter in
heavy water (7). So the overall structure might not be perturbed but loss of activity is possible due to instability of protein in heavy water. If substrate binding is pH sensitive or causes a conformational change, solvent isotope effect would be expected. SIE on microscopic rate constants of enzyme catalyzed reactions would affect steady-state parameters $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$. The best way to determine SIE on $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ is to measure them as a function of substrate. If pH profile of an enzyme is considered in which the enzyme rate follows a bell shaped curve, monitoring the effects of SIE at one pH and one pD will not be enough. When the steady-state parameters of an enzyme do not change over a couple of pH/pD units, the most stable pH region can be chosen and the SIE studies can be done at that pH range. Otherwise wide range of pH range should be selected because SIE requires sensitive measurements.

![Scheme 4.1 Steps of FIH between the resting state and substrate binding which might show solvent isotope effect.](image-url)
SIE measurements in a wide pH range would also give information about the ionization states of enzyme and/or substrate. Substrate binding, enzyme/substrate ionization state and/or product release might all show solvent isotope effects if those steps are pH or solvent dependent.

Examples of solvent isotope studies on nonheme iron enzymes showed no significant effects. An ethylene forming enzyme ACCO showed only background SIE (8) and TauD did not show any SIE (9). Although there is no significant SIE among these enzymes, FIH will be tested for any solvent isotope effects.

In the case of FIH, water release upon CTAD binding is the most interesting step because iron center needs to be five-coordinate for binding and activation of oxygen (Scheme 4.1). If the rate determining step is the water release upon CTAD binding, changing H$_2$O with D$_2$O might show a solvent isotope effect. Properties of normal and heavy water are different in the following ways: First, OH intramolecular bond length is longer than OD by about 0.03 Å or 3%. Second, intermolecular hydrogen bonding distance in H$_2$O is shorter than in D$_2$O by 0.07 Å or 4%. Also the distance between hydrogen atoms on neighboring molecules is about 2% longer for H$_2$O than in D$_2$O. Finally, the number of hydrogen bonds per water molecule is less in H$_2$O than in D$_2$O (3.62 vs. 3.76). So there is an overall change geometrically (7).
If these geometrical differences are applied, hydrogen bonds are stronger in D$_2$O which results in higher density, viscosity and boiling point (3). Zero-point vibrational energy of water molecules are decreased when they are deuterated. In terms of energy, D$_2$O exchange from the Fe(II) center should be slower which would cause normal solvent isotope effects. However, when the hydrogen bonding property of D$_2$O is taken into account, it might exchange faster than normal water. Stronger hydrogen bonding in D$_2$O can weaken Fe-D$_2$O bond causing a faster exchange and slower isotope effect (inverse SIE) with a higher $k_{cat}$ but no change in $K_m$. If this step is not the rate limiting step, SIE=1 would be expected.

Substrate binding might also be affected by heavy water. Because CTAD is a pretty large substrate, hydrogen bonding interactions between CTAD and FIH would change upon changing H$_2$O with D$_2$O. Because D$_2$O forms stronger hydrogen bonds, the interaction between FIH and CTAD would be more pronounced. To test this mechanism, one pH/pD point will not be enough to compare because a solvent isotope effect seen might not be only due to substrate binding step. Interpretation of SIE is generally hard so in addition to wide pH profile control experiments should be done.

4.2 Materials and Methods

4.2.1 Activity assays and pH profile

Initial rate measurements were performed in 50 mM HEPES (pH 6.50, 6.90, 7.40 and 7.80) and incubated at 37 °C in 50 µL reaction volume. Reaction buffer included 2.00 mM ascorbate, 500 µM αKG, 25 µM FeSO$_4$, and 0-300 µM CTAD. The reaction was
initiated by adding the enzyme (0.5 µM), and at certain time points 5 µL aliquots were taken and quenched in 45 µL 0.1% formic acid. For each reaction 10 time points were collected and quenched, then analyzed by LC-ESI-MS. Samples were first loaded onto a C8 column for desalting, and CTAD and hydroxylated CTAD (CTAD\textsuperscript{OH}) were detected by ESI-MS to determine the mole fraction of peptide that had been converted to product, $\chi_{\text{CTAD-OH}}$. Product concentrations were calculated as $[\text{CTAD}\textsuperscript{OH}] = \chi_{\text{CTAD-OH}} \times [\text{CTAD}]_0$, and used to determine initial rates, which were then used for Michaelis-Menten fits. The steady-state parameter $k_{\text{cat}}$ vs. pH was fitted to equation 4.1 to obtain a kinetic pK\textsubscript{a} value of FIH.

$$k_{\text{cat}} = \frac{enzH \times 10^{-pH} + enz \times 10^{-pK_a}}{10^{-pH} + 10^{-pK_a}}$$  Eq. 4.1

### 4.2.2 Solvent isotope effects

For solvent isotope effect studies, MALDI was chosen over LC-ESI-MS. Because in ESI-MS, +3 charge states caused misinterpretation of data. During the HPLC run, deuterated samples were back exchanged to some extent. CTAD and CTAD\textsuperscript{OH} m/z appeared in two places due to H\textsubscript{2}O and D\textsubscript{2}O and this has led the data being not useful. To avoid these problems SIE measurements were performed like in the previous part by using MALDI with the following changes. Reaction buffer (50 mM Hepes pH 7.0) included 2.00 mM ascorbate, 500 µM αKG, 25 µM FeSO\textsubscript{4}, and 0-200 µM CTAD. The reaction was initiated by adding the enzyme (0.5 µM), and at certain time points 5 µL aliquots were taken and quenched in 5-15 µL freshly prepared saturated sinapinic acid solution in 75% ACN+0.2% TFA (in H\textsubscript{2}O). For the measurements in D\textsubscript{2}O, buffer solution of 50 mM Hepes pD 7.0 was prepared by dissolving Hepes salt in D\textsubscript{2}O and adjusting the pH by
NaOH to 6.60 (pD was corrected by adding 0.4 to pH reading). The reaction buffer (50 mM Hepes pD 7.0) included 2.00 mM ascorbate, 500 µM αKG, 25 µM FeSO₄, and 0-200 µM CTAD (all reagents were prepared in D₂O, CTAD was in 50 mM Hepes pD 7.0 buffer). The reaction was initiated by adding the enzyme (0.5 µM in 50 mM Hepes pH 7.0), and at certain time points 5 µL aliquots were taken and quenched in 5-15 µL freshly prepared saturated sinapinic acid solution in 75% ACN+0.2% TFA (in D₂O). Based on the volume percentages, the deuterium content was calculated to be around 90% for SIE measurements. Measurements were done in triplicate.

The matrix quenched samples were then spotted on the target and analyzed by two different MALDI (Bruker Daltonics Omniflex III MALDI TOF and Autoflex III MALDI TOF) mass spectrometers. Omniflex and Autoflex are low and high resolution instruments respectively. The tuning parameters for both instruments were as the following:

<table>
<thead>
<tr>
<th></th>
<th>Omniflex</th>
<th>Autoflex</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ion Source 1</strong></td>
<td>19 kV</td>
<td>19 kV</td>
</tr>
<tr>
<td><strong>Ion Source 2</strong></td>
<td>17.30 kV</td>
<td>17.30 kV</td>
</tr>
<tr>
<td><strong>Lens</strong></td>
<td>9.4 kV</td>
<td>9.4 kV</td>
</tr>
<tr>
<td><strong>Reflector</strong></td>
<td>0 kV</td>
<td>20 kV</td>
</tr>
<tr>
<td><strong>Detector Mode</strong></td>
<td>Linear</td>
<td>Reflective</td>
</tr>
<tr>
<td><strong>Shots</strong></td>
<td>1000</td>
<td>1000</td>
</tr>
</tbody>
</table>

Analysis of the data was done by taking the ratios of hydroxylated CTAD intensity to overall intensity to determine concentration as it was done in the previous part. Calculated and observed masses of CTAD in H₂O and D₂O are summarized in Table 4.1.
Table 4.1 Calculated and observed CTAD mass in H$_2$O and D$_2$O

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Backbone</th>
<th>Fast exchangeable</th>
<th>Sum</th>
<th>Mono mass</th>
<th>Avg mass</th>
<th>Backbone deuterated mass</th>
<th>Fully deuterated mass</th>
<th>Observed mass in H$_2$O</th>
<th>Observed mass in D$_2$O</th>
</tr>
</thead>
<tbody>
<tr>
<td>DESGLPQTLTSYDAEVNAPIQGSRNLLQGEELLRLALDQVN</td>
<td>36</td>
<td>38</td>
<td>74</td>
<td>4252.13</td>
<td>4254.58</td>
<td>4290.81</td>
<td>4329.04</td>
<td>4255</td>
<td>4326</td>
</tr>
</tbody>
</table>

The residues that have fast exchangeable protons: STYCDEHWKQNR

4.3 Results and Discussion

Obtaining a wide pH profile for an enzyme is generally hard due to the limitations of buffer capacities. Two or three component buffers can be used to make the buffer work in a wider pH range. In this case ionic strength should be controlled. Because all of the previous studies have been done in 50 mM Hepes, we have chosen to stay in Hepes within its buffer capacity (useful pH range for Hepes is 6.8-8.2). 50 mM Hepes pH 6.50, 6.90, 7.40 and 7.80 buffers were prepared by dissolving Hepes salt in water and adjusting the pH by NaOH to the according values. The lowest pH point was little off the buffer capacity but at the end of each initial assay the final pH values were checked to make sure reaction is occurred at that pH. The Michaelis-Menten fits can be seen in Figure 4.1.
When the steady-state parameters $k_{\text{cat}}$ and $k_{\text{cat}}/K_M$ were plotted as a function of pH, no drastic change is observed (Figure 4.2 and Figure 4.3). This small pH range did not give a kinetic $pK_a$ so we chose to work around pH 7.0. Log-log plots of $k_{\text{cat}}$ vs. pH was almost linear.

Table 4.2 Steady-state parameters of FIH at different pHs.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{\text{cat}}$, min$^{-1}$</th>
<th>$k_{\text{cat}}/K_M$, µM$^{-1}$min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.5</td>
<td>41.3 ± 4.2</td>
<td>0.52 ± 0.15</td>
</tr>
<tr>
<td>6.9</td>
<td>37.7 ± 3.2</td>
<td>0.57 ± 0.14</td>
</tr>
<tr>
<td>7.4</td>
<td>31 ± 2</td>
<td>0.65 ± 0.15</td>
</tr>
<tr>
<td>7.8</td>
<td>29 ± 2</td>
<td>0.92 ± 0.23</td>
</tr>
</tbody>
</table>
Figure 4.2 \( \log(k_{\text{cat}}) \) vs. pH. FIH showed no pK\( _a \) over pH 6.4-7.8 range.

Figure 4.3 \( k_{\text{cat}}/K_M \) of FIH in pH 6-4-7.8 range.
The steady-state parameters and fitting parameters were summarized in Table 4.2. For the solvent isotope effect studies pH 7.0 was chosen because $k_{\text{cat}}/K_M$ does not change around that pH value. If the $k_{\text{cat}}/K_M$ value deviates within small pH range, that would cause insensitive SIE measurements. If any SIE was to be observed, that might be due to pH changes. So $k_{\text{cat}}/K_M$ should not change drastically within a small region for the control experiments. After preparing 50 mM Hepes pH/pD 7.0 initial assays were done according to the protocol mentioned in methods. $H_2O$ data showed a similar Michaelis-Menten curve with the data in pH profile studies. FIH has a $k_{\text{cat}}$ of 34.1±1.8 min$^{-1}$ and $K_m$ value of 88.2±10.9 µM in 50 mM Hepes pH 7.0 (Figure 4.4). However the $D_2O$ data behaved differently so Michaelis-Menten type kinetics was not observed Figure 4.4. Up to 50 µM CTAD concentration reaction is faster in $D_2O$ than in $H_2O$. When the CTAD concentration was increased, a proinhibition type behaviour was observed. Because there is not any inhibitior in the reaction mixture, CTAD itself inhibited FIH. This type of behaviour due to heavy water could not be addressed; a proinhibition kinetics model (equation 4.2) was used to obtain $K_i$ value for CTAD. The steady-state parameters are summarized in Table 4.3.

$$\frac{V}{E} = \frac{k_{\text{cat}}}{1 + \frac{K_m}{[S]} + \frac{[S]}{K_i}}$$  \hspace{1cm} \text{Eq. 4.2}

Omniflex MALDI instrument gave low resolution spectra in which CTAD and hydroxylated CTAD peaks were not separated enough. The data was still useful to obtain initial rates but to make things sure, SIE measurements were repeated with a high resolution Autoflex MALDI. The comparison of low and high resolution instruments can be seen in Figure 4.5. Interestingly Autoflex MALDI gave the same results. So the
inhibition behaviour seen is not due to resolving issues at high CTAD concentrations. SIE measurements were repeated several times in both high and low resolution instruments, in all of which a proinhibition can be observed (Figure 4.6). To address this proinhibition behaviour, all of the reagents were prepared fresh several times including the buffer. CTAD is also purified, lyophilized and dissolved in pD 7.0 buffer but the results were same.

![Figure 4.4 Solvent Isotope Effects on FIH. H$_2$O data (■); FIH(0.5 µM), αKG(500 µM), Ascrobate (2 mM), Fe (25 µM), CTAD (0-250 µM) were mixed in 50 mM Hepes pH 7. D$_2$O data (●); FIH(0.5 µM), αKG(500 µM), Ascrobate (2 mM), Fe (25 µM), CTAD (0-250 µM) were mixed in 50 mM Hepes pD 7. αKG, Ascrobate and Fe(II) solution were prepared in D$_2$O, CTAD was in 50 mM Hepes pD 7 buffer and FIH was in 50 mM Hepes pH 7 buffer. D$_2$O content is 90%.

Table 4.3 Steady-state parameters of FIH in pH 7.0 and pD 7.0.

<table>
<thead>
<tr>
<th></th>
<th>$k_{cat}$</th>
<th>$K_m$</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 7.0</td>
<td>34.1 ± 1.8</td>
<td>88.2 ± 10.9</td>
<td></td>
</tr>
<tr>
<td>pD 7.0</td>
<td>92.3 ± 34.8</td>
<td>37.1 ± 21.6</td>
<td>29.3 ± 13.9</td>
</tr>
</tbody>
</table>
Figure 4.5 Comparison of low resolution and high resolution MALDI instruments. CTAD m/z is 4326 and CTAD-OH m/z is 4342 in D₂O.

Figure 4.6 FIH(0.5 µM), αKG(500 µM), Ascorbate (2 mM), Fe (25 µM), CTAD (0-250 µM) were mixed in 50 mM Hepes pD 7. αKG, Ascorbate and Fe(II) solution were prepared in D₂O, CTAD was in 50 mM Hepes pH 7 buffer and FIH was in 50 mM Hepes pH 7 buffer. D₂O content is 90%. Autoflex is high resolution MALDI, Omniflex is low resolution MALDI.
4.4 Conclusions and Future Directions

These studies could not be completed due to time limitation. If the substrate inhibition at high concentrations could be addressed, FIH might still show faster rates in heavy water which means an inverse solvent isotope effect. SIE measurements in a wider pH range would also give better answers to our questions. After all for the preliminary efforts, FIH behaves different in heavy water, probably showing an inverse SIE.

CTAD inhibition at high concentrations might be due to stronger hydrogen bonding interactions in heavy water, so product release after hydroxylation might be prevented. To test this behaviour, really high concentrations of CTAD should be tested for any inhibition in H2O.

If the water release upon CTAD binding is the rate limiting step, it would help us to better understand the regulatory role of FIH. It would also elucidate how oxygen is activated. In the absence of substrate, oxygen can still bind to iron center and do slow oxidative reaction like auto-hydroxylation. When the substrate is present, water release opens a coordination site for oxygen binding. Determining the rate limiting step for oxygen activation will provide more insights into oxygen sensor FIH and other nonheme iron enzymes in which oxygen is activated in a controlled manner.

As we have seen in the previous chapters there is not only one factor for oxygen activation. In chapter 2; how activated oxygen is tightly coupled to CTAD hydroxylation in FIH was tested, in chapter 3; the impact of hydrogen bonding network on the second
coordination sphere was studied and in chapter 4; the rate determining step for the oxygen activation was investigated by means of solvent isotope studies.

Overall this thesis provided insights into how oxygen is activated and controlled in FIH which is one of the primary oxygen sensors in human body. Understanding the mechanism of how activated oxygen is controlled in human oxygen sensor FIH would draw new pathways in hypoxia sensing and its related diseases.
4.5 Bibliography


