Role of the Facial Triad in Factor Inhibiting HIF (FIH): Ligand Binding, Substrate Selectivity, and Coupling

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ROLE OF THE FACIAL TRIAD IN FACTOR INHIBITING HIF (FIH):
LIGAND BINDING, SUBSTRATE SELECTIVITY, AND COUPLING

A Dissertation Presented

by

VANESSA D. CHAPLIN

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

DOCTOR OF PHILOSOPHY

September 2018

Department of Chemistry
ROLE OF THE FACIAL TRIAD IN FACTOR INHIBITING HIF (FIH): LIGAND BINDING, SUBSTRATE SELECTIVITY, AND COUPLING

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To my grandparents, those who were able to witness the completion of this dissertation and those who were not. I would not have made it this far without your endless love and support.
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First and foremost, I would like to thank my advisor, Professor Michael J. Knapp for his mentorship. Thank you for your constant support, patience, and advisement, even when I was being stubborn and indignant.

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Thank you to the entire Chemistry department for making my time here so enjoyable. It was an experience I will never forget.
ABSTRACT

ROLE OF THE FACIAL TRIAD IN FACTOR INHIBITING HIF (FIH): LIGAND BINDING, SUBSTRATE SELECTIVITY, AND COUPLING

SEPTEMBER 2018

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Directed by: Professor Michael J. Knapp

Alpha-ketoglutarate (αKG) dependent oxygenases comprise a large superfamily of enzymes that activate O₂ for varied reactions. While most of these enzymes contain a non-heme Fe bound by a His₂Asp facial triad, a small number of αKG-dependent halogenases require only the two His ligands to bind Fe and activate O₂. The enzyme “factor inhibiting HIF” (FIH) contains a His₂Asp facial triad and selectively hydroxylates polypeptides, however removal of the Asp ligand in the D201G variant leads to a highly active enzyme, seemingly without a complete facial triad. Herein, we report on the formation of an Fe-Cl cofactor structure for the D201G variant using x-ray absorption spectroscopy (XAS), which provides insight into the structure of the His₂Cl facial triad found in halogenases. D201G variant supports anion dependent peptide hydroxylation, demonstrating the requirement for a complete His₂X facial triad to support O₂ reactivity. Our results indicate that exogenous ligand binding to form a complete His₂X facial triad was essential for O₂ activation, and provides a structural model for the His₂Cl-bound nonheme Fe found in halogenases.
We also propose that the facial triad ligands couple O₂ activation with hydroxylation in 
FIH, providing insight into the coupling mechanism for this broad class of enzymes. Mutating 
the Asp201 ligand resulted in uncoupled product formation. For the D201X variants, O₂ was 
consumed much faster than primary substrate was hydroxylated, which suggested branching 
in the chemical mechanism. EPR studies established that this branching occurs following the 
formation of the Fe(IV)=O intermediate as the metal centers of WT FIH and D201G were 
triggered by substrate whereas the metal centers of D201E and D201A were unaffected. 
Investigation into the fate of O₂ in the absence of primary substrate identified two products: 
hydrogen peroxide (H₂O₂) and autohydroxylated Trp (TrpOHI). This study provides insight into 
significance of the facial triad in substrate triggering within the αKG oxygenase family of 
enzymes and describes the products of uncoupling.
PREFACE

This dissertation centers around understanding the mechanistic role of the facial triad carboxylate in the Fe(II)/αKG dependent hydroxylase Factor Inhibiting HIF (FIH). Chapter 1 reviews mammalian oxygen sensing mechanisms, with a focus on the regulation of HIF by the HIF hydroxylases. Section 1.5 of this chapter was written by Prof. Michael Knapp. A former Knapp lab undergraduate student, Alexandra Barbato (B.S. UMass Amherst 2017), wrote section 1.6. This chapter has been published by the Royal Society of Chemistry as part of the book Gas Sensing in Cells (2018).

Chapter 2 explores the unique observation of chloride-enhanced substrate hydroxylation activity of the D201G variant of FIH. This chapter is the result of a cumulative effort over the course of several years. A former graduate student in the Knapp lab, Dr. John Hangasky (PhD UMass Amherst 2014), made the D201A/E/G variants of FIH and evaluated the initial rate of enzyme activity in the presence of various anions. He also conducted the UV-Vis assays to check for anion binding to the (Fe+ αKG)FIH enzyme form. The XAS sample was run and analyzed by Hsin-Ting Huang (Maroney lab). Prof. Michael Maroney and Hsin-Ting Huang wrote the XAS results and discussion section and prepared the XAS figure. Ran Duan conducted $K_d$ binding assays for D201E and WT FIH. This chapter is currently being reviewed by Inorganic Chemistry.

Chapter 3 stems from the finding that the D201G variant of FIH binds chloride. In this chapter, we sought to engineer alternate rebound chemistry (such as chlorination) through site-directed mutagenesis of the second coordination sphere of D201G FIH. However, it was discovered that second coordination sphere mutations were not sufficient to position the
halide ligand to outcompete the hydroxide ligand for rebound chemistry. As this project did not yield the desired result, this chapter is not being considered for publication.

Chapter 4 investigates the role of the facial triad carboxylate ligand in coupling O$_2$ reactivity and substrate binding using a variety of kinetic and spectroscopic techniques. This chapter is currently being prepared for publication.

Appendix A details a collaboration with the Rotello lab at UMass Amherst to deliver FIH into mammalian cells in order to control the hypoxic response. Ryan Landis developed the polymer-nanoparticle emulsion delivery system (Rotello lab) and Yiwei Lee (Rotello lab) collected the confocal images.

Appendix B describes the methodology used for screening compound libraries to identify selective inhibitors of the HIF hydroxylases and provides an example screening study.
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CHAPTER 1

MAMMALIAN O₂ SENSING AND SIGNALLING


1.1 Introduction

Mammals sense low pO₂ (hypoxia) by processes over multiple length scales, ranging from cellular-based pathways up through tissue-based pathways. The most prominent O₂ sensing pathway centers on the hypoxia inducible factor (HIF), which induces gene expression under hypoxic conditions. HIF is regulated primarily by the HIF hydroxylases, the Factor Inhibiting HIF (FIH) and Prolyl Hydroxylase Domain (PHD) enzymes, which are nonheme Fe, alpha-ketoglutarate dependent dioxygenases. FIH and PHD recognize selective sequences on HIFα, but they also hydroxylate alternative substrates, such as protein containing ankyrin repeat domains (ARDs) and a variety of proteins involved in cellular stress responses, making the search for new substrates of FIH and PHD a rich area of research. Proposals for additional O₂ sensors, such as H₂S and the F-box and leucine-rich repeat protein 5 (FBXL5), are introduced. The tissue-level responses of vasopermeation and vasoconstriction are discussed, and are put in context with cellular events such as changes in potassium channels.

1.2 Cellular O₂ Sensing

Maintaining the delicate balance of oxygen homeostasis and ensuring adequate O₂ delivery throughout the body is crucial to cell survival. In addition to acting as the terminal electron acceptor for mitochondrial respiration, O₂ is an essential co-substrate for many
enzymatic processes.¹,² Consequently, cells and tissues sense and respond to changes in O₂ levels.³ Cellular O₂ homeostasis is necessary for normal growth and development, including cellular functions such as metabolism and biosynthesis, as well as tissue or organismal-level adaptations such as angiogenesis and erythropoiesis. Unsurprisingly, misregulation of O₂ homeostasis is associated with a variety of pathophysiologies including anemia, ischemia, inflammation and cancer.³-⁵

The scientific literature typically refers to O₂ sensing in terms of cell responses to conditions of hypoxia, or low pO₂, as the hypoxic response is tied to energy balance, iron mobilization, and proliferation.¹ Sensing and responding to changes in O₂ levels is a requirement for survival in aerobic organisms. In addition to balancing metabolic flux between aerobic and anaerobic metabolism, many cellular processes are connected to O₂ levels. Distinct hypoxia sensing mechanisms maintain this delicate balance.⁶ In mammals and most eukaryotes, the master response is mediated by the Hypoxia Inducible Factor (HIF), which leads to a pronounced transcriptional activity that is directly correlated to O₂ levels.⁵

The primary method for sensing hypoxia within cells is through post-translational hydroxylation of the Hypoxia Inducible Factor-1 (HIF-1) which leads to a variety of transcriptional responses that will be discussed below.⁷ In addition to the hydroxylation of the HIF-1 transcription factor, growing evidence suggests that other protein hydroxylations may connect to O₂-dependent pathways. Protein hydroxylation changes protein-protein binding affinities, altering protein stability and downstream enzyme activities.⁷ For example, under normoxia, prolyl hydroxylation of the eukaryotic elongation factor kinase (eEF2K) interferes with the kinase binding to calmodulin, inhibiting eEF2K activity.
eEF2K activity is upregulated in hypoxia, increasing the rate of translation elongation and protein synthesis.\textsuperscript{7,8} This regulation suggests a cytoprotective role for eEF2K and highlights that interaction between hypoxia sensing and broader metabolic response to stress.

As protein hydroxylation is such a simple post-translational modification, there may be additional pathways regulated by hydroxylation, whether or not they are due to the hypoxia response.\textsuperscript{7} While there are a number of protein hydroxylase enzymes that have been identified, there is a need to identify new substrates of these enzymes. Similarly, identifying new hydroxylating enzymes is crucial to pushing this area of research forward. This knowledge could uncover additional O\textsubscript{2} sensing pathways and provide novel therapeutic targets and regulation mechanisms to improve human health.

1.2.1 HIF Transcriptional Regulator

The primary pathway for O\textsubscript{2} sensing in human cells is the HIF pathway, in which changes in [O\textsubscript{2}] lead to transcriptional responses of over 1,000 genes.\textsuperscript{5,9-11} This pathway is essential for normal growth and differentiation of tissues. The products of those genes controlled by HIF are powerful therapeutic targets, both because of their protective roles in responding to varied pO\textsubscript{2} levels as well as their antagonistic impacts on tumor growth. Genes upregulated by HIF are thought to play protective roles in human responses to myocardial ischemia, wound healing, and chronic rejection of organ transplants; conversely, genes upregulated by HIF may play antagonistic roles in hypertension, ocular neovascularization, erythrocytosis, and a variety of cancers.\textsuperscript{2}

The function of many proteins under HIF transcriptional control can be sorted into two categories: those that increase O\textsubscript{2} delivery and those that decrease O\textsubscript{2} consumption.\textsuperscript{12}
Erythropoietin (EPO) and vascular endothelial growth factor (VEGF), for example, are proteins that increase O₂ delivery throughout the body by increasing blood O₂ carrying capacity and vascular permeability respectively. Proteins that decrease O₂ consumption include lactate dehydrogenase A (LDHA) and pyruvate dehydrogenase kinase 1 (PDK1) which are crucial in shifting the cell from oxidative to glycolytic metabolism in response to decrease pO₂. These proteins and resulting biological effects will be discussed in greater detail later in this chapter.

HIF was discovered following the observation that the level of EPO was increased by hypoxia. HIF is a heterodimeric transcription factor that is a member of the PER, ARNT, SIM (PAS) superfamily and is composed of two basic helix-loop-helix proteins, HIFα and HIFβ. This HIFα/β dimer binds to hypoxia response elements (HREs) in DNA that are known to be associated with transcription. HIFβ, also known as ARNT, is a constitutively expressed nuclear protein known to play many roles in biology. HIFα is the more significant protein in relation to oxygen sensing as the abundance of HIFα is decreased when pO₂ is increased.

### 1.2.2 HIFα

HIFα is found as three isoforms in humans and most higher organisms: HIF-1α, HIF-2α, and HIF-3α. All three forms of HIFα are tightly regulated by post-translational modification and alternative splicing. HIF-1α activity is also controlled by its nuclear/cytoplasmic distribution. Both HIF-1α and HIF-2α are regulated by post-translational protein modifications including hydroxylation, acetylation, sumoylation, and phosphorylation in their NODD and CODD domains as well as their catalytically active NTAD and CTAD domains as described in Figure 1. HIF-3α is unique in that its CODD
domain is regulated by alternative splicing. One spliced variant of HIF-3α contains the bHLH and PAS domains, but lacks the CODD and other C-terminal sequences. This spliced version of the protein is referred to as inhibitory PAS protein (IPAS). The extensive nature of HIFα regulation highlights the significance of this protein in oxygen homeostasis.

Figure 1.1 Post-translational modifications of HIF-1α in *homo sapiens*.\textsuperscript{17}

1.2.3 HIF Hydroxylases

The direct link between HIF-1α activity and O$_2$ levels is formed by the action of a small family of non-heme Fe(II) dependent enzymes, which are known as the HIF hydroxylases. This family is composed of FIH and three PHD enzymes. Discovered in 2001 through a combination of sequence analysis and biochemical experiments, these enzymes hydroxylate specific amino acid residues within the sequence of HIF-1α, leading to a decrease in the transcriptional levels of HRE controlled genes.\textsuperscript{18,19}

The HIF hydroxylases are thought to follow the consensus chemical mechanism for the alpha-ketoglutarate (αKG) oxygenase superfamily. Enzymes in this superfamily couple the splitting of O$_2$ to the hydroxylation of primary substrate and oxidative decarboxylation of αKG. Their net chemistry for a simple hydroxylation is:

$$\text{O}_2 + \text{RH} + \alpha\text{KG} \rightarrow \text{CO}_2 + \text{ROH} + \text{succinate}$$

where R is the primary substrate.\textsuperscript{2}
Applying the consensus mechanism to the HIF hydroxylases provides an example of the fundamental steps involved in turnover. The enzyme initially forms a six coordinate Fe(II) complex bound to a two-His one-carboxylate facial triad. As shown in Figure 1.2, binding of primary substrate displaces the H$_2$O ligand coordinated to Fe, leading to the reaction with O$_2$. Oxidative decarboxylation of αKG produces succinate and CO$_2$, and an Fe(IV)=O intermediate. This ferryl intermediate can then oxidize an unactivated C-H bond in the primary substrate (R) allowing for hydrogen atom transfer and rebound chemistry to produce the hydroxylated product (R-OH).

![Figure 1.2 Consensus mechanism for the HIF hydroxylases.](image)

A prominent feature of this catalytic cycle is the multiple changes in geometry about the Fe cofactor, as reported for FIH and several other αKG oxygenases.$^{20-22}$ The cause of this coordination change is not clear, however altered local contacts to the second coordination sphere are thought to induce aquo release.$^{20,23}$ Crystallographic data indicates that the backbone of the hydroxylated residue of HIF-1α is positioned to hydrogen bond to the distal O of the active site Asp residue, which is thought to weaken the hydrogen bond
between Asp and the aquo ligand. In addition, steric clashes within the active site appear to impact the binding of the aquo ligand in the E(Fe+αKG) enzyme form, suggesting that a more constrained active site upon HIF-1α binding may destabilize the aquo ligand.\textsuperscript{24}

Although all of the αKG dependent oxygenases require O\textsubscript{2} as a substrate, both FIH and PHD are O\textsubscript{2} sensors due to their role in regulating HIF-1α. The abundance of all reagents impacts the activity of the HIF hydroxylases, including primary substrate, O\textsubscript{2}, Fe(II), and αKG, resulting in a response that is proportional to substrate and cofactor availability.\textsuperscript{25} A unique feature of FIH and PHD isoymes is the very high Michaelis constant relative to physiological O\textsubscript{2} levels ($K_{M(O2)} \gg [O2]$), leading their activity to be directly proportional to [O\textsubscript{2}] over physiologically relevant values. Interestingly, FIH has a lower $K_{M(O2)}$ than PHD, meaning it has a more pronounced activity response at lower oxygen tensions than PHD. This difference in the kinetic constants between the HIF hydroxylases suggests that they may fulfill distinct cellular functions over a wide range of [O\textsubscript{2}].\textsuperscript{26}

The common protein fold for the catalytic domain of the αKG-dependent oxygenases is the cupin fold, in which 8 β-strands wrap around to form a barrel that binds the Fe cofactor. FIH is a relatively simple example of the cupin fold, however FIH is a homodimer with two catalytic subunits per biological unit. The PHD isoforms exhibit a more complicated structure, as they are composed of a MYND domain as well as a cupin fold.\textsuperscript{27}

PHD2 is thought to be the main prolyl hydroxylase isozyme mediating HIF-1α stability \textit{in vivo} as inactivation of PHD2 alone has been shown to upregulate HIF1α protein levels and HIF target gene expression in several cell lines.\textsuperscript{6,28} PHD1 and PHD3 are active
in the testis and heart respectively whereas PHD2 is ubiquitously expressed.\textsuperscript{9,29,30} The PHD isozymes also exhibit differential cellular distributions, with PHD1 found as a nuclear protein, PHD2 predominately found in the cytoplasm, and PHD3 present in both the nucleus and cytoplasm. Although the reasons for the different localizations of the PHD enzymes is unknown, it is speculated that cytoplasmic HIF hydroxylases control the HIF transcriptional system under normoxia while the nuclear hydroxylases serve as a backstop to downregulate HIF\(\alpha\) following sudden reoxygenation.\textsuperscript{25}

PHDs catalyze hydroxylation of conserved prolyl residues within the N- and C-oxygen degradation domains (N/CODDD) of HIF\(\alpha\) (Figure 1.3). Once hydroxylated at one or both ODDDs, HIF\(\alpha\) is then recognized by pVHL, which targets HIF\(\alpha\) for proteasomal degradation. The action of PHD causes the HIF\(\alpha\) protein levels to decrease in response to elevated [\(O_2\)], leading to a primary control over the transcriptional activity of HIF.\textsuperscript{31,32} As the activity of the PHDs is directly proportional to [\(O_2\)] over the physiological range, simple chemical kinetics provide the main control over the transcriptional activity of HIF, although levels of PHD2 and PHD3 are themselves also upregulated in response to hypoxia.\textsuperscript{25}
Figure 1.3 Regulation of HIF-1α by the HIF hydroxylases.

FIH hydroxylates the β-carbon of a specific asparagine residue in the CTAD of HIF-1α and HIF-2α. This hydroxylation blocks the binding of co-activator p300, preventing HIFα from assembling an active transcriptional complex. p300 and its paralog CBP interact with several transcription factors, regulating their activity. The p300/CBP proteins are composed of several protein-binding domains, including two homologous transcriptional adaptor zinc-binding (TAZ) domains. These TAZ domains are responsible for protein-protein interaction and transcriptional regulation of several cellular pathways; despite sharing sequence homology TAZ1 and TAZ2 are known to bind different groups of transcription factors. Studies of the free TAZ1 domain revealed that the TAZ1 domain is able to form a stable fold without being complexed to a transcription factor, indicating that this domain acts as a preformed scaffold with unique surfaces and grooves to mediate protein-protein recognition. These slight structural differences allow the activation domain
of HIF-1α to distinguish between TAZ1 and TAZ2 and form an active transcriptional complex.

In addition to the HIF hydroxylases, several other enzymes are involved in cellular responses to hypoxia. As hypoxia responses are so closely tied to cellular energetics and proliferation, and a variety of post-translation modifications alter HIFα activity, distinguishing O2-specific responses from other metabolic responses is challenging.34,35 Similarly, there are a growing number of oxygenases linked to gene expression through histone demethylation or DNA repair, which demonstrates that O2-initiating enzymes may regulate genes without being hypoxia sensors.36-39

Figure 1.4 Model for the regulation of VHL and HIF-1α by JMJD6 in trophoblast cells. In normoxia, JMJD6 stabilizes VHL through lysyl hydroxylation, resulting in translocation of VHL into the nucleus where it undergoes SUMOylation by SUMO1. SUMOylated VHL then targets HIF-1α for degradation.40

1.2.4 Other O2 Sensing Enzymes

There is very strong evidence supporting an O2-sensing role for the enzyme Jumonji domain-containing 6 protein (JMJD6). This oxygen sensing role is illustrated in early placental development as JMJD6 expression levels correlate with changes in oxygen
tension.\textsuperscript{40} In normoxia, JMJD6 hydroxylates lysine residues on pVHL, allowing pVHL to translocate into the nucleus where it undergoes SUMOylation and targets HIF-1\(\alpha\) for degradation (Fig. 4).\textsuperscript{40} Under hypoxic conditions, HIF-1\(\alpha\) upregulates JMJD6 expression. However, the dioxygenase activity of JMJD6 is reduced in low oxygen environments, resulting in VHL degradation and accumulation of HIF-1\(\alpha\). The discovery of more oxygen sensing enzymes is anticipated as the regulation of oxygen sensing in mammalian cells is investigated further.

1.3 Discovering new targets for the HIF hydroxylases

HIF hydroxylases react selectively with a target residue within a peptide sequence context; however, there is some variability in these sequences. Both FIH and PHD have been shown to react with proteins other than HIF\(\alpha\) suggesting that there are additional substrates remaining to be discovered for these enzymes.

1.3.1 HIF-1\(\alpha\)

HIF-1\(\alpha\) is the established physiological substrate for FIH and the PHD isozymes with the specific reactions being asparaginyl hydroxylation within the C-terminal transactivation domain (CTAD) for FIH, and prolyl hydroxylation in the C/N terminal oxygen-dependent degradation domain (ODDD) for PHD.\textsuperscript{10,16,25} The protein sequence of HIF-1\(\alpha\) is highly conserved in these domains highlighting the specific nature of these modifications. As shown in Figure 1.5 there is little diversity in the CTAD region of HIF-1\(\alpha\) across numerous species. The CTAD sequence of DXEVNAP, with Asn\textsuperscript{803} being the residue hydroxylated by FIH, is highly conserved indicating that these residues are crucial for regulation of HIF-1\(\alpha\) by FIH.
Figure 1.5 Logo representation of the CTAD consensus sequence of HIF-1α across several species. 9 eukaryotic HIF-1α protein sequences of various phylums and classes were aligned using Clustal Omega, then modelled using the web logo program developed at the University of California, Berkeley. The N803 is the site of hydroxylation. Aligned species (accession number): Homo sapiens (NP_001521.1), Rattus norvegicus (NP_077335.1), Myotis davidii (ELK27249.1), Canis lupus familiaris (NP_001274092.1), Bos grunniens (AAT39520.1), Branchiostoma floridae (AGX25238.1), Tribolium castaneum (EFA04586.2), Expaipptasia pallida (KXJ20783.1), and Saccoglossus kowalevskii (ADB22425.1).

The conserved recognition site in CTAD correlates with the area of CTAD that binds in the active site pocket of FIH as seen in Figure 1.6 The alpha helical and loop residues which reside outside of the FIH active site are not responsible for substrate recognition and subsequent hydroxylation; therefore, there is a higher tolerance of variability at these sites.

Figure 1.6 Crystal structure of CTAD (magenta) in the active-site pocket of FIH (wheat). Residues that are conserved in the CTAD consensus sequence are displayed and labeled (cyan). CTAD residues 807-811 are not resolved in the crystal structure. PDB Code: 1H2K.41
The PHD recognition sequence within the ODDD displays a greater tolerance for residue variability. Both Pro\textsuperscript{402} and Pro\textsuperscript{564} are targeted for hydroxylation and subsequent regulation of HIF-1\(\alpha\) by the PHD isozymes. This variability in recognition sequence may explain why the PHD isozymes hydroxylate two different proline residues with the ODDD. As seen in Figure 1.7 using the same HIF-1\(\alpha\) sequences as the CTAD HIF-1\(\alpha\) alignment, the NODDD PHD binding site has a consensus sequence of LTXXAPAAAGD whereas the CODDD consensus sequence is even more variable: LXXXAPYIXX.

![Figure 1.7 Logo representation of the NODDD (top) and CODDD (bottom) consensus sequence of HIF-1\(\alpha\) across several species. The same eukaryotic HIF-1\(\alpha\) protein sequences of various phylums and classes as used in Figure 1.5 were aligned using Clustal Omega, then modelled using the web logo program developed at the University of California, Berkeley. The hydroxylated residues are P402 (NODDD)and P564 (CODDD). Aligned species (accession number): Homo sapiens (NP_001521.1), Rattus norvegicus (NP_077335.1), Myotis davidii (ELK27249.1), Canis lupus familiaris (NP_001274092.1), Bos grunniens (AAT39520.1), Branchiostoma floridae (AGX25238.1), Tribolium castaneum (EFA04586.2), Expaiptasia pallida (KXJ20783.1), and Saccoglossus kowalevskii (ADB22425.1).](image)

As seen with the FIH/CTAD adduct, the CODDD residues that bind in the active site of PHD are conserved (Fig. 1.7 & 1.8). Fewer residues of CODDD fit in the PHD active site pocket, which leads to a greater tolerance for variability in the rest of the
CODDD chain (Fig. 1.8). Close inspection of the HIF hydroxylase recognition sites in HIF-1α suggests the FIH/CTAD binding is more selective than PHD2/CODDD as there are more conserved residues in the CTAD consensus sequence.

Figure 1.8 Crystal structure of CODD (magenta) in the active-site pocket of PHD2 (wheat). CODD residues that are conserved in the consensus sequence are displayed and labeled (cyan). PDB Code: 5L9B.42

1.3.2 Ankryin Repeat Domains

FIH also hydroxylates a limited set of target residues within proteins containing ankyrin repeat domains (ARDs). ARDs are composed of repeating ~33 amino acid segments in a helix-loop-helix conformation with a β-hairpin loop connecting adjacent repeats. These ARDs are found in numerous biologically significant proteins and can exist on their own as a single domain or in tandem with other domains to compose a larger protein.43 ARD-containing proteins mediate a wide variety of protein-protein interactions in the areas of cell-cell signaling, transcription and cell cycle regulation, inflammatory response, and development.43
FIH displays a broad tolerance for several target residues within the ARD sequence context, in contrast to the specificity for Asn within the CTAD context.\textsuperscript{44} FIH will hydroxylate Asn, His, Leu, Ile, Ser, and Asp target residues within a consensus ARD peptide sequence \textit{in vitro}, indicating that polar and hydrophobic target residues are accepted.\textsuperscript{26} In addition to the \textit{in vitro} peptide substrates, biological assays revealed hydroxylation at Asn, Asp, and His target residues.\textsuperscript{45, 46} The ARD substrates are the only known example of non-asparaginyl substrate hydroxylation by FIH.

![Scheme 1.1](image)

\textbf{Scheme 1.1} Substrate hydroxylation by FIH.

Hydroxylation of ARD substrates by FIH likely follows the same consensus mechanism as CTAD hydroxylation (Scheme 1.1). The added hydroxyl group forms a hydrogen bond to nearby aspartyl residues stabilizing the ARD fold in solution.\textsuperscript{47} Hydroxylation of His is less efficient than the other residues, which may reflect the positioning of the target residue within the active site of FIH.

![Figure 1.9](image)

\textbf{Figure 1.9} Logo representation of known ARD-containing substrates for asparaginyl hydroxylation by FIH. Protein sequences were aligned using Clustal Omega. The hydroxylated residue is N16. Aligned human proteins (hydroxylated residue): ASPP2 (N986), IκBα (N210), IκBα (N244), p105 (N678), p19-INK4d (N101), ILK-1 (N94), FEM1β (N526), FGIF (N168), GABP-B1 (N98), Gankyrin (N100), Myotrophin (N62), RNaseL (N233), MYPT1 (N67), Notch1 (N1956), Rabankyrin-5 (N316), AnkyrinR (N105), Tankyrase-1 (N864), Tankyrase-2 (N203).
As exemplified in Figure 1.9, the 33 amino acid structure of the ARDs is well conserved. Despite the high degree of sequence conservation of the ARDs, insertions in the loop regions of the ARDs are not uncommon. Structure alignments of the p105 and IκBα proteins, which were the first ARD-containing proteins confirmed as FIH substrates, revealed that the hydroxylated residue resides at the apex of the hairpin loop which connects the ARD repeating units. As the loop region containing the target residue is semi-conserved throughout the ARD family, it was hypothesized that more ARD containing proteins would also be suitable FIH substrates. This hypothesis was confirmed through biochemical assays and modeling, resulting in ARDs becoming the largest class of hydroxylation substrate to date. All of the current known ARD and HIF substrates of FIH are listed in the table below.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Peptide Sequence</th>
<th>Function</th>
<th>Substrate Class</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>YDCEVNAPIQ</td>
<td>HIF transcriptional regulation</td>
<td>HIF</td>
<td>Wilkins 2009</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>YDCEVNAAPVL</td>
<td>HIF transcriptional regulation</td>
<td>HIF</td>
<td>Wilkins 2009</td>
</tr>
<tr>
<td>ASB4</td>
<td>NNAEVRARDD</td>
<td>Ubiquitin-mediated proteolysis</td>
<td>ARD</td>
<td>Ferguson 2007, Cockman 2009</td>
</tr>
<tr>
<td>ASPP2</td>
<td>FGVNNAADS</td>
<td>Regulates cell-cell contacts and maintenance of tight junctions</td>
<td>ARD</td>
<td>Janke 2013</td>
</tr>
<tr>
<td>FEM1β</td>
<td>CGAEVNAVDN</td>
<td>Unknown</td>
<td>ARD</td>
<td>Cockman, 2006</td>
</tr>
<tr>
<td>GABP-β</td>
<td>HGADVNAKDM</td>
<td>Unknown</td>
<td>ARD</td>
<td>Cockman, 2006</td>
</tr>
<tr>
<td>Gankyrin</td>
<td>KGAQVNAVQ</td>
<td>Unknown</td>
<td>ARD</td>
<td>Cockman, 2006, Wilkins 2012</td>
</tr>
<tr>
<td>IκBα</td>
<td>LGADVNAQEP</td>
<td>NF-κB signaling</td>
<td>ARD</td>
<td>Cockman 2006</td>
</tr>
<tr>
<td>Gene</td>
<td>Sequence</td>
<td>Function</td>
<td>Source</td>
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<td>---------</td>
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<tr>
<td>ILK-1</td>
<td>YKADINAVNE</td>
<td>Unknown</td>
<td>ARD Cockman, 2006</td>
<td></td>
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<td>Myotrophin</td>
<td>KGADINAPDK</td>
<td>Unknown</td>
<td>ARD Cockman, 2006</td>
<td></td>
</tr>
<tr>
<td>MYPT-1</td>
<td>AGYDVNIKDV</td>
<td>Actin-myosin contractility</td>
<td>ARD Cockman 2009, Webb Anyi 2009</td>
<td></td>
</tr>
<tr>
<td>Notch-1</td>
<td>ASADANIQDN</td>
<td>Proliferation, differentiation, apoptosis</td>
<td>ARD Cockman 2009, Coleman 2007, Wilkins 2009</td>
<td></td>
</tr>
<tr>
<td>Notch-3</td>
<td>AGADTNAQDH</td>
<td>Proliferation, apoptosis</td>
<td>ARD Cockman 2009, Coleman 2007, Wilkins 2009</td>
<td></td>
</tr>
<tr>
<td>P19-INK4d</td>
<td>GADVNPDG</td>
<td>Unknown</td>
<td>ARD Cockman, 2006</td>
<td></td>
</tr>
<tr>
<td>p105 (NFkB1)</td>
<td>AGADVNAQE</td>
<td>NF-κB signaling</td>
<td>ARD Cockman 2006, Webb Anyi 2009</td>
<td></td>
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<tr>
<td>Rabankryin-5</td>
<td>HQADINVRTQ</td>
<td>Endocytosis, macropinocytosis</td>
<td>ARD Cockman, 2009</td>
<td></td>
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<tr>
<td>RIPK-4</td>
<td>PGVSVNAQLT</td>
<td>Influences RIPK4 stability and downstream signaling</td>
<td>ARD Rodriguez, 2016</td>
<td></td>
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<td>RNaseL</td>
<td>HGADVNVRGE</td>
<td>Endoribonuclease</td>
<td>ARD Cockman 2009, Webb Anyi 2009</td>
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<tr>
<td>Tankyrase-1</td>
<td>HGADVNAQDK</td>
<td>Telomere, vesicle regulation</td>
<td>ARD Cockman 2006, Webb Anyi 2009</td>
<td></td>
</tr>
<tr>
<td>Tankyrase-2</td>
<td>TPLNVNCHAS</td>
<td>Telomere, vesicle regulation</td>
<td>ARD Cockman, 2009</td>
<td></td>
</tr>
</tbody>
</table>
Hydroxylation of the ARDs does not induce a conformational change in the ARD structure as show by crystallography of the Notch-1 substrate.\(^4\) The proposed function of ARD hydroxylation is to induce localized changes in the ankyrin repeat stability in order to regulate its function. This hypothesis is based on studies of IκBα which indicated that changes in ARD stability influence NF-κB association and effect the half-life of the uncomplexed protein.\(^5\)

While the biological significance of FIH hydroxylation of ARDs is unknown, there is some speculation that ARD proteins are competitive inhibitors of the CTAD FIH substrate.\(^2\)\(^5\),\(^4\)\(^7\) Evidence to support this theory includes the tighter binding of FIH to ARD than to CTAD (resulting in a lower \(K_{M(ARD)}\) than \(K_{M(CTAD)}\)).\(^2\)\(^7\) Interestingly, FIH has been shown to bind some ARDs without hydroxylating them, suggesting that ARDs may serve to buffer the availability of FIH to sense O\(_2\). Further, when co-expressed in transfected cells, ARD proteins compete with CTAD for hydroxylation.\(^2\)\(^7\) In addition, the FIH-ARD complex reacts slower with O\(_2\) than FIH-HIFα.\(^2\)\(^6\) Exploring the scope of ARD hydroxylation by FIH will provide further insight into the protein-protein interactions these ARDs mediate and may identify other pathways regulated by FIH.
1.3.3 Alternate Substrates of PHD

Several non-HIF substrates have been identified for the PHD isozymes, many of which play a significant role in the biological pathways activated during hypoxia. For example, IKKβ activates the NF-κB pathway which is critical to the inflammatory response. Hydroxylation of Rpb1 by PHD1 and PHD2 promotes tumor growth, which is known to be unregulated during certain cancers. A complete list of the known hydroxylation substrates of the PHD isozymes is presented in Table 1.2 The wide range of substrate hydroxylation by the PHD isozymes implies that there are a host of HIF-independent responses to changes in oxygen tension within the cell.

Table 1.2 Known substrates hydroxylated by the PHD isozymes.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Peptide Sequence</th>
<th>PHD Isoform</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt</td>
<td>FRSGS PSDNSG</td>
<td>PHD2</td>
<td>Decrease in Akt-mediated cell proliferation and tumorigenesis</td>
<td>Guo 2016</td>
</tr>
<tr>
<td></td>
<td>TFCGT PSEYLAP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B(2)AR</td>
<td>CEDLPGTEDFV</td>
<td>PHD3</td>
<td>Regulation of receptor degradation</td>
<td>Wong 2013, Xie 2009</td>
</tr>
<tr>
<td></td>
<td>GHQGTV-PSDNI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B and γ actin</td>
<td>ITALAPSTMK</td>
<td>PHD3</td>
<td>Impaired actin polymerization</td>
<td>Luo 2014</td>
</tr>
<tr>
<td></td>
<td>GTTMYPGIAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CEP192</td>
<td>LSSLAPPYVK</td>
<td>PHD1</td>
<td>Control of cell cycle progression</td>
<td>Moser 2013</td>
</tr>
<tr>
<td>Protein</td>
<td>Site(s)</td>
<td>PHD(s)</td>
<td>Regulation/Activity</td>
<td>Reference</td>
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<td>---------</td>
<td>--------------------------</td>
<td>--------</td>
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<td>-----------------</td>
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<tr>
<td>CERKL</td>
<td>Unknown</td>
<td>PHD1,3</td>
<td>Regulation of CERKL protein stability</td>
<td>Chen 2015</td>
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<td>DYRKI</td>
<td>Unknown</td>
<td>PHD1</td>
<td>HIF-2α destabilization</td>
<td>Lee 2016</td>
</tr>
<tr>
<td>A/B</td>
<td></td>
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<td>EPOR</td>
<td>LLRPWTLCPE</td>
<td>PHD3</td>
<td>Decrease in erythropoiesis and EPO induced JAK-STAT signaling</td>
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<td></td>
<td>LPPTPHLKL</td>
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<tr>
<td>FLNA</td>
<td>Multiple Pro residues</td>
<td>PHD2</td>
<td>Inhibition of p53 transcriptional activity</td>
<td>Segura 2016</td>
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<td>FOXP3</td>
<td>SGLGSPTSSF</td>
<td>PHD1,3</td>
<td>Prevents binding of USP9x</td>
<td>Zheng 2014</td>
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<td></td>
<td>STVFQPSSLN</td>
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<td></td>
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<td>HCLK2</td>
<td>LAQLGEPELRL</td>
<td>PHD3</td>
<td>Promotes DNA damage response</td>
<td>Wong 2013, Xie 2012</td>
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<td>HIF-1α</td>
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<td>PHD1,2,3</td>
<td>HIF transcriptional regulation</td>
<td>Tarhonskaya 2015</td>
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<td>LTLAAPAAGD</td>
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<td>PHD1</td>
<td>Activation of NF-κB</td>
<td>Cummins 2006</td>
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<td>MAPK6</td>
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<td>PHD3</td>
<td>Stabilization of MAPK6 protein</td>
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<td>Gene</td>
<td>Motif/Peptide</td>
<td>PHD Domain</td>
<td>Effect</td>
<td>References</td>
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<td>---------------------------------------------</td>
<td>-------------------------------------</td>
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<tr>
<td>Myogenin</td>
<td>Unknown</td>
<td>PHD3</td>
<td>Stabilization of myogenin protein</td>
<td>Wong 2013, Fu 2007</td>
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<td>NDRG3</td>
<td>CGGLPQVVQP</td>
<td>PHD2</td>
<td>Decrease in RAF-ERK1/2 kinase signaling</td>
<td>Lee 2015</td>
</tr>
<tr>
<td>p53</td>
<td>Unknown</td>
<td>PHD1</td>
<td>Phosphorylation of Ser15 on p53, binding with p38</td>
<td>Deschoemae-ker 2015</td>
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<td>PDE4D</td>
<td>SSSLNSSIPRP</td>
<td>PHD2</td>
<td>Regulation of intracellular cAMP levels</td>
<td>Wong 2013, Huo 2012</td>
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<td>PHD3</td>
<td>HIF activation</td>
<td>Wong 2013, Luo 2011</td>
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<td>Rbp1</td>
<td>P1465</td>
<td>PHD1,2</td>
<td>Activation of Rbp1 and tumor growth and promotion</td>
<td>Wong 2013, Mikhaylova 2007, Kuznetsova 2003</td>
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<td>Spry2</td>
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<td>PHD2,3</td>
<td>Increased transcription of PLN</td>
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<td>LKNLRPEFMQ</td>
<td>PHD1,2,3</td>
<td>Negative regulation of ion channel activity</td>
<td>Takahashi 2011, Wong 2013</td>
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1.4 New Target Identification

Despite the physiological role for FIH and PHDs as HIF hydroxylases, there is still much to be learned about new hydroxylation targets of these enzymes. Identifying new targets may include pathways involved in O₂ sensing independent of HIF, or complementary to HIF. Alternative targets have been discovered for both FIH and the PHD isozymes, as described in the previous section; additional substrates are likely to be discovered.

Identifying new substrates for this group of enzymes will provide insight into other roles of the HIF hydroxylases, such as the potential for HIF-independent O₂ sensing. Substrate discovery is challenging due to limited methods for naïve screening to identify substrate hydroxylation. Unlike many other post-translational modifications, hydroxylation does not significantly alter the molecular weight of the target, as occurs in ubiquitination; nor does it alter the pI of a peptide, as is the case for acetylation or phosphorylation.⁵²,⁷¹ This means that bulky methods, such as column enrichment used for phosphopeptides, do not work to selectively enrich hydroxylated peptides. Consequently, there is a reliance on lower throughput methods, such as biological binding assays or high-resolution mass spectrometry (MS).

The general approach for detecting hydroxylation is summarized as follows: identify the hydroxylated peptide, quantify hydroxylation prevalence with and without hydroxylase present, then evaluate the molecular and biological function of the hydroxylation.⁷,⁵² In addition to sequencing analysis to identify putative substrates based on sequence similarity, there are a variety of chemical and biochemical approaches to assay for hydroxylation which will be discussed in detail in this section.
An unbiased method to identify hydroxylation is based on a radio-tracer decarboxylation assay.\textsuperscript{65} It is well known that hydroxylation by the αKG oxygenases results in the decarboxylation of αKG and release of CO$_2$. This technique takes advantage of the decarboxylation chemistry as the alpha carbon of αKG is labeled with radioactive $^{14}$C resulting in the release of radioactive $^{14}$CO$_2$ in the presence of a substrate as described in Figure 1.10.\textsuperscript{14}CO$_2$ release can then be quantified as a measure of enzyme activity, which is typically stimulated by binding primary substrate. A strength of this assay is that it is very widely applicable, independent of substrate identity; weaknesses include the low-throughput nature and complications of uncoupled decarboxylation that is prominent in some αKG oxygenases.

![Figure 1.10 αKG decarboxylation assay design. *=14C labelled.](image)

This approached yielded multiple hits from a library of proteins expressed from 1,000 cDNAs.\textsuperscript{65} One target in particular, FOXO3a, was thoroughly characterized for its role in oxygen sensing. Hydroxylation of FOXO3a by PHD2 inhibits binding of the deubiquitinase USP9x which results in proteasomal degradation of FOXO3a. In hypoxia, FOXO3a is not hydroxylated leading to its accumulation in cells and subsequent suppression of the mammary proliferation protein Cyclin D1.\textsuperscript{65}

Biophysical binding assays for new substrates can be quite powerful, provided that the enzyme/substrate binding affinity is high enough to be directly probed. One
type of binding assay used to successfully identify new substrates for PHD is the yeast 2-hybrid assay, which probes the interactions between a fusion protein (ie. Gal4) and a reporter gene. The binding domain of the fusion protein recognizes the unique upstream activator sequence (UAS) in the reporter gene and targets the enzyme-BD fusion protein. Interaction between the enzyme and substrate bring the BD and activation domains (AD) together spurring expression of the reporter gene. A specific example of a yeast 2-hybrid assay design is described in Figure 1.11.

This technique is powerful as it is amenable to high throughput screening of substrate libraries in order to identify substrates which bind to a protein of interest. However, one glaring limitation of the yeast 2-hybrid method is that the assay tests for target binding to the enzyme, but does not test for the ability of enzyme to hydroxylate the substrate. In addition, low affinity binding is challenging to observe, meaning that many substrates may be overlooked. Several HIF hydroxylase binding partners, such as ATF-4, have been identified via MBP pull-down assays; hydroxylation could not be confirmed by MS suggesting that protein-protein binding may involve the non-catalytic domains of the HIF hydroxylase.
Heir and colleagues took a more specific approach to identifying potential substrates of the PHD isozymes using FLAG-tagged coimmunoprecipitation. The EPO receptor (EPOR) was known to interact with VHL during polyubiquitination, suggesting that EPOR may play a role in oxygen homeostasis by an unknown mechanism. Heir hypothesized that one of the PHD isozymes bound to EPOR, leading to an oxygen-dependent modification of EPOR. Flag-tagged gene products which precipitated with a PHD isozyme were pooled as potential substrates for subsequent screening by MS. PHD3, but not PHD1 or PHD2, preferentially precipitated with EPOR-FLAG, suggesting that only the PHD3 isozyme reacted with EPOR. Subsequent MS-detected activity assays confirmed prolyl hydroxylation at two sites in EPOR. While this method would not work for screening pools of potential substrates, it does work well for identifying interaction partners for a specific target.

A very precise method to identify new substrates is through the use of targeted MS based proteomics to detect the increased mass from hydroxylation on a target
analyte. To use this tactic, a specific substrate library of interest must be identified. For the HIF hydroxylases, screening FIH or PHD against consensus sequence or peptides containing ARDs are common approaches. Several groups have screened PHDs against the –LXXLAP- binding motif\(^2,10\) of the PHD substrates.\(^{59,60,68}\) Several new substrates have been identified using targeted libraries. It was shown that subunit 1 of RNA polymerase II (Rbp1) was modified by prolyl hydroxylation. Hydroxylation of Rbp1 led to pVHL binding and subsequent ubiquitination of Rbp1, which regulated the elongation of *TH* gene mRNA.\(^{68}\)

Luo and coworkers applied this technique to the pyruvate kinases isoforms PKM1 and PKM2 and discovered that PKM2 was a substrate for the PHD3 isozyme. PKM2 plays a prominent role in cancer cells contributing to tumorigenesis and altering glucose metabolism; hydroxylated PKM2 exhibited enhanced binding affinity towards HIF-1\(\alpha\) suggesting PKM2 may play a role in oxygen sensing by promoting HIF-1 transactivation.\(^{59}\) Moser and coworkers identified the centrosome protein Cep 192 as a substrate of PHD1 by screening for the –LXXLAP- motif. Here, cell cycle progression is halted based on changes in PHD activity, which can result from oxygen deprivation or metabolic alterations. Hydroxylation of Cep 192 by PHD1 identified a new target which linked cell cycle progression to environmental changes.\(^{60}\)

Since FIH is known to hydroxylate ARDs,\(^{44-47}\) screening for the ARD motif has been a successful method for identifying new FIH substrates, however this is biased towards substrates which contain an ARD. In what can be viewed as a generalizable approach (Figure 1.12), a substrate library based on the ARD sequence was screened for binding to FIH in the presence of enzyme inhibitor. Proteins that could bind to FIH
were trapped, immunoprecipitated (IP), and subsequently identified by MS.\textsuperscript{47,50} Numerous FIH substrates have been identified using this method including Tankyrase-2\textsuperscript{47} and the deubiquitinase ovarian tumor domain containing ubiquitin aldehyde binding protein (OTUB1).\textsuperscript{53} Possible roles of Tankyrase-2 hydroxylation include vesicle trafficking and telomere regulation.\textsuperscript{47} Hydroxylation of OTUB1 promotes its interaction with metabolic proteins; removal of the hydroxylation site alters these interactions suggesting a link between metabolic regulation and oxygen sensing.

![Substrate library + FIH + DMOG](image)

**Figure 1.12** A generalized workflow for identification of novel FIH-ARD interactors by means of substrate trapping with DMOG.\textsuperscript{47}

Combining two methods can increase the scope while searching for new substrates. Quantitative interaction proteomics combines Cockman’s substrate trapping approach with an unbiased search for new substrates, not reliant on screening for a particular consensus sequence or motif.\textsuperscript{52} As before, DMOG “traps” inactive hydroxylase-substrate complexes enriching the pool of hydroxylated proteins from which to screen and increasing the number of bound hydroxylases-substrate complexes. Substrate screening begins by overexpressing the hydroxylase of interest
in cells, then precipitating the hydroxylase along with any proteins bound to it (Figure 1.13). MS followed by label-free quantitation algorithms, such as MaxQuant (MaxLFQ), is then implemented to identify and quantify IP proteins.\textsuperscript{75} The intensity of the MaxLFQ readout correlates to relative protein abundance and changes in protein interaction. Ingenuity pathway analysis is then used to propose the biological significance of new hydroxylase-substrate interactions through mapping precipitated proteins onto various signaling pathways.\textsuperscript{52}

\textbf{Figure 1.13} Schematic illustration of the mass spectrometry-based hydroxylase screen. Cells are transfected with hydroxylase enzyme and treated with the inhibitor DMOG. Hydroxylases (E) were immunoprecipitated (IP) along with any bound protein targets (S), digested, and analysed via liquid chromatography mass spectrometry (LC-MS).\textsuperscript{52}

Rodriguez and coworkers identified two new hydroxylase substrates using this method. The PHD3 isozyme hydroxylates Pro\textsuperscript{25} of MAPK6, stabilizing hydroxylated MAPK6 and protecting this protein from proteasomal degradation. FIH hydroxylates the signaling protein RIPK4, which was shown to regulate RIPK4 kinase-dependent phosphorylation.\textsuperscript{52}

These experiments highlight the successes in identifying new hydroxylase targets to further understand the extent of HIF hydroxylase oxygen sensing activity
within the cell. The development of high resolution mass spectrometry and improved mass accuracy has greatly improved peptide fragment analysis and hydroxylation site assignment, however there is still a need for a more efficient substrate enrichment and screening method for hydroxylation.

1.5 Selected Hypoxia Sensitive Pathways

One of the greatest impacts of oxygen availability on biological pathways is seen in cellular metabolism. As the amount of oxygen in the cell determines the balance between oxidative and glycolytic metabolism, metabolic O₂ consumption must be equilibrated to O₂ supply. Cellular hypoxia induces a cascade of reactions which affect the metabolic pathway: (1) The accumulation of HIF-1α leads to increased transcription of genes that encode glucose transporters and glycolytic enzymes, (2) HIF-1α induces PDK1 which has the effect of suppressing the Krebs cycle and oxidative phosphorylation within the mitochondria. (3) Suppression of the Krebs cycle leads to a shift in mitochondrial glutamine metabolism from oxidative to reductive carboxylation.¹⁴,⁵⁹

HIF-1α mediated alterations of cellular metabolism during hypoxia result in a reduction of mitochondrial O₂ consumption and an increase in O₂ independent ATP production.¹⁴ Key genes induced by HIF-1α during this process include the glucose transporters GLUT1 and GLUT3, LDHA which converts pyruvate to lactate regenerating NAD⁺ for glycolysis, and monocarboxylate transporter 4 (MCT4) which then transports lactate out of the cell. In cancer cells, pyruvate kinase M2 (PKM2) plays a role in reprogramming glucose metabolism. Located in the nucleus, PKM2 stabilizes HIF-1 binding to DNA and helps recruit p300 to form an active transcriptional
complex.\textsuperscript{59} Through this interaction, PKM2 aids in upregulating the genes controlled by HIF-1. There is speculation that PKM2’s interaction with HIF-1\(\alpha\) and cofactor p300 may aid HIF-1\(\alpha\) in avoiding negative regulation by the HIF hydroxylases.\textsuperscript{59}

The impact of HIF-mediated gene regulation on the glycolytic pathway is extensive. In normoxia, pyruvate is converted to acetyl CoA by pyruvate dehydrogenase (PDH) linking glycolysis to the Krebs cycle; however, under hypoxia conditions, pyruvate is converted to lactate.\textsuperscript{76} PDH activity is regulated by two enzymes, the phosphorylating kinase PDK which inactivates PDH and pyruvate dehydrogenase phosphatase which dephosphorylates PDH into its active form. These transcriptional changes result in pyruvate being exported from the mitochondria, instead of being directed to the Krebs cycle, decreasing the efficiency of the electron transport chain. Overall, it is important to recognize that the metabolism of pyruvate drastically changes under hypoxia.

Substantial cross-talk occurs between the oxygen sensing and inflammatory pathways. Both pathways are activated in response to environmental stress and can spur disease states when deregulated. As in the oxygen sensing pathway, the inflammatory pathway is highly regulated through small peptides, glycoproteins, and transcription factors.\textsuperscript{77} The transcription factor NF-\(\kappa\)B is a main player in the inflammatory response. Disregulated NF-\(\kappa\)B is apparent in several disease states including inflammatory bowel disease, asthma, cancer, and HIV.\textsuperscript{77}

Parallel to HIF in the hypoxic response, NF-\(\kappa\)B is one of the main regulators of the inflammatory pathway. HIF and NF-\(\kappa\)B are linked at both the molecular level and in their resulting physiological responses.\textsuperscript{77} Unactivated NF-\(\kappa\)B resides in the cytosol
bound to an inhibitory protein IκB. Stimuli (such as the pro-inflammatory cytokine tumor necrosis factor α) activates the IκB kinase resulting in phosphorylation and degradation of IκB, and allowing NF-κB to translocate to the nucleus where it binds to specific DNA sequences, forming an active transcriptional complex as described in Figure 1.14. At the molecular level, NF-κB works in concert with HIF1β to stabilize HIF1α levels in hypoxia. Furthermore, HIF1α has been shown to suppress NF-κB both in vivo and in vitro during inflammation.

**Figure 1.14** Overview of the canonical NF-κB pathway. Activated in response to stimuli, IKK mediates phosphorylation of IκB, targeting it for degradation. Once IκB is released from NF-κB, NF-κB translocates into the nucleus where it binds DNA to form an active transcriptional complex.

Crosstalk between HIF and NF-κB is evident in the systemic autoimmune disorder rheumatoid arthritis (RA), among other disease states. Hallmarks of RA include chronic inflammation of synovial membranes in joint tissues at multiple sites in the body which results in localized joint destruction and deformity. In the context of
RA, NF-κB, along with several other transcription factors, is deregulated. NF-κB is over-activated in the RA synovium leading to the formation of a positive feedback loop due to NF-κB’s activation of pro-inflammatory cytokines, chemokines, metalloproteins, and metabolic proteins. This enhanced activation of pro-inflammatory signals manifests as chronic and persistent inflammation.

HIF is a known potential therapeutic target for RA as it is responsible for tackling the severe hypoxic state of the synovial tissue. Hypoxia activates HIF1α and HIF2α stimulating angiogenesis and activating pro-inflammatory cytokines respectively. A misregulated hypoxic response to RA gives rise to cellular dysfunction, eventually resulting in cell death. It is important to note that HIF1α is known to repress the NF-κB pathway playing an anti-inflammatory role whereas HIF2α stimulates inflammation. Taking into consideration that NF-κB is an activator of the HIF transcription factors, one might infer that NF-κB may play a role in differentiating the responses of HIF1α and HIF2α in the presence of hypoxia or inflammation, or a combination thereof.

In order to fully decipher the intricate link between the hypoxia and inflammatory pathways, an improved inflammatory model in which both pathways and their respective stimuli can be controlled is necessary. Insight into the regulation of the hypoxia and inflammatory pathways will allow for the development of more successful therapeutic strategies against disease states where hypoxia and inflammation are prominent, such as RA.
1.5.1 Hydrogen Sulfide and Hypoxia

A controversial proposal for hypoxia sensing is that speciation of S between oxidized and reduced pools, most notably, hydrogen sulfide (H$_2$S), directly senses acute hypoxia in many tissues of higher organisms.$^{89,90}$ There are a number of correlations between pO$_2$ and H$_2$S biochemistry, and responses to increased [H$_2$S] resemble those caused by hypoxia.$^{89}$ It has been proposed that the increased speciation of sulfur into reduced pools serves as a signal for decreased pO$_2$, with an unknown signal transduction mechanism leading to a cellular response.$^{91}$

Sulfur speciation between reduced and oxidized pools depends on the availability of O$_2$, correlating hypoxia and elevated [H$_2$S].$^{91}$ Cytosolic metabolism of S-containing compounds produces H$_2$S whereas mitochondrial oxidation of H$_2$S to SSO$_3^{2-}$ and SO$_4^{2-}$ provides a dynamic system responsive to pO$_2$. However, there are other sources of H$_2$S, such as H$_2$S production from glutathione,$^{92}$ and sinks of H$_2$S, such as the consumption of H$_2$S by ROS,$^{93-95}$ that are additional drivers of H$_2$S levels. This interplay between ROS, glutathione, pO$_2$, and [H$_2$S] makes a clear link between hypoxia and H$_2$S elusive.

The transsulfuration pathway is a major source of cellular H$_2$S.$^{93,94,96,97}$ H$_2$S is produced from cysteine, homocysteine, and cystathionine by the cytosolic enzymes cystathione β-synthase (CBS) and cystathione γ-lyase (CSE).$^{98}$ An additional source of H$_2$S from Cys is the path from cysteine aminotransferase (CAT) and mercaptopyruvate sulfotransferase (MST).$^{99}$ Sulfur excretion occurs via the oxidation of H$_2$S to SO$_4^{2-}$ in the mitochondria. The fate of cytosolic H$_2$S is unclear, however correlations between
H₂S and glutathione levels imply that the cytosolic pool of H₂S is fed into the mitochondria for oxidation.

As H₂S is oxidized by enzymes in the mitochondrial matrix, H₂S connects to respiration via the inner membrane quinone pool.⁹⁶ This has led to the proposal that that H₂S oxidation rather than H₂S production is the key step in hypoxia sensing.¹⁰⁰ The enzyme sulfide quino-oxidoreductase (SQR) oxidizes S²⁻ to S⁰ forming a persulfide with glutathione (GSSH).⁹⁶ Subsequent oxidation to sulfite by persulfide dioxygenase (ETHE1)⁹⁶,⁹⁷,¹⁰¹ is a key step, with the terminal oxidation to sulfate or thiosulfate (SSO₃²⁻).

![Oxidation reactions of sulfur species in the mitochondria. Key species include glutathione (GSSH), hydrogen sulfide, sulfite, thiosulfate, and sulfate.](image)

**Figure 1.15** Oxidation reactions of sulfur species in the mitochondria. Key species include glutathione (GSSH), hydrogen sulfide, sulfite, thiosulfate, and sulfate.

The dynamic metabolism of thiols leads to connections between pO₂, H₂S, and SO₄²⁻ with GSH, thiosulfate, and sulfite as enzymatically produced intermediates. As H₂S consumption decreases in bovine tissues when [O₂] drops below ~ 20 μM,⁹¹ acute hypoxia appears to lead to elevated [H₂S]. Notably, the non-enzymatic reduction of thiosulfate by GSH rapidly produces H₂S under hypoxic conditions, suggesting that
this non-enzymatic reaction may play a major role in determining the levels of several sulfur species under acute hypoxia.\textsuperscript{89} Another potential checkpoint which may lead to elevated H\textsubscript{2}S levels under hypoxia is the enzyme ETHE1, as this enzyme consumes stoichiometric O\textsubscript{2} while oxidizing H\textsubscript{2}S to sulfite.\textsuperscript{101} Although the proposal that H\textsubscript{2}S levels report on acute hypoxia is plausible through the above correlations, the molecular basis for transducing the signal of elevated [H\textsubscript{2}S] remains the largest unanswered question for this model of hypoxia sensing.

Exogenously administered H\textsubscript{2}S stimulates a hypoxic response in tissues,\textsuperscript{91} suggesting that the responses to elevated [H\textsubscript{2}S] and hypoxia may be complementary.\textsuperscript{94} However, as mentioned above, the molecular pathways linking H\textsubscript{2}S levels to tissue hypoxia responses have not yet been identified. Although the transcriptional effects of chronic H\textsubscript{2}S in \textit{C. elegans} requires HIF-1, the genes regulated by chronic H\textsubscript{2}S are distinct from those regulated by hypoxia.\textsuperscript{102} It is possible that better discrimination of responses to acute hypoxia from those due to chronic hypoxia would help to clarify the specific effector pathways induced by H\textsubscript{2}S.

1.5.2 \textit{FBXL5}

F-box and leucine-rich repeat protein 5 (FBXL5) has been proposed as an O\textsubscript{2} sensor, due to its O\textsubscript{2}-dependent role in cellular responses to iron ions. A hemerythrin (Hr)-like domain in FBXL5 makes the stability of FBXL5 dependent upon [O\textsubscript{2}], although it is unclear whether or not this protein responds directly to O\textsubscript{2} or to Fe\textsuperscript{2+}. The molecular connection between O\textsubscript{2} and Fe\textsuperscript{2+} arises because FBXL5 binds IRP2 to a ubiquitin ligase, leading to proteasomal degradation of IRP2 in the presence of both Fe\textsuperscript{2+} and O\textsubscript{2}.\textsuperscript{103,104}
FBXL5 contains a Hr-like domain which reversibly binds a diferrous cofactor, similar to that found in typical Hr\textsuperscript{105,106}. Unfolded FBXL5 is in equilibrium with the \([\text{Fe}^{2+}\text{Fe}^{2+}]\) form of FBXL5, which allows for this form of the protein to be degraded by the cell, causing IRP2 to increase in abundance. The FBXL5 cofactor is oxidized in the presence of \(\text{O}_2\) to the tightly-bound \([\text{Fe}^{3+}\text{Fe}^{3+}]\) cofactor, irreversibly stabilizing the folded structure of FBXL5. This oxidized FBXL5 is the form that binds both IRP2 and an ubiquitin ligase, causing ubiquitinylation of IRP2 and subsequent degradation. Although it is not clear whether FBXL5 responds to physiologically-relevant changes in \([\text{O}_2]\), it is clear that this protein connects Fe and \(\text{O}_2\) homeostasis.

1.6 Tissue Signaling

1.6.1 Acute Hypoxia Sensing by Mammalian Tissue

Mammals utilize very distinct sensing strategies for acute hypoxia (on the time scale of minutes) versus chronic hypoxia (on the time scale of hours to days). Acute hypoxia sensing begins at the tissue level with specialized cells, including neuroepithelial bodies (NEBs) and type 1 glomus cells, responding to changes in \(\text{pO}_2\) by opening and closing ion channels to alter the membrane potential of the cells\textsuperscript{107-109}. Cellular hypoxia sensing leads to the fundamental physiological responses of vasodilation and vasoconstriction, which serve to regulate oxygen delivery to tissues by changing vascular tension to modify blood flow. Vasodilation is the dilation of blood vessels and results in increased perfusion of blood to the affected tissue\textsuperscript{110}. Vasoconstriction, which ultimately results in the opposite response as vasodilation, is a regulatory mechanism that constricts blood vessels in the pulmonary vasculature.
allowing the redistribution of blood flow to better ventilated portions of the lung.\textsuperscript{111} Although these acute responses are crucial for tissues experiencing hypoxia and are thought to be mediated within the cell by ion channel signaling pathways, the specific molecular mechanisms linking changes in pO\textsubscript{2} to ion channel activity are not completely understood.

The ability of tissues to sense and respond to changes in oxygen availability has countless biological impacts, such as the protection against ischemic brain injury through hypoxic preconditioning.\textsuperscript{112} Hypoxic preconditioning occurs upon the repetitive short-term exposure of an organism to mild hypoxia, leading to ischemic tolerance and stroke protection in both mice and in a neonatal rat model.\textsuperscript{113,114} It is thought that this preconditioning signals to the organism to prepare for more intense hypoxia. It has been shown that the initial preconditioning occurs through the acute oxygen sensing pathways.\textsuperscript{112} There are three phases of responses to acute hypoxia. The first consists of the immediate chemical responses to hypoxia, which change intracellular ion concentrations,\textsuperscript{115} stabilize HIF-1 due to increased succinate concentration, and remodel the mitochondrial electron-transport chain to favor complex II.\textsuperscript{116} The second phase consists of early biochemical responses to hypoxia which regulate signal transduction, such as the activation of glutamatergic, calcium, phosphoinositide, and cyclic AMP regulatory systems\textsuperscript{115} and modification of ion channels which lead to secondary messengers.\textsuperscript{117} The third phase responses to hypoxia are the regulation of gene expression by transcription factors activated by the secondary messengers.\textsuperscript{112} Many of these pro-adaptive gene products are involved in intracellular plasticity and aid in vital neuronal function and structural integrity.\textsuperscript{112}
Long-term hypoxic tolerance results from chronic hypoxia responses, such as the desensitization of HIF, which serve to protect cells from necrosis, and changes in metabolic homeostasis (Fig. 1.15). In contrast to hypoxic preconditioning, research into hypoxic postconditioning, the exposure of an organism to mild hypoxia after injury, is more disputed due to controversial results and variation in experimental parameters between different groups.

1.6.2 Neuroepithelial Bodies (NEBs) and Type 1 Glomus Cells

The acute hypoxia response is facilitated by specific chemosensory cells termed neuroepithelial bodies (NEBs) and type 1 glomus cells of the carotid body. NEBs line the mucosa of mammalian airways, working to sense $O_2$ and transduce signals to the central nervous system (CNS) in response to different stressors. In addition to

Figure 1.16 Cellular responses to acute versus chronic hypoxia.
sensing changes in pO₂, NEBs also sense hypercarbia (elevated pCO₂), mechanical stretch, and certain stimulants, while type 1 glomus cells also sense pCO₂, pH, temperature, and osmolality. The enzyme NADPH oxidase (Nox) mediates the hypoxic response in both NEBs and type 1 glomus cells through a path linking ROS generation to the CNS.

There are seven isoforms of Nox, including Nox1-5 and Duox1-2, which are expressed in different tissues and cell types with varying expression, localization, and regulation. This distribution suggests a broad and varied response to changes in pO₂. All Nox isoforms produce ROSs, however they do so in different manners; Nox1-3 and Nox5 reduce oxygen to superoxide (O₂⁻) and Nox4 and Duox1-2 directly produce H₂O₂. Most of the isoforms of Nox are regulated post-translationally with certain activators, such as Protein Kinase C (PKC), or inhibitors of Nox turned on or off in response to various stimuli. One isoform that is thought to be mainly transcriptionally regulated, Nox4, was recently found to be negatively regulated via phosphorylation by the tyrosine kinase FYN under conditions of oxidative stress.

In various cell types, Nox has been shown to be regulated by changes in transcriptional levels of Protein Kinase C, which activates Nox.

In NEBs under normal pO₂, Nox rapidly converts superoxide to H₂O₂, which signals O₂-sensitive K⁺ channels to remain open. There is evidence that certain Nox isoforms activate specific types of O₂-sensitive K⁺ channels, such as Nox2 activating the Kv class of K⁺ channels and Nox4 activating TASK channels. Whether the chemical signal to K⁺ channels is O₂⁻ or H₂O₂ is ambiguous as O₂⁻ can dismutate to H₂O₂. When NEBs experience hypoxia, however, the reduced availability of oxygen
results in decreased generation of ROS by Nox and closure of these K⁺ channels. This channel inhibition causes depolarization of the membrane, activating voltage-gated Ca²⁺ channels to increase Ca²⁺ influx and neurotransmitter release (Fig. 1.16).¹²⁸ These neurotransmitters relay information to the CNS to elicit a physiological response to hypoxia.¹²¹ Although activation of Nox by PKC led to hypoxia sensitive K⁺ channel closure in the immortalized model NEB H146 cell line, Nox inhibitors failed to fully suppress this response, suggesting that multiple mechanisms may be utilized by these cells to sense O₂.¹²⁹
Figure 1.17 Comparison of oxygen response in normoxic and hypoxic NEBs. In normoxic cells, high ROS generation by Nox keeps K⁺ channels open. In hypoxic cells, low ROS generation by Nox inhibits K⁺ channels, leading to their closure, membrane depolarization, and Ca²⁺ influx, which triggers neurotransmitter release.

Oxygen sensing by type 1 glomus cells differs subtly from that in NEBs. When type 1 glomus cells experience normal pO₂, oxygen-sensitive K⁺ channels remain open as in NEBs, however the exact molecular mechanism of what activates and inhibits these channels in type 1 glomus cells is not well understood. In type 1 glomus cells experiencing hypoxia, oxygen-sensitive K⁺ channels, such as TASK and maxi-K⁺ channels, close. Neurotransmitters relay information to the carotid-sinus nerve, which then conveys the information to the CNS. In the glomus cells of mice both
deficient of TASK channels and with blocked maxi-K⁺ channels, hypoxia depolarization occurs just as it does in cells with those channels, suggesting that there are multiple subtypes of potassium channels involved in Ca²⁺ entry and neurotransmitter release in the hypoxic response.¹³¹ There is a great dispute as to the role of Nox in oxygen sensing in type 1 glomus cells. Some propose that Nox suppresses K⁺ channel closure, in contrast to its role in NEBs,¹³² while others propose that Nox is not directly involved in type 1 glomus cell oxygen sensing.¹³⁰ The lack of clarity surrounding Nox’s role in type 1 glomus cells suggests that the signaling transduction pathway for oxygen sensing may be complex and tissue specific. In addition to the complexity of this signaling pathway, it has also been hypothesized that instead of the ROSs being the signal for NEBs and type 1 glomus cells, the reduced pyridine nucleotides NADH and NADPH are in fact the signal that triggers K⁺ channel closure and eventual neurotransmitter release,¹³³ however this hypothesis has not been rigorously tested in cells. This follows the broader theme of interrelatedness between metabolic status and O₂ abundance.

1.6.3 Smooth Muscle Cells (SMCs)

Smooth muscle cells (SMCs) work in concert with NEBs and type 1 glomus cells to facilitate tissue-specific responses to acute hypoxia. In pulmonary artery SMCs, acute hypoxia closes K⁺ channels, causing membrane depolarization and Ca²⁺ influx.¹¹¹,¹³⁴ This causes muscle cell contraction and results in vasoconstriction, which results in the redistribution of blood flow to better ventilated portions of the lung.¹¹¹ In systemic SMCs, acute hypoxia causes ATP-sensitive K⁺ (K_ATP) channels to open and hyperpolarize the membrane, inhibiting Ca²⁺ influx.¹³,¹³⁵ This causes muscle cell
relaxation and vasodilation to increase perfusion of blood to the affected tissue.\textsuperscript{110} Recent findings implicate transient NO as a regulator of both vasodilation and vasocontraction, however the exact mechanism is not well understood.\textsuperscript{110,111} The most convincing argument links the regulation of vasodilation by NO to S-nitrosohemoglobin-dependent bioactivity in red blood cells.\textsuperscript{136}

Both vasoconstriction and vasodilation work together to regulate oxygen delivery to tissues by changing vascular tension to modify blood flow depending on the affected tissue.\textsuperscript{137} Although they have opposite effects, both result from changes in membrane channels that cause myocyte contraction or relaxation.\textsuperscript{111,138} In this manner, adaptation to acute hypoxia relies on a carefully orchestrated response that involves neuroepithelial bodies, type 1 glomus cells, and smooth muscle cells. The cellular machinery that compose the acute hypoxic response are just now emerging and understanding of the molecular details awaits further research.

1.7 Conclusions

Oxygen sensing in mammals occurs over multiple scales, ranging from the molecular-level sensing proposed for reduced sulfur species to the well-established chemical sensing by the HIF hydroxylases, leading to well-established tissue-level responses. The most exciting areas involve discovery of new targets for the established $O_2$-sensing enzymes, and new enzymes that may sense $O_2$. Identifying new targets for FIH and the PHD isozymes would uncover pathways that can lead to cellular changes independent of the HIF transcriptional response. New enzymes would similarly add richness to the $O_2$-dependent responses of cells and tissues. A further complexity is
the interplay between $O_2$ levels, metabolism, and stress responses, which simply underscores the richness of the field.
1.8 References


CHAPTER 2

LIGAND SUBSTITUTION SUPPORTS O₂ ACTIVATION IN THE FACIAL TRIAD VARIANTS OF FIH, AN αKG DEPENDENT OXYGENASE

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2.1 Introduction

The αKG dependent oxygenases functionalize a wide variety of substrates using a non-heme Fe(II) coordinated by a His₂(Asp/Glu) facial triad. The consensus mechanism of αKG dependent oxygenases is proposed to follow the HAG mechanism involving H-atom transfer to form a substrate radical, with rebound of an Fe-bound ligand (Scheme 2.1). Typical enzymes in this superfamily hydroxylate substrates by the transfer of an O-atom from O₂, whereas a subset of αKG oxygenases perform halogenation chemistry (Scheme 2.1). As seen with both the hydroxylases and halogenases, the presence of the organic substrate (R-H) is necessary to trigger O₂ activation leading to the formation of an Fe(IV)=O intermediate, giving rise to subsequent rebound chemistry. Gaining insight into the structural factors needed to trigger O₂ activation are of paramount interest to the field.
Scheme 2.1 Mechanism of Fe(II)/αKG hydroxylases and Fe(II)/αKG halogenases. R = substrate; for hydroxylases X = Asp/Glu; for halogenases X = halide.

The resting enzyme form for hydroxylases contains a ferrous ion coordinated by a His$_2$(Asp/Glu) facial triad, a bidentate αKG, and an aquo ligand. In contrast to the hydroxylases, halogenases naturally have a His$_2$(Ala/Gly) motif, but bind a halide ion to support halogenase chemistry. The crystal structure of chloride-bound SyrB2 provided the first evidence of a halide directly coordinated to the metal in this family of enzymes, supporting earlier studies with model complexes which indicated that metal-bound ligands were transferred in non-heme Fe mediated halogenation. While XAS studies of CytC3 and SyrB2 further confirmed the presence of a Fe-bound halide in these halogenases, bound chloride was not observed crystallographically, suggesting that a protein conformational change was needed to create the chloride binding site.

Several lines of inquiry have shown that a Cl$^-$ ligand is essential for the rebound step in halogenases to form halogenated product, but it remains unclear whether a complete His$_2$X facial triad is essential for the initial step of O$_2$ activation. Electronic structure calculations of a model halogenase cofactor predicted a modest kinetic barrier for OH rebound, whereas Cl rebound would be barrier-less, suggesting a kinetic advantage for halogenation. Reports demonstrate the potential to interconvert hydroxylases and
halogenases through changing the disposition of substrate relative to the hydroxide and halide ligands.\textsuperscript{15-17} While the above demonstrate that the Cl\textsuperscript{−} ligand is essential for rebound chemistry, it is not clear whether or not a complete facial triad would be necessary for the preceding step of O\textsubscript{2} activation. As hydroxylation does not involve the facial triad ligands in an obvious manner, hydroxylating enzymes with incomplete His\textsubscript{2}X facial triads are of interest,\textsuperscript{18-21} as these examples suggest that a complete facial triad is not necessary for O\textsubscript{2} activation chemistry.

FIH is an αKG dependent oxygenase which hydroxylates the sidechain of Asn803 within the C-terminal transactivation domain (CTAD) of the hypoxia inducible factor (HIF-1\textalpha). While the Fe cofactor of WT FIH has the His\textsubscript{2}Asp facial triad, prior reports indicated that D201A/E/G variants of FIH supported intriguing O\textsubscript{2} activation and hydroxylation chemistry making these variants excellent starting points to test the link between His\textsubscript{2}X facial triads and O\textsubscript{2} activation. Here, we demonstrate that exogenous anion binding to form a complete His\textsubscript{2}Cl facial triad is both selective and essential for the D201G variant of FIH to support O\textsubscript{2} activation chemistry. We also provide metrical details of the Fe-Cl cofactor in the D201G variant, revealing an excellent mimic of the halogenase geometry found in SyrB2. These results indicate that a complete His\textsubscript{2}X facial triad is essential for O\textsubscript{2} activation, and suggest that an elaborate halide binding pocket is not needed to replicate the chloroferrous cofactor found in halogenases.
2.2 Methods

2.2.1 Materials

All materials, except for the synthetic peptide CTAD and the FIH variants, were purchased from commercial vendors and used without further purification. CTAD was purchased from EZBiolab (Carmel, IN, USA) as desalted peptide and purified using RP-HPLC to >95% purity. The CTAD peptide sequence, DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN, corresponded to that of the C-terminal activation domain of human HIF-1α(788-826) with the Asn803 residue targeted by FIH in bold, and a Cys800 → Ala to prevent unwanted oxidation reactions.

2.2.2 Protein expression and purification

WT-FIH and the D201A/E/G variants were overexpressed in Escherichia coli with an N-terminal His6 tag. Following lysis of the cell pellet using sonication, WT-FIH and each variant were found in the soluble fraction. Centrifugation of the cell lysate (14,000 x g, 30 minutes) pelleted the cellular debris and the supernatant was buffer exchanged into 10 mM Tris pH 8.00 to remove the EDTA from the lysis buffer. The supernatant was centrifuged before being loaded onto a Ni-NTA column. The column was washed with 5 column volumes each of 100% A buffer (50 mM Tris pH 8.0, 300 mM NaCl, 15 mM Imidazole) and 15% B buffer (50 mM Tris pH 8.0, 300 mM NaCl, 250 mM Imidazole) to remove contaminating proteins before eluting the His6-FIH protein with 100% B buffer. The subsequent thrombin cleavage of the His6 tag was performed in thrombin cleavage buffer for 36 hrs at 4 °C and resulted in >80% cleavage. The Ni-NTA column was used to remove the His6 tag as well as any remaining uncleaved His6-FIH. Overnight incubation
with EDTA (50mM) removed exogenous metals. The FIH dimer was obtained following size exclusion chromatography and the purity (>95%) was assessed by SDS-PAGE.

2.2.3 Activity Assays

Activity assays used to screen for the halide dependent hydroxylation of CTAD contained αKG (100 µM), FeSO₄ (25 µM), CTAD (100 µM), ascorbic acid (2 mM), WT FIH or an D201 variant (0.5–2.5 µM) and NaX (X = F, Cl, Br or I; 100 mM) in 50 mM HEPES pH 7.00. Similarly, the reaction buffer for activity assays that screened for the pseudo halide dependent hydroxylation of CTAD contained αKG (100 µM), FeSO₄ (25 µM), CTAD (100 µM) ascorbic acid (2 mM), FIH (0.5–2.5 µM) and the sodium salt of the pseudo halide (2 mM) in 50 mM HEPES pH 7.00. All reactions were initiated by the addition of enzyme and were performed at 37.0 °C.

To determine the enzyme activity, reaction mixture aliquots (5 µL) were quenched in 75% acetonitrile/0.2% TFA saturated with 3,5-dimethoxy-4-hydroxycinnamic acid (20 µL) over a predefined time course spanning several minutes. Each quenched aliquot was analyzed for formation of both peptide hydroxylation and (pseudo)-halogenation products using a Bruker MicroFlexII MALDI-TOF-MS (Supplemental Figs. 2.5 and 2.6). Initial rates were calculated based on the rate of product formation (Supplemental Fig. 2.7).

2.2.4 O₂ Consumption Assays

The rate of oxygen consumption was monitored using a Clark-type electrochemical O₂ sensor. Each day, a new membrane was prepared and the electrode was calibrated using air saturated water and dithionite. Ascorbate (50 µM), αKG (100 µM), FeSO₄ (25 µM), and CTAD (80 µM) in 50 mM HEPES pH 7.00 were equilibrated in the reaction vessel at
37.0 °C until a stable baseline was achieved. The reaction was then initiated with cold enzyme (1–10 µM) using a gas-tight Hamilton syringe. O₂ consumption over time was recorded until the rate of O₂ consumption approached the baseline slope.

2.2.5 UV-Vis absorption spectroscopy

All reagents were prepared anaerobically using multiple cycles of evacuation and N₂ flush. Samples consisting of FIH (400 µM), FeSO₄ (375 µM) and αKG (400 µM) in 50 mM HEPES pH 7.00 at 23 °C containing either a sodium salt halide (100 mM) or pseudohalide (2 mM) were prepared in an anaerobic glove box. UV-Vis spectra of each sample were recorded anaerobically using an Avantes UV-Vis spectrometer with an integration time of 25 ms and averaging of 25 spectra. The spectrum of (Fe)FIH was subtracted from the spectrum of (Fe+αKG)FIH to produce the reported spectra.

2.2.6 X-ray Absorption Spectroscopy

A sample of 1 mM FeSO₄ was prepared in 50 mM HEPES, pH 7.00, 100 mM NaCl and metal concentration was determined using a Perkin Elmer Optima DV4300 ICP-OES instrument. This metal sample was used to reconstitute the D201G variant of FIH anaerobically with FeSO₄ (0.9 eq.), N-oxalylglycine (NOG) (1.0 eq.), and excess NaCl (100 eq.) in 50 mM HEPES, pH 7.00.

Data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beam line 9-3 using a 20 element Ge x-ray fluorescence detector on samples containing 1 mM protein held at liquid He temperature. Beamline set-up and ring conditions are as previously described²² and are further detailed in the Supplemental Information (section 2.5). Internal energy calibration was performed by collecting spectra simultaneously in
transmission mode on Fe foil to determine the first inflection point on the edge, which was set to 7111.2 eV.

XANES analysis was performed using OriginPro 2015 to fit the edge background and pre-edge features as pseudo-Voigt peaks, as previously described. These features were analyzed assuming $O_h$ local symmetry for the Fe site. Data processing and analysis using the SixPack, Artemis, and FEFF6 software packages has been previously described and further information regarding it is available in the Supplemental Information (section 2.5). Assessment of the goodness of fit was performed as previously described.

Reported data sets were fit with separate sets of $\Delta r_{\text{eff}} (r - r_{\text{eff}})$ or the change in metal-ligand bond distance with respect to the input path distance, and $\sigma^2$ for the Cl and nitrogen-donors including imidazole rings with initial values of 0.0 Å$^2$ and 0.003 Å$^2$, respectively. Each fit was initiated with a universal $E_0$ (7125 eV for Fe) and $\Delta E_0 = 0$ eV, which was allowed to vary for each fit. Histidine ligands were fit as geometrically rigid imidazole rings. Multiple-scattering parameters for imidazole ligands bound to Fe were generated using the FEFF6 software package with the imidazole input obtained from average bond lengths and angles gathered from crystallographic data, as previously described. All multiple scattering paths with path lengths outside the fitting window ($r = 1 – 4$ Å) were discarded. Histidine ‘counting’ was performed by adding integer numbers of histidine imidazole ligands to fits and assessing the effect on the $R$-factor and reduced $\chi^2$. NOG was modeled as a geometrically rigid molecule in an identical manner to the histidines.

2.2.7 Metal Titrations

The $K_d$ of metal binding to FIH was measured by intrinsic tryptophan fluorescence quenching using a PTI fluorimeter. Samples containing 20 µM FIH, 500 µM αKG, and 1
mM sodium citrate in 50 mM HEPES pH 7.00 were prepared in a fluorescence cuvette. Sodium citrate was used to buffer the free metal in solution. The titrating solution contained 1 mM CoSO₄ and 1 mM sodium citrate in 50 mM HEPES pH 7.00. Co(II) was used as an Fe(II) mimic to avoid auto-oxidation. Increasing amounts of metal were added to the cuvette solution and incubated for 7 minutes at room temperature while stirring. Following incubation, samples were excited at 285 nm with emission monitored from 300-400 nm. The intensity of the fluorescence peak at 330 nm after each metal addition was used to determine the relative fluorescence intensity, which was fit to the following equation to determine the $K_d$(Co) binding for each protein variant.32

$$y = y_{min} + \frac{y_{max} - y_{min}}{1 + 10^{(x-(log K_d)B)}}$$

The stability constant of the free Co$^{2+}$ ion, [Co(H₂O)₆]$^{2+}$ in citrate was used to calculate the buffered concentration of free Co$^{2+}$ in solution.33

### 2.3 Results and Discussion

The reactivity of WT FIH and D201A/E/G variants were previously compared to test the impact of altering the His₂Asp facial triad carboxylate on FIH, however those experiments used Tris/HCl buffer20 or HEPES with 100 mM NaCl to control ionic strength.21 When preparing protein samples for structural analysis by XAS, we observed that initial activity screens of the D201G variant in HEPES buffer showed batch-to-batch variation in the rate for hydroxylating the CTAD peptide. We subsequently found that prolonged dialysis into chloride-free HEPES buffer led to a protein that had a low rate for
CTAD\textsuperscript{OH} formation, suggesting that chloride was essential for O\textsubscript{2} activation by this variant. This raised the potential for Cl\textsuperscript{−} as an essential exogenous anion to complete a His\textsubscript{2}Cl facial triad in this variant, and for anion-selective rescue of activity for the other enzyme variants. This was intriguing as anion rescue failed in two αKG-dependent peptidyl hydroxylases\textsuperscript{18, 20} and limited rescue of activity was observed in αKG-dependent hydroxylases utilizing smaller substrates\textsuperscript{16, 19, 34}.

2.3.1 Anion dependent oxygen activation

FIH variants were screened for anion selective hydroxylation of the CTAD peptide, which was used in place of the full-length HIF-1α protein as a substrate for FIH\textsuperscript{31, 35}. Each FIH variant was exhaustively dialyzed into 50 mM HEPES/NaOH, pH 7.00 to remove loosely associated anions from the apo-protein sample, then tested for activity at 37.0 °C. Reactions were monitored over several minutes by MALDI-TOF-MS in order to detect the +16 mass gain due to peptide hydroxylation.
Figure 2.1 Activity screens of WT FIH, D201E, D201G and D201A in the presence of sodium halides (100 mM). (A) The initial rate for CTAD$^{\text{OH}}$ formation as measured by MALDI-TOF. (B) O$_2$ consumption of D201G in the presence of NaX salts.

MALDI analysis revealed that all variants produced the hydroxylated CTAD peptide (CTAD$^{\text{OH}}$) albeit with widely different rates for product formation (Fig. 2.1A). As we used MALDI for product analysis, we could observe whether products contained an additional O-atom (+16) or halogen atom (eg: replacing an H with a Cl would increase mass by +34.5 and +36.5); the only product observed was the +16 product corresponding to peptide hydroxylation (Supplemental Figure 2.5). WT-FIH exhibited robust hydroxylation activity under all tested conditions (Fig. 2.1A); notably, NaX was not required for activity; the diminution of activity in the presence of NaI was attributed to
partial quenching of the ferryl intermediate by iodide. Notably, the conservative D201E variant, which bound the Fe by a His\textsubscript{2}Glu facial triad, exhibited anion independent hydroxylation (Fig. 2.1A) albeit at roughly 10% the rate of WT-FIH.\textsuperscript{21}

Both of the variants containing a nonpolar sidechain at position 201, D201A/G, exhibited anion selective hydroxylation activity with contrasting anion requirements (Fig. 2.1A). The D201G variant was nearly as active as WT-FIH toward hydroxylating CTAD, provided that NaCl was present. The D201A variant was prepared to mimic the ligands found in αKG-dependent halogenases such as SyrB2, however this variant hydroxylated CTAD only in the presence of F\textsuperscript{−}. The anion-selectivity indicated that an exogenous anion was essential for substrate hydroxylation, implying that a complete His\textsubscript{2}X facial triad formed in these enzyme variants. This further suggested a complementarity between the size of the anion and the bulk of the non-coordinating D201A/G sidechain.

As forming CTAD\textsuperscript{OH} requires successful completion of both half reactions, it was possible that the exogenous anion was needed for either the initial O\textsubscript{2} activation, or for the subsequent peptide hydroxylation. To gain insight into which step in the consensus mechanism was influenced by the anion, O\textsubscript{2} consumption by the D201G variant was assessed using a Clark-type O\textsubscript{2} sensor. The O\textsubscript{2} sensor is an electrochemical cell, requiring KCl electrolyte under an O\textsubscript{2}-permeable membrane, which separates the enzyme reaction from the electrode. The rate of O\textsubscript{2} consumption by D201G was significantly enhanced by the addition of chloride to the assay buffer (Fig. 1B) which showed that the chloride anion stimulated the O\textsubscript{2} consumption step. Relative rates of O\textsubscript{2} consumption in the presence of other NaX salts were consistent with the relative rates of CTAD hydroxylation, indicating anion binding was essential for the initial O\textsubscript{2} activation half-reaction.
The αKG dependent halogenase, SyrB2, supported incorporation of alternate anions into the product, including Cl\(^-\), N\(_3\)\(^-\), and NO\(_2\)\(^-\),\(^{34}\) suggesting that richer chemistry may be possible with varied exogenous ligands in enzymes with a His\(_2\)X facial triad. The anion-selective FIH variants D201A/G were screened for anion incorporation using the sodium salts of several mono-anions including pseudo-halides, using MALDI-TOF analysis to identify the product masses. Only two of the tested anions supported hydroxylation activity above a basal level to form CTAD\(^{OH}\), but none of the tested anions were incorporated into the product (Fig. 2.2A). The D201G variant had significant hydroxylation activity for \(X^- = N_3^-\) and interestingly, both the D201G and D201A variant showed near WT activity when \(X^- = OCN^-\). The higher activity with OCN\(^-\) may reflect the increased charge asymmetry of OCN\(^-\) relative to N\(_3\)\(^-\).

There have been very few examples of exogenous ligands binding to and rescuing activity in αKG-dependent oxygenases. The most prominent example of exogenous anion binding to the Fe cofactor is from the αKG-dependent halogenase SyrB2, which showed tolerance for a variety of anions to bind in place of the natural Cl\(^-\) anion.\(^{34}\) However, SyrB2 naturally binds Cl\(^-\) to form a His\(_2\)Cl facial triad, and is therefore likely to tolerate other ligands. On the other hand, rescuing activity of a natural hydroxylase following mutation of the Asp/Glu facial triad ligand is rare. The D201A variant of taurine dioxygenase (TauD) exhibited a trace level of turnover as isolated, which was increased by binding formate implying that formate bound to the Fe. However, the overall rate of turnover remained low (<< 1%) relative to WT-TauD.\(^{19}\) Facial triad variants of Prolyl-4-hydroxlyase were inactive and could not be rescued by addition of exogenous ligands.\(^{18}\) The single success was the N-substituted l-amino acid dioxygenase (SadA), which
normally forms hydroxylated products. The D157G variant of SadA was able to both bind Cl⁻/Br⁻ as well as perform alternate rebound chemistry, forming a mixture of products containing an R-OH and R-X bonds. Although structural data for this SadA variant was not presented, the chemical competence and requirement of X⁻ clearly implicated formation of a complete His₂X facial triad.

2.3.2 Anion binding to the D201G variant

Anion binding to the facial-triad variants was monitored by UV-Vis absorption spectroscopy in an anaerobic glovebox using a diode-array spectrophotometer. D201A/E/G variants reconstituted with Fe(II) and αKG exhibited modest shifts in the MLCT band from that of WT-FIH ($\lambda_{\text{max}} = 500$ nm), with the largest shift observed for the D201G variant ($\lambda_{\text{max}} = 485$ nm) (Supplemental Fig. 2.8). These electronic transitions indicated that there were modest changes in the orbital energies of the Fe(II) + αKG chromophore due to removal of the facial triad D201 ligand. The MLCT transitions shifted by less than 5 nm upon addition of 100 mM NaCl to the FIH variants presumably due to displacement of an aquo ligand, as similarly small shifts were reported for Cl⁻ binding to the αKG-dependent halogenase SyrB2, reflecting the comparable positions in the spectrochemical series for H₂O and Cl⁻ ligands. Addition of OCN⁻ or N₃⁻ to the D201G variant red-shifted the MLCT transition by roughly 15 nm, indicating that these pseudo halide ligands bound to the Fe(II), forming a His₂X facial triad within the FIH active site (Fig. 2.2B).
Figure 2.2  (A) Activity screens of WT FIH, D201E, D201G and D201A in the presence of pseudo halides (2mM). Each reaction contained αKG (100 µM), FeSO₄ (25 µM), CTAD (100 µM), and ascorbic acid (2 mM). The rate for CTAD hydroxylation is denoted. (B) Anaerobic UV-Vis spectra of the MLCT region for the pseudo halides (2 mM) in complex with (Fe⁺ αKG)FIH variants (400 µM) constituted with FeSO₄ (375 µM) and αKG (400 µM) in 50mM HEPES pH 7.00.

2.3.3 X-ray spectroscopy of (Fe⁺NOG+Cl)D201G

Definitive structural data for the His₂Cl facial triad was obtained by Fe K-edge X-ray absorption spectroscopy on the D201G variant of FIH constituted anaerobically with FeSO₄ (1.0 eq.), N-oxalyl-glycine (NOG, 0.9 eq.), and excess NaCl (100 eq.) in 50 mM
HEPES, pH 7.00. NOG is isostructural to αKG, and has been used in several crystallographic studies of FIH to prevent O₂ activation. The X-ray absorption edge energy reflects the electron density around the metal center, and can indicate the metal oxidation state when compared to similar coordination environments. The edge energy (7123.3 eV) fell between that observed for Fe(III)WT-FIH (7125.0 eV)³¹ and that observed for the Fe(II) centers in SyrB2 (7120.9 eV)¹³ and HIF-prolyl hydroxylase (7121.2 eV)³⁷ suggesting that the present FIH sample corresponded to the ferrous state.

X-ray absorption near-edge structure (XANES) analysis was used to determine the coordination number and geometry of the metal center. Metal ions such as Fe(II) that have vacancies in the 3d orbitals and exhibit pre-edge features involving 1s→3d electronic transitions, the number and intensity of which vary with coordination number and geometry. The pre-edge data for FIH reveals partially resolved features arising from multiple excited states.²³ Three excited states are expected to arise from the ⁵T₂g ground state of O_h high-spin Fe(II) centers (⁴T₁g, ⁴T₂g, and ⁴T₁g) and can generally be fit to the data between 7111 and 7114 eV. In the case of FIH, only two peaks are required to fit the data, similar to the situation for solid state FeI₂, where transitions to the lower energy excited states are not resolved. These two peaks are observed at 7111.1 and 7113.0 eV and have areas of 5.0 x 10⁻² eV and 0.9 x 10⁻² eV, respectively, giving a total pre-edge peak area of 5.9 x 10⁻² eV, which is somewhat greater than typically observed for O_h high-spin Fe(II) (3.1 – 4.4 x 10⁻² eV), possibly reflecting a lowering of the symmetry that would allow for a small amount of 3d-4p mixing. The peak intensity ratio (area of lowest energy peak/total area) is 0.83, and is similar to that observed for high-spin model Fe(II) complexes (0.72 – 0.85), as is the energy difference between the resolved peaks (1.9 eV; 1.8 – 2.4 eV in
The results of the XANES analysis are consistent with a 6-coordinate distorted $O_h$ Fe(II) center, as expected for the His$_2$Cl facial triad, a bidentate NOG, and a coordinated aquo ligand.

Extended x-ray absorption fine structure (EXAFS) analysis was used to measure the distance to Fe-bonded atoms with a precision of $\sim \pm 0.02$ Å. EXAFS also provides information on the atomic number of scattering atoms, readily distinguishing period 2 (N/O) ligands from period 3 (S/Cl) ligands, as well as a second estimate on the coordination number. The EXAFS data were fit using progressively more detailed models to arrive at a...
best fit. The data were first fit with a single shell of N donor atoms with coordination numbers ranging from 4 - 6 followed by a fit with a single shell of S-donor atoms with coordination number between 4 - 6. Neither single shell fit generated a fit with a % R-value below 5%. The data were then fit with a mixed shell of N and S donors, with all possible combinations of N and S donors at various distances with coordination number 6. This improved the fit, and the best fit (N: S), %R = 6.01 is shown in Supplemental Table 2.4. Considering the scattering from only the primary ligands in the mixed shell fit did not result in a fit with a % R-value of < 5%, scattering from two rigid imidazole rings for His199 and His279 was then included. The best EXAFS fits obtained for the D201G data are six-coordinate, consistent with the XANES analysis, and feature one S/Cl-donor ligand, three N/O-donors and two imidazole rings (%R = 3.0). However, this fit did not accurately portray the sample composition as NOG is a bidentate ligand with rigid atoms that were likely to contribute to scattering. NOG was subsequently included in the model, reducing the %R to 2.4% for the best fit (Table 2.1). The best fit of the EXAFS corresponded to an Fe(His)2(O/N)3Cl center, in agreement with the pseudo-octahedral geometry shown by XANES. Notably, it was not possible to obtain an acceptable fit (R < 5%) without including a Cl scattering atom in the model (Supplemental Table 2.4).

### Table 2.1 Best fit to EXAFS data for the D201G variant of FIH.

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<th>n</th>
<th>Radius Å</th>
<th>σ² (x10⁻³ Å²)</th>
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<td>3(3)</td>
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<td>3(3)</td>
<td></td>
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<tr>
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<td>2.01(9)</td>
<td>6(6)</td>
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</tr>
</tbody>
</table>
The Fe-Cl distance of the D201G variant (2.36 Å) compares well with Fe-Cl distances found in the αKG-dependent halogenases SyrB2\textsuperscript{13} (2.31 Å, EXAFS), and WelO5\textsuperscript{16} (2.1 – 2.3 Å, crystallography) indicating that the D201G variant of FIH replicated the structure of the halogenase cofactor (Table 2.2). Further, as the O\textsubscript{2} activation chemistry (Fig. 2.1) and the other metrical details match the halogenase cofactors (Table 2.2), the D201G variant is an excellent mimic of the halogenase structures.

<table>
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<td>1.95 – 2.11</td>
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</table>


\textsuperscript{d}. Present work

\textit{2.3.4 Metal binding in D201A/E/G variants}

The presence of an Fe-Cl bond in the D201G variant raised the question of whether mutations at this site impair metal binding and if the anionic ligand would increase metal
binding affinity. Comparison of crystal structures of D201G (Zn bound), D201A (Fe bound) and WT FIH showed that the polar contacts from the D201 were lost in the variant proteins, but that the metal retained pseudo-octahedral geometry in the active site by binding bicarbonate, implying that the aspartate ligand was not required for metal binding. Additional crystallographic studies with D201E (Zn and Fe bound structures) confirmed this conservative mutation retained polar contacts from the facial triad and the ability to bind metal. Previous studies conducted by Hangasky, et al. demonstrated the binding of αKG to Fe(II) by the D201A/E/G variants using UV-Vis absorption spectroscopy to observe the expected Fe$^{2+}$(t$_{2g}$) $\rightarrow$ αKG($\pi^*$) MLCT of the (Fe+αKG)FIH chromophore. While the above indicates that the M+αKG can bind to each of the D201A/E/G variants, the binding affinity of these variants has not been reported.

Intrinsic tryptophan fluorescence quenching was monitored using a PTI fluorimeter to determine the equilibrium dissociation constant (K$_d$) of metal binding to the D201A/E/G variants in the presence of a large excess of αKG (Fig. 2.4A). Co(II) was used as an Fe(II) mimic in order to conduct the assays aerobically, as was done previously for WT FIH. Samples were excited at 285 nm and emission was monitored at 330 nm as increasing amounts of Co(II) were titrated into the protein solution. We observed that the K$_d$(Co) for WT FIH (K$_d$ = 2.0(2) x 10$^{-6}$ M) increased by 80-fold in the D201A variant (Table 2.3). This difference in K$_d$(Co) is much greater than the 6-fold increase in K$_d$ seen between the αKG dependent oxygenase TfdA and its D116G active site variant. The lower metal binding affinity for the D201A variant suggests that the Ala substitution sterically impinges on the metal center, and may explain the inability of this variant to hydroxylate CTAD.
Figure 2.4. $K_d$(Co) determination based on intrinsic tryptophan fluorescence quenching for (A) D201A (green), D201E (blue), D201G (red) and WT FIH (black). (B) The D201G sample with (red) 100 mM NaCl in the assay buffer and without (black). Assays contained the following: 20 µM FIH, 500 µM αKG, and 10 mM sodium citrate in 50 mM HEPES pH 7.0. The titrating solution was composed of 1 mM CoSO$_4$, and 1 mM sodium citrate or 10 mM CoSO$_4$, and 10 mM sodium citrate in 50 mM HEPES pH 7.0.
<table>
<thead>
<tr>
<th>Table 2.3</th>
<th>(K_d(\text{Co})) values.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(K_d(\text{Co}), \times 10^{-6} \text{ M})</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>D201A</td>
<td>157 ± 30</td>
</tr>
<tr>
<td>D201E</td>
<td>5.5 ± 0.8</td>
</tr>
<tr>
<td>D201G</td>
<td>4.5 ± 0.7</td>
</tr>
</tbody>
</table>

We observed that the metal binding affinity for the D201E and D201G variants were very similar to that of WT FIH, varying by less than three-fold (Table 2.3). This suggests that these variants likely retain pseudo-octahedral geometry about the bound metal ion. Further, the high metal binding affinity for these variants is consistent with their competence to hydroxylate CTAD. Unexpectedly, addition of chloride to the D201G variant had no effect on the \(K_d(\text{Co})\) (Fig. 2.4B). This Co(II) titration was repeated for D201G with both CoSO\(_4\) and Co(ClO\(_4\))\(_2\) (Supplemental Fig. 2.9) to test the possibility that the sulfate group was binding in place of the Cl\(^-\) anion in the chloride-free sample. The reproducibility of the \(K_d(\text{Co})\) for the D201G variant implies that exogenous ligand binding to form a complete His\(_2\)X facial triad is essential for catalytic activity of the variants, but not metal binding.

### 2.4 Conclusion

The D201G variant of FIH requires a His\(_2\)Cl triad to activate \(\text{O}_2\), as shown by the low catalytic activity in the absence of Cl\(^-\). This is of crucial importance to the \(\alpha\text{KG}\) dependent oxygenase family of enzymes, as it suggests larger role for a complete His\(_2\)X
facial triad in the chemical steps. Previous studies of this enzyme family have identified the requirement of a Cl\(^-\) anion in the active site to form halogenated product, however the role of the His\(_2\)X facial triad in O\(_2\) activation was not explored. The data presented here provide evidence that a full His\(_2\)X triad is an absolute requirement for O\(_2\) activation in the αKG oxygenases. The XAS metrics for the Fe-Cl bond provide insight into the requirements for the His\(_2\)Cl facial triad structure found in halogenases. This study further indicates that an extended anion binding site is not needed to form an Fe-Cl center, as suggested from crystallography of CytC\(_3\).\(^{12-13}\) Overall, this work advances knowledge of the requirements for anion binding to trigger O\(_2\) reactivity, and role of the His\(_2\)Cl facial triad structure in the αKG dependent oxygenases.

### 2.5 Supplemental Information

#### 2.5.1 Site directed mutagenesis

The primers used to mutate the pET28a-WT FIH construct are found below. Each mutated plasmid was sequenced (Genewiz, NJ, USA) to ensure only the desired mutation was present before being transformed into BL21 (DE3) competent cells for protein expression.

**D201A**

forward, 5’-GACACCTGCTCCTATGCTGAGCAGCAGAACTTTT-3’; reverse, 5’-AAAAGTTCTGCTGCTCAGCATAGTGAGCAGGTGTC-3’.

**D201G**

forward, 5’-GTGACACCTGCTACTATGGCGAGCAGCAGAATTTTTT-3’; reverse, 5’-GTTCTGCTGCTGCTCAGCATAGTGAGCAGGTGTCAC-3’. 
forward, 5’-GACACCTGCTCACTATGAGGAGCAGCAGAACTTTTTT-3’;
reverse, 5’-AAAAAAGTTCTGCTGCTCCTCATAGTGAGCAGGTGTC-3’.

2.5.2 Activity assays

Figure 2.5 MALDI-TOF spectra of quenched FIH reaction aliquots showing an increase in CTADOH. [CTAD+H]$^+$ $^{obs} = 4256$ m/z ([CTAD+H]$^+$ $^{calc} = 4254$ m/z), [CTADOH+H]$^+$ $^{obs} = 4272$ m/z ([CTADOH+H]$^+$ $^{calc} = 4270$ m/z) [CTAD+Na]$^+$ $^{obs} = 4278$ m/z ([CTAD+Na]$^+$ $^{calc} = 4276$ m/z).
Figure 2.6 Zoomed-in MALDI spectra. Peaks corresponding to the chlorination of CTAD (in a 3:1 ratio of $^{35}$Cl and $^{37}$Cl isotopes), $[\text{CTAD}^{\text{Cl}+\text{H}}]^{+}_{\text{calc}} = 4288$ and $4290$ m/z, are not observed. 4278 m/z = $[\text{CTAD}^{+\text{Na}}]^{+}_{\text{obs}}$, 4294 m/z = $[\text{CTADOH}^{+\text{Na}}]^{+}_{\text{obs}}$, 4298 m/z = $[\text{CTAD} + \text{Acn}^{+\text{H}}]^{+}_{\text{obs}}$.

Figure 2.7 Initial rate of CTAD hydroxylation determined from the relative intensities of $[\text{CTAD}^{\text{OH}+\text{H}}]^{+}$ and $[\text{CTAD}^{+}\text{H}]^{+}$. 

$m= \nu_{\text{obs}} = 25.3 \mu\text{M min}^{-1}$
2.5.3 UV-Vis spectroscopy

Figure 2.8 Anaerobic UV-Vis spectra of the MLCT region for (Fe+αKG)FIH variants (400 µM) constituted with FeSO₄ (375 µM) and αKG (400 µM) in 50mM HEPES pH 7.00.

2.5.4 X-ray absorption spectroscopy

Data were collected at the Stanford Synchrotron Radiation Laboratory (SSRL) on beam line 9-3. For data collected at SSRL, the samples were cooled to ~10 K using a liquid helium cryostat (Oxford Instruments). The ring conditions were 3 GeV and 450-500 mA. Beam line optics included a Si(220) double-crystal monochromator. X-ray fluorescence data were collected using a 100 element detector (Canberra). Soller slits with a Z-1 element filter were placed between the sample chamber and the detector to minimize scattering. Internal energy calibration was performed by collecting spectra simultaneously in transmission mode on the corresponding metal foil to determine the first inflection point on the edge, which was set to 7111.2 eV for Fe. X-ray absorption near-edge structure (XANES) data were collected from -200 to +200 eV relative to the metal K-edge. Extended X-ray absorption fine structure (EXAFS) data were collected to 15k above the reference edge energy.
The SixPack software program was used to remove bad channels, average the data, and to perform energy calibrations, in addition to data reduction and normalization. Background correction was performed using a Gaussian pre-edge function and a quadratic polynomial for the post-edge region followed by normalization of the edge jump. A seven section, fourth-order polynomial spline between \( k = 2 \) Å\(^{-1}\) and \( k = 14 \) Å\(^{-1}\) was used for EXAFS baseline correction. The Artemis software program was used for EXAFS analysis by utilizing the EXAFS equation with parameters generated using FEFF6. The EXAFS equation is defined as:

\[
\chi(k) = \sum N_i f_i(k) e^{-2k^2\sigma_i^2} \frac{1}{k^2 r_i^2} \sin[2kr_i + \delta_i(k)]
\]

where \( f(k) \) is the scattering amplitude, \( \delta(k) \) is the phase-shift, \( N \) is the number of neighboring atoms, \( r \) is the distance to the neighboring atoms, and \( \sigma^2 \) is the disorder to the nearest neighbor. The data were converted to \( k \)-space using the \( k = \left[ \frac{2m_e(E-E_0)}{\hbar^2} \right]^{1/2} \) relationship, where \( m_e \) is the mass of an electron and \( \hbar \) is Plank’s constant divided by \( 2\pi \). All data sets were Fourier-transformed using a Kaiser-Bessel window over the range \( k = 2 \) – 14 Å, and fit in \( r \)-space over the range \( r = 1 \) – 4 Å in order to assess multiple-scattering contributions from histidine imidazole rings, and employing an amplitude reduction factor \( (S_0) \) value of 0.9. The reported Fourier-transformed spectra were not phase-corrected. Reported data sets were fit with separate sets of \( \Delta r_{\text{eff}}(r - r_{\text{eff}}) \) or the change in metal-ligand bond distance with respect to the input path distance, and \( \sigma^2 \) for the Cl and nitrogen-donors including imidazole rings with initial values of 0.0 Å\(^2\) and 0.003 Å\(^2\), respectively. Each fit was initiated with a universal \( E_0 \) (7125 eV for Fe) and \( \Delta E_0 = 0 \) eV, which was allowed to vary for each fit. Histidine ligands were fit as geometrically rigid imidazole rings with...
varied angles of rotation (\(\alpha\)), with \(\alpha\) being defined as the rotation around an axis perpendicular to the plane of the ring and going through the coordinated nitrogen. The distances of the five non-hydrogen atoms in the imidazole ring were fit in terms of a single \(\Delta r_{\text{eff}}\) for various angles \((0 – 10^\circ)\) so that all atoms in the ring move with the Fe-N distance.\(^5\)\(^7\) Multiple-scattering parameters for imidazole ligands bound to Fe were generated using the FEFF6 software package with the imidazole input obtained from average bond lengths and angles gathered from crystallographic data, as previously described.\(^8\)\(^9\) All multiple scattering paths with path lengths outside the fitting window \((r = 1 – 4 \text{ Å})\) were discarded.

Histidine ‘counting’ was performed by adding integer numbers of histidine imidazole ligands to fits and assessing the effect on the \(R\)-factor and reduced \(\chi^2\). \(N\)-oxalylglycine (NOG) was modeled as a geometrically rigid molecule in an identical manner to the histidines. However, NOG was modeled as either the entire structure (Whole) or utilizing only the two oxygen atoms that traditionally bind bidentate to iron as well as the two backbone carbon atoms attached to them (Ring). The tail of the NOG structure is both spatially mobile in correlation with the iron atom as well as distant from the iron center and so should contribute little to nothing to the scattering pattern and can be easily omitted.

To assess the goodness of fit from different fitting models, the fit parameters \(\chi^2\), reduced \(\chi^2\) (\(\text{red} \chi^2\)), and \(R\)-factor were minimized. Increasing the number of adjustable parameters is generally expected to improve the \(R\)-factor; however \(\chi^2\) may go through a minimum, with the increase indicating the model is over-fitting the data. These parameters are defined as follows:

\[
\chi^2 = \frac{N_{\text{idp}}}{N_{\varepsilon^2}} \sum_{i=1}^{\frac{t}{N}} \left( \text{Re}[\bar{\chi}_{\text{data}}(R_t) - \bar{\chi}_{\text{theory}}(R_t)]^2 + \text{Im}[\bar{\chi}_{\text{data}}(R_t) - \bar{\chi}_{\text{theory}}(R_t)]^2 \right)
\]
And

\[ \text{red} \chi^2 = \frac{\chi^2}{N_{\text{idp}} - N_{\text{var}}} \]

Where \( N_{\text{idp}} \) is the number of independent data points defined as
\[ N_{\text{idp}} = \left(\frac{2\Delta r \Delta k}{\pi}\right), \]
\( \Delta r \) is the fitting range in \( r \)-space, \( \Delta k \) is the fitting range in \( k \)-space, \( N_{\text{var}} \) is the number of refining parameters and represents the degrees of freedom in the fit, \( N_{\chi^2} \) is the number of uncertainties to minimize, \( \text{Re}() \) is the real part of the EXAFS Fourier-transformed data and theoretical functions, \( \text{Im}() \) is the imaginary part of the EXAFS Fourier-transformed data and theoretical functions, and \( \tilde{\chi}(R_i) \) is the Fourier-transformed data or theoretical function.

Additionally, IFEFFIT calculates the R-factor for each fit, which is directly proportional to \( \chi^2 \) and a measure of the absolute misfit between the data and theory given by:

\[ R = \frac{\sum_{i=0}^{n} \left\{ \text{Re}[\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i)]^2 + \text{Im}[\tilde{\chi}_{\text{data}}(R_i) - \tilde{\chi}_{\text{theory}}(R_i)]^2 \right\}}{\sum_{i=0}^{n} \left\{ \text{Re}[\tilde{\chi}_{\text{data}}(R_i)]^2 + \text{Im}[\tilde{\chi}_{\text{data}}(R_i)]^2 \right\}} \]

Selected fits to the data are shown in Supplemental Table 2.4; the best fit is at the bottom of the table.
Table 2.4 Selected fits of EXAFS for (Fe+Cl+NOG)D201G using varied ligand composition.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Radius</th>
<th>$\sigma^2$</th>
<th>$\Delta E_0$</th>
<th>R</th>
<th>nvar</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Å</td>
<td>(x10^-3 Å²)</td>
<td>(eV)</td>
<td>factor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6N/O</td>
<td>2.13(1)</td>
<td>3(1)</td>
<td>-5(1)</td>
<td>0.0818</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>5N/O</td>
<td>2.12(1)</td>
<td>0(1)</td>
<td>-5(1)</td>
<td>0.0601</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>1S/Cl</td>
<td>2.40(3)</td>
<td>5(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4N/O</td>
<td>2.12(1)</td>
<td>0(1)</td>
<td>-5(2)</td>
<td>0.0597</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>2.08(11)</td>
<td>10(16)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>2.25(4)</td>
<td>1(5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3N/O</td>
<td>2.09(1)</td>
<td>2(1)</td>
<td>-9(1)</td>
<td>0.0300</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>1S/Cl</td>
<td>2.38(3)</td>
<td>4(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>1.95(3)</td>
<td>2(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>2.16(3)</td>
<td>3(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1N/O</td>
<td>2.09(1)</td>
<td>4(1)</td>
<td>-10(2)</td>
<td>0.0236</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>1S/Cl</td>
<td>2.36(4)</td>
<td>5(4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>1.95(4)</td>
<td>2(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1Imd</td>
<td>2.16(4)</td>
<td>3(3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1NOG(ring)</td>
<td>2.01(9)</td>
<td>6(6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.11(9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.5.5 Metal titrations

To control for the possibility that the sulfate group may be binding to the active site of the D201G variant without added chloride, resulting in a similar $K_a$(Co) (5 µM) for the chloride-saturated sample, additional assays were conducted using Co(ClO$_4$)$_2$. Changing the counter ion of the metal salt did not affect the $K_a$(Co).

![Figure 2.9](image)

**Figure 2.9** $K_a$(Co) determination based on intrinsic tryptophan fluorescence quenching for the D201G sample with (red) and without (black) 100 mM NaCl in the assay buffer. Assays contained the following: 20 uM FIH, 500 uM αKG, and 10 mM sodium citrate in 50 mM HEPES pH 7.0. The titrating solution was composed of 1 mM Co(ClO$_4$)$_2$, and 1 mM sodium citrate or 10 mM Co(ClO$_4$)$_2$, and 10 mM sodium citrate in 50 mM HEPES pH 7.0. Samples were mixed for 7 minutes following addition of metal, then were excited at 285 nm. Maximum emission was monitored at 330 nm.
2.6 References


85


32. Mills, S. A.; Marletta, M. A., Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from Escherichia coli. *Biochemistry* 2005, 44 (41), 13553-13559.


CHAPTER 3
SECOND COORDINATION SPHERE INFLUENCES SUBSTRATE SELECTIVITY IN FACTOR INHIBITING HIF (FIH)

3.1 Introduction

Bio-orthogonal chemistry has led to a powerful set of tools that enable chemists to study biological processes within living cells.¹ Two prominent tools are the Staudinger ligation² and the Huisgen 1,3 cycloaddition (“click”) reaction;³,⁴ both reactions couple an organic azide with another functional group. The small size and inherent lack of reactivity with enzymes makes azides attractive for biomolecular recognition.¹ However, organic azides cannot be biosynthesized,¹ limiting their applications. Thus, there is a need to hijack the molecular machinery of enzymes to produce non-natural peptide modifications, such as azidation, in order to facilitate bio-orthogonal efforts.

The αKG dependent halogenase SyrB2 was engineered to facilitate azidation and nitration of substrate through radical rebound chemistry of an exogenous anion, suggesting other αKG dependent oxygenases could support this chemistry.⁵ However, attempts at reprogramming the chemistry of other αKG dependent hydroxylases have not been successful. In TauD and prolyl-4-hydroxylase, mutations to the facial triad carboxylate to permit exogenous anion binding did not support formation of the active (Fe+αKG) complex, preventing the enzymes from reacting with O₂.⁶,⁷ Recently, the αKG dependent hydroxylase SadA was reported to halogenate a small molecule substrate with simply an Asp→Gly mutation of the facial triad carboxylate residue,⁸ suggesting non-natural peptide modifications of hydroxylases are possible through manipulation of enzyme structure.
provided that an exogenous anion can bind and the substrate is positioned correctly. While
the halogenases are thought to follow the same consensus mechanism of the hydroxylases,
the differences which govern halogenation versus hydroxylation are still being
elucidated.9–11

We proposed to engineer FIH to perform radical rebound chemistry with exogenous
anions, to produce peptides with varied modifications. Recently, our group demonstrated
that the D201G variant of FIH is only active in the presence of chloride and that the active
site of this variant contains an Fe-Cl bond, forming a His2Cl facial triad reminiscent of the
αKG dependent halogenases (Chapter 2), demonstrating 3 out of the 4 criteria for radical
rebound chemistry. Having the chloride positioned in the active site of the enzyme may
allow for alternate rebound chemistry to occur if the substrate and anion binding sites are
arranged to favor anion rebound over •OH rebound (see Chapter 2, Scheme 2.1 for more
details). Chloride binding to the active site of D201G raises the potential for binding of
other anions, such as azide.

Engineering the second coordination sphere within the D201G variant of FIH to
allow for an exogenous anion to undergo radical rebound could induce novel C-X bond
formations, which could then be used to prepare peptides for bio-orthogonal conjugations.
This thesis reports on the anion dependent substrate hydroxylation by FIH variants in initial
efforts to achieve non-natural peptide modification. Our results from Chapter 2 indicate
that FIH could be engineered to bind exogenous anions and perform anion-selective
hydroxylation chemistry, achieving the preliminary steps necessary for alternate rebound
chemistry.
The extensive hydrogen bonding network between the second coordination sphere residues and the active site has been reported to play a role in chloride binding in the halogenases CtyC3 and SyrB2, and aids in coupling O\textsubscript{2} activation with substrate hydroxylation in FIH. We hypothesized that the second coordination sphere residues in the αKG dependent oxygenases govern Fe-X orientation relative to substrate through hydrogen bonding interactions. In the search for non-natural peptide modifications, various ARD sequences were screened. FIH hydroxylates several types of amino acids at the target residue within ARD domains whereas Asn is the only known target residue for CTAD. Changing the target residue could alter substrate positioning, promoting alternate rebound chemistry. Instead, some variants exhibited substrate selectivity disfavoring the Asn residue, suggesting target residue preference could be modified through second sphere interactions within FIH. Overall, this study exemplifies the robust hydroxylase nature of FIH and high tolerance for chemical diversity within its second coordination sphere.

3.2 Methods

All reagents were purchased from commercial vendors and used as received, with the exception of CTAD substrate. CTAD was purchased (EZBiolab, Carmel, IN, USA) as a desalted peptide and further purified by reverse phase HPLC methods (described in section 3.2.1).

3.2.1 CTAD purification

Desalted CTAD peptide representing HIF-1α\textsuperscript{788-826} with the sequence DESGLPQLTSYDAEV\textsubscript{N}APIQGRSLQGEELLRALDQVN (N803 hydroxylation site
underlined) was stored at -20 °C upon arrival. A 2.5 mg/mL solution of desalted peptide in 20% HPLC Grade Acetonitrile (ACN)/0.1% trifluoroacetic acid (TFA) was prepared and filtered with a 0.22 µm syringe filter. Solvents were prepared as follows: Solvent A= ddH2O with 0.1% TFA; Solvent B= ACN with 0.1% TFA. A Proto C18 semi prep column (Higgins Analytical) was attached to an Agilent HPLC 1100 system. The following Agilent ChemStation method was used to isolate pure CTAD from its impurities (current method named “CTAD PREP 12.21.16):

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>% Solvent B</th>
<th>Flow rate (mL/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>25</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
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<td>28</td>
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</tr>
<tr>
<td>30</td>
<td>25</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 3.1 CTAD purification HPLC method.

To start the method, 1.8 mL of impure CTAD solution was loaded into the manual injector using a blunt HPLC injection needle on the end of a 2 mL syringe and the manual injector was flipped from “load” to “inject” automatically starting the method. The semi-pure CTAD peak was manually collected around 9 minutes post injection. Following completion of the method, a new injection of impure CTAD solution was made until all the impure CTAD was run over the column. Semi-pure CTAD was then flash frozen using liquid nitrogen and lyophilized overnight. The semi-pure CTAD was then re-dissolved in 20% ACN/0.1% TFA and run over the column for a second time using the same method.
The pure CTAD peak was manually collected about 10 minutes following injection. Pure CTAD was again flash frozen using liquid nitrogen and lyophilized overnight to remove ACN. Lyophilized pure CTAD was dissolved in a minimal amount (300 µL) of fresh 50 mM HEPES buffer pH 7.00 for activity assays. 10 µL of pure CTAD in HEPES was diluted in 110 µL of 0.1 M NaOH and the concentration of CTAD was determined using UV-Vis spectroscopy at 293 nm (ε= 2,400 M⁻¹cm⁻¹). CTAD was stored at a high concentration at -20 ºC.

3.2.2 Site directed mutagenesis

Degenerate primers were designed according to guidelines from Integrated DNA Technologies (IDT) (detailed in section 3.5.1). PCR was conducted using the degenerate primers and D201G plasmid DNA to develop the library of second coordination sphere variants. PCR reactions included Phusion master mix (Fisher), 125 ng/µL forward primer, 125 ng/µL reverse primer, 100 ng/µL D201G plasmid DNA, 3% DMSO, and sterile H₂O. PCR cycling conditions were as follows:

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Time</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>98 ºC</td>
<td>1 minute</td>
<td>1 cycle</td>
</tr>
<tr>
<td>Denature</td>
<td>98 ºC</td>
<td>15 seconds</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Anneal</td>
<td>62 ºC</td>
<td>30 seconds</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Extend</td>
<td>72 ºC</td>
<td>3.5 minutes</td>
<td>30 cycles</td>
</tr>
<tr>
<td>Final extension</td>
<td>72 ºC</td>
<td>10 minutes</td>
<td>1 cycle</td>
</tr>
</tbody>
</table>

DNA amplification was confirmed using agarose gel electrophoresis and PCR products were transformed into XL1-Blue cells. DNA from individual colonies on the XL1-Blue plates was isolated using a GeneJet miniprep kit (Fisher) and sequenced.
Colonies were picked, miniprepped, and sequenced until all expected mutations possible with the degenerate primers were found. Sequenced plasmid DNA stocks were labeled and stored at -80 °C.

3.2.3 Crude cell lysate screen

Plasmid DNA for each variant was transformed into BL21-DE3 cells. Individual 2 mL 2xYT overnight cultures were inoculated with a single colony from the agar plates. Overnight cultures were incubated at 37 °C while shaking at 225 rpm for 12-16 hours. Variants were then expressed in BL21-DE3 cells grown in 5 mL cultures of 2xYT media. Cultures were inoculated with 100 µL of overnight culture and grown at 37 °C while shaking at 225 rpm until optimal cell density was achieved. Protein expression was induced with 0.5 M Isopropyl β-D-1-thiogalactopyranoside (IPTG) at [math]\text{OD}_{600}= 0.6-0.8[/math] and incubated for 5 hours at 37 °C while shaking at 225 rpm. Cells were harvested by centrifugation (Sorvall Legend RT with swinging buckets) at 3,441 x g for 15 minutes and stored at -20 °C. Cell pellets were lysed for activity screens using 1 mL of 1 X lysis buffer (25 mM Tris Base, 200 µM EDTA, 1 mg/mL lysozyme, and 1 µg/mL of AEBSF) pH 8.0. Lysates were vortexed vigorously, flash frozen using liquid nitrogen, and stored at -80 °C for one hour. Lysates were then thawed at room temperature and centrifuged (Eppendorf, FA-45-18-11 rotor) at 16,900 x g for 25 minutes to pellet cell membrane components. Supernatant was removed and stored at -20 °C for activity assays.

Supernatants were thawed on ice in preparation of activity assays. Activity assays were conducted with ascorbate (2 mM), αKG (200 µM), FeSO₄ (100 µM), CTAD or ARD substrate (50 µM) and 10% crude cell lysate in 50 mM HEPES/100 mM NaX pH 7.00. All
assay components were mixed to a total volume of 50 µL and incubated at 37 ºC overnight. The next morning, 5 µL of each assay was quenched in 20 µL of matrix (3,5-dimethoxy-4-hydroxycinnamic acid saturated in 75% ACN/0.2% TFA) and analyzed by MALDI-TOF-MS (Ultraflex, Bruker) monitoring the +1 charge state of the substrate mass and any mass additions to the substrate peak. Hydroxylated substrate resulted in a +16 mass shift due to loss of a hydrogen followed by 'OH rebound from FIH to the peptide substrate. Anion rebound modifications were expected to produce a mass shift of +34.5/36.5, +80, and +42 for chlorination, bromination, and azidation respectively based on the mass of the element added to the mass of the peptide, minus loss of a hydrogen.

### 3.2.4 Protein purification

Selected variants were expressed and purified as previously described (section 2.2.1). Briefly, His tagged variants were expressed in BL21-DE3 cells and cell pellet was harvested. Cell pellet was lysed using sonication and centrifuged (Sorvall RC6, SS-34 rotor, 14,000 rpm). Supernatant was dialyzed into 10 mM Tris pH 8.0 and run over a Ni-NTA column to remove contaminating proteins. Restriction grade thrombin was then used to cleave the His tag. Cleaved protein was loaded on to the Ni-NTA column again to remove the His tag. Cleaved protein was found in the flow through. Flow through was treated with 50 mM EDTA overnight at 4 ºC to remove endogenous metals. Protein was then isolated from thrombin and EDTA using size exclusion chromatography (Superdex 75 10/300, GE Life Sciences). Eluted protein was buffer exchanged into 50 mM HEPES pH 7.00 and stored at -20 ºC. Protein purity was checked using SDS PAGE and mass spectrometry.
3.2.5 O$_2$ consumption

An Oxygraph Plus System (Hansatech) was used to monitor the amount of oxygen consumed by the enzyme. This oxygen sensor contains a central reaction vessel surrounded by a water jacket, with an oxygen electrode disc at the bottom of the reaction vessel. The electrode disc consists of a central platinum cathode and a concentric silver anode. To conduct signal across the electrode disc, a KCl bridge is formed between the two electrode elements with the aid of a fine paper wick and a layer of PTFE membrane, which is selectively permeable to oxygen molecules. A new membrane was prepared and calibrated each day with air saturated water and dithionite. 400 µL reactions were equilibrated at atmospheric O$_2$ in the reaction vessel at 37 °C until a stable baseline was achieved. Assays were initiated with cold purified enzyme (10 µM) using a Hamilton syringe. The rate of O$_2$ consumption was monitored over time until the rate of O$_2$ consumption resembled the baseline slope. Assays contained 50 µM ascorbate, 100 µM αKG, 50 µM FeSO$_4$, 80 µM CTAD and 10 µM FIH in 50 mM HEPES 100 mM NaCl pH 7.00. Typical activity assay conditions include 2 mM ascorbate, however ascorbate consumes O$_2$ and resulted in a steep baseline slope before purified enzyme was added to the reaction mixture. Therefore, controls were done to determine the optimal amount of ascorbate that could be used to minimize baseline slope while keeping the Fe$^{2+}$ reduced. 50 µM ascorbate fit both of these conditions as it was equimolar to the amount of FeSO$_4$ (50 µM) and allowed for the amount of O$_2$ consumed upon enzyme addition to be distinguished from the baseline signal.
3.2.6 Thermal shift assay

Thermal shift assay experiments were conducted using a BioRad CFX Connect Real-Time PCR Detection System instrument. Protein solutions were dispensed in 50 µL aliquots into clear 96-well PCR plates and sealed to prevent evaporation. The PCR plates were placed in the thermal block of the PCR instrument and heated at a rate of 0.5 °C/s. Fluorescence was measured and an image was collected every 0.5 °C. Reference wells contained (Fe⁺αKG) in HEPES buffer without enzyme. Relative fluorescence intensity data was obtained from the instrument, plotted for comparison to control samples, and the sigmoidal portion of the data was fit using the Boltzman equation in Origin to obtain the TM.

\[
y = \frac{A_1 - A_2}{1 + e^{(x-x_0)/d_x}} + A_2
\]

Samples were composed of 100 µM αKG, 5 µM CoSO₄, 5 µM FIH and 5x Sypro Orange dye in 50 mM HEPES 100 mM NaCl pH 7.00. The concentration of enzyme, metal, and αKG were previously optimized to obtain a strong fluorescent signal during the inhibitor screens described in Appendix B. Samples were denatured using a temperature gradient. An increase in fluorescence intensity correlated to an increase in denaturation state of the protein. ΔTM was calculated by subtracting the TM of apo FIH from the TM of FIH variants.

\[1\] A₁ = initial temperature  
A₂ = final temperature  
x₀ = midpoint (TM)  
d_x = time constant
3.3 Results and Discussion

3.3.1 Second sphere mutagenesis

Target residues were selected for mutation using a rational design approach to promote anion rebound over hydroxide rebound. The second sphere interactions of FIH were modeled against those of SyrB2 to establish the nearest contacts for the metal center orientation relative to substrate. In both enzymes, an extensive hydrogen bonding network positions the active site residues, however the residues involved differ significantly between the hydroxylase and the halogenase. Sequence alignments of FIH and the αKG dependent halogenases (Table 3.2) identified three sites which form polar contacts to the primary ligands coordinating the metal center in distinct ways between the two enzyme classes. Positions 205, 294, and 296 (bolded in Table 3.2) were chosen as targets for manipulating FIH active site configuration to promote alternate rebound chemistry. As seen in Figure 3.1, these three residues form hydrogen bonds with the primary sphere ligands in both FIH and SyrB2, suggesting these sites are important in positioning.

**Figure 3.1** Second coordination sphere interactions with primary ligands in (A) D201G FIH (PDB Code: 3D8C,\(^{19}\) Cl\(^-\) modeled) and (B) SyrB2 (PDB Code: 2FCT\(^{20}\)).
<table>
<thead>
<tr>
<th>Site</th>
<th>199</th>
<th>201</th>
<th>205</th>
<th>279</th>
<th>294</th>
<th>296</th>
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<td>H</td>
<td>D</td>
<td>N</td>
<td>H</td>
<td>N</td>
<td>W</td>
</tr>
<tr>
<td>SyrB2</td>
<td>H</td>
<td>A</td>
<td>T</td>
<td>H</td>
<td>A</td>
<td>R</td>
</tr>
<tr>
<td>CytC3</td>
<td>H</td>
<td>A</td>
<td>T</td>
<td>H</td>
<td>A</td>
<td>R</td>
</tr>
<tr>
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<td>H</td>
<td>N</td>
<td>S</td>
<td>H</td>
<td>G</td>
<td>F</td>
</tr>
<tr>
<td>CurA</td>
<td>H</td>
<td>G</td>
<td>S</td>
<td>H</td>
<td>V</td>
<td>R</td>
</tr>
</tbody>
</table>

A library of FIH second sphere variants at these three positions in the D201G construct was developed using degenerate primers in order to broadly sample chemical space at each position, while minimizing PCR reactions. Initially, 15 double variants within the D201G construct were made containing a mutation at either position 205, 294, or 296. An additional library of 58 quadruple variants were also made within the D201G construct in which the residues at positions 205, 294 and 296 were varied (Supplemental Table 3.4). These variants were then sequenced (Genewiz, South Plainfield, NJ, USA) and over expressed in *E. coli*. A freeze-thaw process was used to lyse the cells. Lysate was then centrifuged to remove membrane components as described in section 3.2.2 of this chapter; the supernatant, referred to as crude cell lysate, was used to assess endpoint activity of the variants, the results of which are elaborated on below. Protein expression was verified using SDS PAGE gel electrophoresis of crude cell lysate (Supplemental Fig. 3.9)

3.3.2 Anion rebound screens

Bollinger *et al* established four conditions which facilitate halide rebound chemistry: (i) halide anion coordination to Fe(II) cofactor, (ii) O₂ activation coupled to decarboxylation to form the haloferryl intermediate, (iii) hydrogen atom abstraction and
(iv) transfer of halogen atom to substrate radical. These conditions were used in Chapter 2 to evaluate the novel Fe-Cl bond identified in the D201G variant of FIH. However, initial activity studies of this single variant did not reveal anion rebound chemistry, prompting the site directed mutagenesis study presented in this chapter. Further structural analysis of SyrB2 reactivity revealed halogenases position the substrate target residue distal to the oxo moiety of the ferryl intermediate, such that halide rebound is facilitated. These requirements would need to be met by the D201G second sphere variants in FIH to achieve alternate rebound chemistry.

In order to quickly evaluate the rebound chemistry potential of the D201G variants, a crude cell lysate activity screen was developed. Initial screens were conducted in the presence of 100 mM NaCl as Cl⁻ was previously shown to bind to the active site of D201G (Chapter 2). Variants were incubated with ascorbate, αKG, FeSO₄, CTAD in 50 mM HEPES/100 mM anion at pH 7.00 and 37 °C overnight to allow for trace products to accumulate. MALDI-TOF-MS was used to detect substrate modifications following overnight incubation. Screens without any added anion were conducted in parallel as a control. Additional screens with Br⁻ or N₃⁻ were conducted to determine if anions of varying size could bind to the Fe(II) as Cl⁻ was shown to do and rebound to substrate. As each of these modifications results in a unique mass shift, MALDI-TOF-MS was ideal for detecting all possible substrate rebound products.

We hypothesized that varying the second sphere residues would position the anion ligand to compete with the hydroxide ligand for rebound chemistry, leading to alternate substrate modifications. However, this was not observed. All the variants minimally hydroxylated the CTAD substrate with and without added Cl⁻. The hydroxylated +16 mass
shift was seen with all variants, irrespective of anion. Mass shifts of possible alternate rebound modifications include chlorination (+34.5/36.5 in a 3:1 ratio), bromination (+79/81 in a 1:1 ratio), and azidation (+42). No substrate modifications with these mass shifts were seen for any of the variants under the given conditions. The double variant halide rebound screens are shown in Figure 3.2.
Figure 3.2. Endpoint halide rebound screens in crude cell lysate using the CTAD substrate. (A) 205 variants (B) 294 variants and (C) 296 variants. Assays included ascorbate (2 mM), αKG (200 µM), FeSO₄ (100 µM), CTAD substrate (50 µM) and 10% crude cell lysate in 50 mM HEPES/100 mM NaX pH 7.00.
Less than 5% hydroxylated CTAD was detected for all the variants except GNTW (residues at positions 201, 205, 294, 296 respectively) which was 15% hydroxylated in the presence of Cl\textsuperscript{-}. This was surprising as halogenases prefer small, nonpolar side chains at the analogous 294 position, however the Trp\textrightarrow{}Thr mutation was a conservative change within FIH. From this screen, we concluded that all of the second coordination sphere double variants impaired CTAD hydroxylation. This low CTAD hydroxylation was likely due to impaired Fe/X\textsuperscript{-} binding, with the GNTW mutation having a lesser effect on Fe/X\textsuperscript{-}.

In addition, the second sphere had a small effect on anion selectivity. Only CTAD hydroxylation by GNTW was enhanced by the addition of NaCl to the assay buffer. Some variants have a 1-2% increase in hydroxylation activity in the presence of NaBr, however the total hydroxylation was less than 5%. Activity screens with added NaN\textsubscript{3} had a much lower signal to noise ratio than the other NaX additives, therefore the low amount of CTAD hydroxylation seen in Figure 3.2 with NaN\textsubscript{3} was not deemed significant enough to pursue. The increased noise with NaN\textsubscript{3} was thought to be due to impaired ionization of the matrix quenched samples. An example of a MALDI-TOF-MS spectra is shown below (Fig. 3.3), depicting the unmodified CTAD peak ([R+H]\textsuperscript{+}) and the hydroxylated CTAD peak ([R+OH]\textsuperscript{+}). No other peaks are seen in the spectra with the exception of the [R+Na]\textsuperscript{+} peak which is present in all of the spectra as the pH of the HEPES buffer is adjusted with 1 M NaOH. Overall, these screens suggested that these second coordination sphere variants did not increase the flexibility of the active site to allow the X\textsuperscript{-} ligand to compete with the hydroxide for substrate modification.
Along with hydroxylating Asn803 of CTAD, FIH is known to hydroxylate multiple target residues within ARD domains, however the biological significance of this hydroxylation is unknown.\textsuperscript{24,25} Target residues span all facets of diversity including polar, neutral, acidic, basic, hydrophobic and hydrophilic residues such as Asn, Asp, His, Ile, Leu, and Ser.\textsuperscript{26,27} The ARD peptide recognition sequence is about half the number of amino acids as CTAD; using a shorter substrate may allow for more flexibility in the active site. As FIH is highly promiscuous towards these substrates, we hypothesized that the ARD substrates may facilitate rebound chemistry over CTAD.

Purified (>98\%) 19-mer ARD peptides containing each of these target residues at position 16, the known site of hydroxylation, were purchased from EZBiolab (Carmel, IN, USA). Working stocks of each peptide were prepared in 50 mM HEPES/10\% ACN pH 7.00 and the substrate concentration was verified by BCA assay. Crude cell lysate screens
of the D201G second sphere variants at either positions 205, 294, or 296 with Asn, His, Leu or Ser ARD substrates were conducted in the presence of 100 mM NaCl as described above. Similar to the screen conducted with CTAD substrate, none of the ARD substrates displayed a +34.5/36.5 doublet peak indicative of Cl− rebound. Surprisingly, the variants hydroxylated the ARD substrates more efficiently than CTAD.
Figure 3.4 Endpoint halide rebound screens in crude cell lysate using various ARD target residues as substrate. (A) 205 variants (B) 294 variants and (C) 296 variants. Assays included ascorbate (2 mM), αKG (200 µM), FeSO$_4$ (100 µM), ARD substrate (50 µM) and 10% crude cell lysate in 50 mM HEPES/100 mM NaX pH 7.00.
As seen in Figure 3.4, the Asn target residue is not favored by the D201G second sphere variants as it is in WT FIH. His was generally preferred by the variants, especially when position 205 was varied. However, there were a few cases where the variants are more inclined to hydroxylate Ser or Leu. For example, the GNNY variant containing a W296Y mutation strongly preferred the hydrophobic Leu side chain; GNRW and GNNT also prefer Leu to a lesser extent than GNNY. Overall second coordination sphere variants at positions 205, 294, or 296 in the D201G construct retain some activity towards ARD substrates, but lose CTAD reactivity. The observed loss of CTAD reactivity with the variants is ideal for those wishing to identify loss of function mutations for FIH, however this was not our goal. From this screen, we conclude that the second coordination sphere of FIH imparts substrate selectivity; substrate selectivity does not solely rely on the identity of the metal center.

3.3.3 SyrB2 mimic purified variants

SyrB2 catalyzes the O$_2$-dependent chlorination of threonine on syringomycin E biosynthesis.$^{28}$ Crystal structures of SyrB2 revealed the chloride ion replaces the facial triad carboxylate to fill the vacant coordination site,$^{20}$ as reported for the D201G variant of FIH (Chapter 2). Other known rebounding anions of SyrB2 include bromination,$^{29}$ azidation and nitrination,$^5$ making this an ideal enzyme to model our FIH engineering studies. As the crude cell lysate screens for alternate rebound chemistry with the D201G second sphere variants did not yield the desired result, a more focused study was conducted with purified variants. Variants that mimicked the SyrB2 residues at positions 205, 294, and 296 were expressed and purified as described in section 3.2.4 to investigate the effects of the
point mutations on stability and reactivity. Notably, position 205 was mutated to a Ser instead of a Thr as seen in the analogous position of SyrB2 because studies of the halogenase WelO5 implicated the Ser residue at the position in stabilizing the halogenated product by hydrogen bonding to the ferryl intermediate, resulting in complete substrate halogenation.\textsuperscript{21} The S189A mutation in WelO5 yielded an equal amount of hydroxide and halide products, highlighting the significance of this residue in rebound chemistry.

![Figure 3.5](image)

**Figure 3.5** Anion screens with purified SyrB2-mimic variants and CTAD substrate. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively. Assay conditions included ascorbate (2 mM), αKG (100 µM), FeSO₄ (50 µM), CTAD substrate (50 µM) and FIH (2 µM) in 50 mM HEPES/100 mM NaX pH 7.00.

Anion rebound screens were again conducted with the CTAD substrate, this time controlling the purity and amount of enzyme used. Similar to the results described in Figure 3.2, all the SyrB2 mimic variants had little to no hydroxylation activity and no other substrate modifications were detected by MALDI-TOF-MS (Fig. 3.5). Consistent with the crude cell lysate screen, the purified SyrB2 mimic variants were most active in the presence of added chloride.
To gain a deeper understanding as to how the stability and reactivity of these variants was affected by manipulation of the second coordination sphere interactions, additional assays were conducted. First, protein stability was assessed using a thermal shift assay in which hydrophobic portions of the protein were labeled with a fluorescent Sypro orange dye that monitors protein unfolding. As the protein was denatured with increasing temperature, hydrophobic portions of the protein were exposed, resulting in an increase in fluorescence. From this data, the unfolding temperature (\(\Delta T_M\)) was determined and compared to that of WT FIH. We hypothesized that the SyrB2 mimic variants would have decreased stability than WT FIH due to manipulation of the second sphere interactions and that the stability trend would correlate to the hydroxylation screens; i.e. variants with more activity would have a (\(\Delta T_M\)) closer to that of WT FIH.

![Figure 3.6](image.png)

**Figure 3.6** Thermal stability of SyrB2 mimic variants. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively. Samples were composed of 100 µM \(\alpha\)KG, 5 µM CoSO\(_4\), 5 µM FIH and 5x Sypro Orange dye in 50 mM HEPES 100 mM NaCl pH 7.00.

Figure 3.6 shows the thermal melt results for (Co+\(\alpha\)KG)WT FIH and the SyrB2 mimic variants. Co\(^{2+}\) was used in place of Fe\(^{2+}\) to avoid oxidation at ambient O\(_2\). In
agreement with our hypothesis, all of the variants substantially destabilized the protein, resulting in a lower $T_M$ than WT FIH. Analysis of the $\Delta T_M$ values relative to WT FIH revealed that there was not a trend between the $\Delta T_M$ and the percent CTAD hydroxylation seen in Figure 3.5; in fact, the single D201G mutation was the among the most destabilized despite having the most hydroxylation activity (Table 3.3). The increased stabilization of the GSAR variant, a quadruple variant, relative to the double variants suggests that extensive site directed mutagenesis within the second coordination sphere is not detrimental to enzyme stability.

**Table 3.3.** Thermal stability values for variants. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively.

<table>
<thead>
<tr>
<th></th>
<th>$T_M$, °C</th>
<th>$\Delta T_M$, °C from WT FIH</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FIH</td>
<td>59.54 ± 0.09</td>
<td>--</td>
</tr>
<tr>
<td>GNNW</td>
<td>52.77 ± 0.16</td>
<td>-6.77</td>
</tr>
<tr>
<td>GSNW</td>
<td>52.48 ± 0.12</td>
<td>-7.06</td>
</tr>
<tr>
<td>GNAW</td>
<td>54.28 ± 0.10</td>
<td>-5.26</td>
</tr>
<tr>
<td>GNRR</td>
<td>52.98 ± 0.31</td>
<td>-6.56</td>
</tr>
<tr>
<td>GSAR</td>
<td>55.56 ± 0.14</td>
<td>-3.98</td>
</tr>
</tbody>
</table>

Although the SyrB2 mimic variants are less stable than WT FIH, some retain their ability to hydroxylate substrate to a low fraction of conversion. In light of these results, initial O$_2$ consumption studies of the SyrB2 mimic variants were conducting using a Clark-type O$_2$ sensor. The amount of O$_2$ consumed by the variants ($\Delta$O$_2$) was calculated from the difference between the baseline O$_2$ consumption of the reaction mixture represented by the black dashed line in Figure 3.7 and each colored line 300 seconds after enzyme initiation.

---

2 Samples were composed of 100 μM αKG, 5 μM CoSO$_4$, 5 μM FIH and 5x Sypro Orange dye in 50 mM HEPES 100 mM NaCl pH 7.00.
Figure 3.7 Endpoint O$_2$ consumption of purified SyrB2 mimic variants from baseline (black dotted line). Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively. GSNW (red), GNAW (blue), GNNR (purple), GSAR (green) 50 µM ascorbate, 100 µM αKG, 50 µM FeSO$_4$, 80 µM CTAD, 10 µM FIH in 50 mM HEPES 100 mM NaCl pH 7.00.

As seen in Figure 3.7, GNNR and GSAR did not consume any measurable O$_2$ during the given time course upon enzyme addition, suggesting that O$_2$ activation is impaired by the W296R mutation, possibly due to unproductive formation of the (Fe+αKG)FIH enzyme state. The GNNR double variant was previously expressed and purified by Schofield, et al. They also reported that Asn803 CTAD hydroxylation with this variant was not detectable, presumably due to the substitution of the hydrophobic Trp residue with the polar/charged Arg residue disrupting the interaction with Val802 in the CTAD substrate. While this experiment highlighted the essential nature of the W296 residue, it did not bring us closer to the goal of alternate rebound chemistry as it killed reactivity. GSNW consumed a considerable amount of O$_2$ compared to hydroxylated substrate, suggesting that reactivity of this variant with O$_2$ is largely uncoupled to substrate hydroxylation. Activity of the GNAW variant was more closely coupled than GSNW based
on the relative amount of O$_2$ consumed to hydroxylated CTAD. These results provide a rationale for the lack of rebound activity seen with the GNNR and GSAR variants as they do not consume O$_2$ and suggests positons 205 and 294 are more tolerant of mutations.

The intriguing ARD hydroxylation results from the crude cell lysate screens (Fig. 3.4) encouraged us follow up on this altered substrate preference. Overnight endpoint activity assays in the presence of ARD substrates with varying target residues were conducted with the purified SyrB2 mimic variants. As shown in Figure 3.8, for all the purified variants Ser was the least hydroxylated target residue suggesting the hydroxyl group is not tolerated well in the active site. All variants favored Asn hydroxylation over His and Leu with the exception of GNAW which showed a preference for His/Leu target residues in both the crude cell lysate and purified variant activity screens. Also in all cases, the amount of hydroxylated ARD was greater than the amount of hydroxylated CTAD in the presence of 100 mM NaCl, suggesting an altered substrate preference for the second coordination sphere variants.
Figure 3.8 Hydroxylation screen with purified SyrB2-mimic variants and ARD substrates. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively. Assays included 2 mM ascorbate, 100 µM αKG, 50 µM FeSO₄, 50 µM ARD substrate and 2 µM FIH in 50 mM HEPES 100 mM NaCl pH 7.00.

3.4 Conclusion and Future Directions

In the end, this study did not yield the anticipated results. The second coordination sphere variants described here did not facilitate alternate rebound chemistry. Presumably, mutations to the hydrogen bonding network did not induce enough flexibility to the active site of FIH to position the halide ligand closer to the substrate, allowing it to compete with the hydroxide ligand for rebound chemistry. Conservative mutations to the second coordination sphere residues displayed modest hydroxylation activity, however the amount of hydroxylated product was less than that of the single D201G mutation in all cases. Curiously, variation to the second sphere altered substrate preference for the N16N ARD peptide over the CTAD peptide. Some variants even showed a preference for target residues other than Asn. However, there was no apparent trend as to how this target residue preference was mediated. More investigation is needed to determine the specifics of how
the second coordination sphere residues mediate substrate selectivity. Overall, it was concluded that substrate positioning is key for rebound chemistry. FIH is a robust enzyme which is specifically hydroxylates substrate. As FIH targets large protein substrates as opposed to small molecule substrates, manipulating the structure of the enzyme enough to facilitate alternate rebound chemistry may not be possible.

3.5 Supplemental Information

3.5.1 Degenerate primer design

Overhanging degenerate primers were designed using guidelines provided by IDT.

N205 Degenerate Primers

5' - GTC CAC TAT GGT GAG CAG CAG RYC TTT TTT GCT CAG-3'

3' - CCA CTC GTC GTC YRG AAA AAA CGA GTC TAT TTT CC-5'

R= A/G; Y= C/T

N294 Degenerate Primers

5' - GG GGG ATT ACC ATC ACT GTG RSR TTC TGG TAT AAG-3'

3' - GG TAG TGA CAC YSY AAG ACC ATA TTC CCC CGA G-5'

R= A/G; S= C/G; Y= C/T

W296 Degenerate Primers

5' - G ATT ACC ATC ACT GTG AAC TTC A HY TAT AAG GGG GC-3'

3' - GA CAC TTG AAG TDR ATA TTC CCC CGA GGG TGG G-5'

H= A/C/T; Y= C/T; D= A/G/T; R= A/G
3.5.2 Confirmation of double variant expression in E. coli

As a control to ensure that protein was being expressed in all of the double variants, SDS PAGE gel electrophoresis (Fig. 3.9) was conducted before checking enzyme activity using the crude cell lysate screening method. Running the assays to completion (overnight) helped control for the differences in expression levels.

![Figure 3.9 SDS PAGE analysis of crude cell lysate following expression of variants in E. coli. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively.](image)

3.5.2 Quadruple variant library

After the second coordination sphere double variants were shown to not induce alternate rebound chemistry, additional variants with mutations at positions 205, 294, and 296 were made within the D201G construct to further increase active site flexibility. However, only low hydroxylation (< 5%) was seen with these variants. Variants are coded by single letter amino acid abbreviations at each position in the following order: 201, 205, 294, 296.
**Table 3.4.** Second sphere mutagenesis within D201G construct of FIH. Single letter amino acid abbreviations represent residues at positions 201, 205, 294, and 296 respectively.

<table>
<thead>
<tr>
<th>Quadruple variants</th>
<th>Quadruple variants</th>
<th>Quadruple variants</th>
<th>Quadruple variants</th>
<th>Quadruple variants</th>
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<tbody>
<tr>
<td>GNRN</td>
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<td>GIRI</td>
<td>GSRY</td>
</tr>
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<td>GIRN</td>
<td>GSRS</td>
</tr>
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<td>GATS</td>
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</tr>
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</table>
3.6 References


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CHAPTER 4

FACIAL TRIAD IS ESSENTIAL FOR COUPLING HYDROXYLATION TO O₂ ACTIVATION IN FIH

4.1 Introduction

As a member of the vast alpha-ketoglutarate (αKG) dependent non-heme Fe(II) oxygenase family, Factor Inhibiting Hypoxia Inducible Factor (FIH) catalyzes the hydroxylation of a variety of peptide substrates upon molecular oxygen (O₂) activation. Other functions of this enzyme family include halogenation, desaturation, epimerization, and most recently endoperoxidation.¹² These enzymes oxidatively decarboxylate αKG producing succinate and CO₂, generating a reactive O₂-derived species in the process.³ This species then oxidizes a third substrate, known as the primary substrate, resulting in post-translational substrate modification. Maintaining tight control over these two half reactions is crucial to proper functioning in this broad class of enzymes.

In the αKG dependent oxygenases, the process of O₂ activation is regulated by a “substrate triggering” effect. Substrate triggering dictates that primary substrate (R-Scheme 4.1) must be bound in a catalytically relevant orientation in order for O₂ binding/activation to occur.⁴⁵ Rapidly following O₂ activation is the irreversible formation of an Fe(IV)=O intermediate and substrate rebound chemistry. This process results in a one-to-one stoichiometry of oxygen consumed to modified substrate. Deviations in this stoichiometry suggest an alternate O₂ activation pathway for O₂ consumption.⁴
While a variety of catalytic outcomes are possible within this class of enzymes upon reacting with O$_2$, nonproductive reactions with oxygen are also seen. “Uncoupled turnover” results when an enzyme inactivates following αKG decomposition into CO$_2$ and succinate, rendering the substrate unchanged.$^1$ Several groups have reported ascorbate-reversible uncoupling of αKG oxygenases as oxidation of the metal led to inactivation.$^{6,7}$ Other alternate pathways implicated in enzyme uncoupling include autohydroxylation and reactive oxygen species (ROS) production.$^3,8$ This uncoupled reactivity is thought to serve as a proof-reading tool to reject improper substrate positioning or incorrect substrate binding.$^9$ While multiple cases of uncoupling have been reported for the αKG dependent oxygenases, mechanistic details regarding how and why these enzymes uncouple remain unknown.

The active site of FIH contains the hallmark αKG dependent hydroxylase His$_2$(Asp/Glu) facial triad and follows a well-defined consensus mechanism.$^{1,10,11}$ Initial O$_2$ activation studies of the facial triad carboxylate variants in FIH indicated that the D201 residue is not required for O$_2$ activation and instead aids in coupling O$_2$ activation with substrate hydroxylation.$^{12}$ This observation allowed us to investigate the role of the facial
triad carboxylate ligand in coupling O₂ reactivity and substrate binding. Previous site-directed mutagenesis studies conducted with D201 hypothesized that this residue is not required for metal binding or hydroxylation activity of FIH. However, more recently, it was revealed that non-polar mutations at the D201 site bind a halide anion, completing the facial triad and permitting hydroxylation activity (Chapter 2). While the kinetics and structure of the D201X variants have been reported by our lab and others, questions remain over how and why the D201X variants inactivate. Herein we report a branched mechanism of enzyme uncoupling for the D201X variants without substrate triggering and describe the fate of the oxidant in the absence of primary substrate, which results in autohydroxylation and formation of hydrogen peroxide (H₂O₂).

4.2 Methods

4.2.1 Materials

The prime substrate for FIH, the C-terminal transactivation domain of HIF-1α (CTAD) was purchased (EZBiolab, Carmel, IN, USA) as a desalted 39-residue peptide representing HIF-1α⁷⁸⁸-⁸₂⁶ with the sequence DESGLPQLTSYDAEVNAPIQGSRNLLQGEELLRALDQVN (N803 hydroxylation site underlined). This peptide was unmodified at the peptide termini and contained a C800A point mutation to prevent peptide oxidation. The desalted peptide was further purified by reverse phase HPLC before use (see Chapter 3, section 3.2.1 for detailed methods). All other reagents were purchased from commercial vendors and used as received.
4.2.2 Protein expression and purification

Point mutations to the 201 site were made using the QuickChange mutagenesis kit (Stratagene) in the pET28a-FIH construct. The resulting plasmid DNA was sequenced (Genewiz, NJ, USA) to confirm the point mutation. His$_6$-WT FIH and the His$_6$-D201X variants were overexpressed in BL21-DE3 *E. coli* and purified as previously described (Chapter 2). Briefly, cell pellets were lysed using sonication, centrifuged, then dialyzed into 10 mM Tris pH 8.00 to remove EDTA from the lysis buffer. Centrifugation of the supernatant post dialysis further clarified the lysate prior loading on to a Ni-NTA column. After loading the lysate, the column was washed with 5 column volumes each of 100% A buffer (50 mM Tris pH 8.0, 300 mM NaCl, 15 mM Imidazole) and 15% B buffer (50 mM Tris pH 8.0, 300 mM NaCl, 250 mM Imidazole) to remove contaminating proteins before eluting the His$_6$-FIH protein with 100% B buffer. Thrombin was then added to the eluent to cleave the His$_6$ tag for 36 hours at 4 °C, resulting in >80% cleavage. The cleaved protein was loaded on to the Ni-NTA column again to remove the His$_6$ tag and any remaining uncleaved protein. This was followed by overnight incubation with 50 mM EDTA to remove exogenous metals. Size exclusion chromatography was then used to separate the protein from the thrombin and EDTA. Purified protein was aliquotted and stored at 20 °C in 50 mM HEPES pH 7.00. Protein purity (>95%) was evaluated using SDS-PAGE.

4.2.3 O$_2$ consumption assays

The amount of oxygen consumed by the enzyme was monitored using an Oxygraph Plus System (Hansatech). Each day, a new membrane was prepared with subsequent electrode calibration. Ascorbate (50 µM), αKG (100 µM), FeSO$_4$ (50 µM), and CTAD (80 µM) in 50 mM HEPES pH 7.00 (400 µL total) were equilibrated at atmospheric O$_2$ in the
reaction vessel at 37°C until a stable baseline was achieved. The reaction was then initiated with cold enzyme using a Hamilton syringe, with a final concentration of 10 µM FIH in solution. O₂ consumption over time was recorded until the rate of O₂ consumption resembled the baseline slope.

4.2.4 Steady-state kinetics varying O₂

Assays were conducted as described for the O₂ consumption experiment, with the amount of O₂ in the system being controlled for. Ratios of N₂ and O₂ gasses were mixed using a flow-meter to provide a wide range of O₂ concentrations. Assays were conducted at 37 °C and contained ascorbate (50 µM), αKG (100 µM), FeSO₄ (50 µM), CTAD (80 µM) and FIH (1-5 µM) in 50 mM HEPES pH 7.00. D201G assay buffer contained 100 mM NaCl. Upon consumption of O₂, either the slope of the O₂ trace was monitored to determine the initial rate of O₂ consumption or a sample of the reaction mixture was taken and quenched in matrix (α-Cyano-4-hydroxycinnamic acid dissolved in 75% ACN/0.2% TFA). At 20 second intervals, 5 µL of assay mixture were quenched in 20 µL of matrix (3,5-dimethoxy-4-hydroxycinnamic acid saturated in 75% ACN/0.2% TFA). The ratio of hydroxylated to unhydroxylated CTAD substrate was measured using MALDI-TOF-MS (Bruker Ultraflextreme MALDI TOF/TOF mass spectrometry system) to determine the mole fraction of hydroxylated product at each time point. The initial rates versus O₂ concentration were then plotted in Origin and fit to the Michaelis-Menten equation (below) to ascertain the steady-state parameters for each substrate. Equation 1 was used to define $k_{cat}$ and $K_M$ under the given conditions. Equation 2 defines $k_{cat}/K_M$ with greater precision.
Equation 1\(^1\): \[ y = \frac{v_{\text{max}}x}{K_M + x} \]

Or

Equation 2\(^2\): \[ y = \frac{(V/K)x}{1 + (x/K_M)} \]

---

\(1\) \(v_{\text{max}}\) = maximal rate of reaction
\(K_M\) = concentration of substrate when reaction velocity is equal to one half the maximal velocity of the reaction (\(V_{\text{max}}\))

\(2\) \((V/K)\) = \(v_{\text{max}}\) divided by \(K_M\) defines \(k_{\text{cat}}/K_M\), a measure of enzyme efficiency
\(K_M\) = concentration of substrate when reaction velocity is equal to one half the maximal velocity of the reaction (\(V_{\text{max}}\))
4.2.5 Autohydroxylation assays

Autohydroxylation studies were conducted as previously described.\textsuperscript{15,16} Briefly, samples containing αKG (500 µM), FeSO\textsubscript{4} (100 µM) and FIH (100 µM) in 50 mM HEPES pH 7.00 were prepared anaerobically in a quartz cuvette and sealed. Once out of the glove box, slow oxidation was stimulated by removal of the cuvette cap. Absorbance was monitored over time upon exposure of the sample to oxygen using an Agilent HP 8453 diode-array UV-Vis spectrophotometer.

4.2.6 H\textsubscript{2}O\textsubscript{2} assays

H\textsubscript{2}O\textsubscript{2} induced oxidation of 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) was coupled with 1 U/mL horseradish peroxidase (HRP) to detect H\textsubscript{2}O\textsubscript{2} production by FIH.\textsuperscript{17} The reactions contained αKG (500 µM), FeSO\textsubscript{4} (50 µM), CTAD (80 µM), ABTS (150 µM), and FIH (20 µM) in 50 mM HEPES pH 7.00. H\textsubscript{2}O\textsubscript{2} production was monitored by absorbance at 405 nm. Ascorbate was omitted from the reaction mixtures to avoid reduction of ABTS\textsuperscript{+}. The amount of ABTS\textsuperscript{+} produced by the reactions was calculated using Beer’s Law (\(\varepsilon_{\text{ABTS}^{+}} = 36,100 \text{ M}^{-1}\text{cm}^{-1}\))\textsuperscript{18} and fit (Origin) to a single exponential equation.

\[
\text{H}_2\text{O}_2 + 2 \text{ABTS} \rightarrow 2 \text{ABTS}^{+}
\]

2.2.7 EPR

Spectra of the vanadyl-substituted WT FIH and the D201X variants were collected on a Bruker Elexsys-500 EPR spectrometer using Q-Band (QT403 cavity). Samples were positioned under vacuum in a liquid-nitrogen cryostat. Samples were composed of 1 mM
FIH, 0.9 mM VOSO$_4$ in 10 mM H$_2$SO$_4$, 0.9 mM succinate in water, and 0.9 mM CTAD (when noted) in 50 mM HEPES pH 7.00. Metal was added in 0.5 uL increments to prevent precipitation. Samples were loaded into EPR tubes and flash frozen with LN$_2$ for analysis. Spectra were collected at 90 K with the following settings: 34 MHz frequency, 0.61 mW power, 2.5 G modulation amplitude, and 40.96 ms time constant and conversion time. Data was simulated using XSophe software (Bruker). Error was determined by visual differences in the simulated spectra upon incrementally increasing/decreasing each simulation parameter.
4.3 Results and Discussion

4.3.1. Catalytic activity of D201X variants

Proper O₂ activation is required for coupled catalytic activity of αKG dependent oxygenases. It is well known for this class of enzymes that the binding of primary substrate “triggers” the release of an aquo ligand from the Fe(II) cofactor, providing a vacant coordination site for O₂ to bind (Scheme 4.1).¹⁹ The strength of the Fe-OH₂ bond mediates this substrate triggering mechanism and subsequent O₂ activation. Upon O₂ activation, αKG is oxidatively decarboxylated to produce succinate and CO₂. Uncoupled chemistry results when the half reaction of decarboxylation occurs faster than that of product formation and has been noted for several αKG dependent oxygenases.⁸,¹¹,¹⁵,¹₆,₂⁰,₂¹

Tight coupling between CTAD binding and subsequent O₂ activation is required for effective hypoxia sensing by FIH as evidenced by changes in the coordination geometry of the Fe(II) cofactor.¹⁴ In addition, this O₂ activation step is rate limiting, supporting FIH’s role as an O₂ sensor.²² This was verified by an inverse solvent isotope effect (SIE) indicating that there was a pre-equilibrium aquo release followed by an irreversible step in the consensus mechanism.¹⁴ Previous studies of the D201X variants identified uncoupling between succinate formation and CTAD⁰H.¹² In order to gain insight into the substrate triggering effect and the resulting uncoupled O₂ activation chemistry in the D201X variants, steady-state kinetics assays were conducted varying O₂ and monitoring O₂ consumption and CTAD⁰H in tandem.

The kinetic studies varying O₂ using a Clark-type O₂ sensor (Fig. 4.1) showed pronounced uncoupling between the two half reactions of the D201X variants, whereas the
rates of O₂ consumption and CTAD^{OH} were equal for WT FIH. The exceedingly low reactivity of D201A made it difficult to distinguish O₂ consumption from the instrument noise. \( k_{\text{cat}} \) of CTAD^{OH} production for both the D201E and D201G variants was much slower than \( k_{\text{cat}} \) of O₂ consumption (Supplemental Table 4.4), supporting uncoupled catalytic activity. As \( k_{\text{cat}} \) measures turnover when CTAD and other substrates are saturating, a better measure of branching is \( k_{\text{cat}}/K_{M(O₂)} \) (Table 4.1). If O₂ consumption were tightly coupled to CTAD^{OH}, \( k_{\text{cat}}/K_{M(O₂)} \) for CTAD^{OH} formation divided by the \( k_{\text{cat}}/K_{M(O₂)} \) for O₂ consumption would equal 1. Based on the \( k_{\text{cat}}/K_{M(O₂)} \) values reported in Table 4.1, this is only the case for WT FIH. The ratios of \( k_{\text{cat}}/K_{M(O₂)} \) for D201E (0.05) and D201G (0.03) were nowhere near unity, confirming that O₂ activation is largely uncoupled. The 20-35 fold changes between the \( k_{\text{cat}}/K_{M(O₂)} \) seen with D201G and D201E for O₂ consumption versus CTAD^{OH} suggested there was an alternate pathway for the fate of O₂. This data indicate that the reaction pathway is branched, and that O₂ can be consumed without substrate present.
Figure 4.1 Steady-state kinetics varying $O_2$ using an oxygen sensor monitoring $O_2$ consumption (filled shapes) versus CTAD$^{\Delta H}$ (empty shapes). Assays were conducted at 37 °C and contained ascorbate (50 µM), αKG (100 µM), FeSO$_4$ (50 µM), CTAD (80 µM) and FIH (1-5 µM) in 50 mM HEPES pH 7.00. D201G assay buffer contained 100 mM NaCl.
Table 4.1 $k_{\text{cat}}/K_M(O_2)$ values for O$_2$ consumption and CTAD$^{\text{OH}}$ formation.

<table>
<thead>
<tr>
<th></th>
<th>$d[O_2]/dt$ (µM$^{-1}$ min$^{-1}$)</th>
<th>$d[\text{CTAD}^{\text{OH}}]/dt$ (µM$^{-1}$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FIH</td>
<td>0.097 ± 0.008</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>D201G</td>
<td>0.08 ± 0.03</td>
<td>0.004 ± 0.003</td>
</tr>
<tr>
<td>D201E</td>
<td>0.07 ± 0.01</td>
<td>0.002 ± 0.001</td>
</tr>
</tbody>
</table>

To further explore the mechanistic branching in the D201X variants, O$_2$ consumption assays were conducted in the presence and absence of CTAD, and the fate of activated O$_2$ was measured. αKG dependent oxygenases typically react sluggishly with O$_2$ until triggered by substrate binding. For example, the halogenase SyrB2 reacts with O$_2$ ~5000 times faster upon binding of primary substrate. A more closely related enzyme, PHD2, has been reported to react 30 times faster with O$_2$ in the presence of primary substrate. Due to known structural differences in the substrate-binding intermediate of the facial triad variants (II-Scheme 4.1) and the increased rate of autohydroxylation compared to WT FIH, we hypothesized that the D201X variants may be primed to react with O$_2$ before CTAD binds.

O$_2$ consumption was monitored using a Clark-type electrode upon addition of enzyme to the reaction mixture. As seen in Supplemental Figure 4.6, WT FIH, D201E and D201G all consumed O$_2$ when initiated with enzyme. Without CTAD, D201E rapidly consumed O$_2$ (Fig. 4.2A), whereas D201G and WT FIH slowly consume O$_2$ at relatively the same rate (Table 4.2). D201A only consumed 2-5 µM O$_2$ regardless of primary substrate indicating that removing the facial triad ligand at this site weakens O$_2$ activation, supporting the lack of hydroxylation activity seen with this variant. In the absence of CTAD, D201G and WT
FIH consumed 5 µM and 8 µM O₂ respectively, several fold less than in the presence of CTAD, which suggested these variants were substrate trigged. The similar reactivity of D201E (~20 µM O₂ consumed) with or without CTAD supported the hypothesis that D201E can react with O₂ without substrate triggering.

Figure 4.2 (A) O₂ consumption in the absence of CTAD substrate for all D201X variants and WT FIH. (B) Steady state kinetics varying O₂ for D201E in the absence of CTAD using an oxygen sensor monitoring O₂ consumption. Assays were conducted at 37 ºC and contained ascorbate (50 µM), αKG (100 µM), FeSO₄ (50 µM), CTAD (80 µM) and FIH (1-5 µM) in 50 mM HEPES pH 7.00. D201G assay buffer contained 100 mM NaCl.
Additional assays were conducted varying O₂ while monitoring O₂ consumption to define the kinetics of O₂ consumption in the absence of CTAD for D201E (Fig. 4.2B). Interestingly, $k_{\text{cat(O2)}}$ without CTAD present (3.0 min⁻¹) is about equal to $k_{\text{cat(O2)}}$ with CTAD (3.6 min⁻¹) (Supplemental Information, Table 4.4). The similarities in $k_{\text{cat}}$ suggest that D201E is primed to react with O₂ before it is presented with primary substrate, resulting in little to no CTAD⁰⁸H. This reactivity is supported by structural evidence reported in Solomon et al., in which we described that D201E is mostly five coordinate Fe(II) before it encounters CTAD, suggesting there is an open coordination site for O₂ to bind whereas WT FIH remains six coordinate until CTAD is present. This change in coordination environment resulting in uncoupled turnover has also been reported for TauD.²⁴

Table 4.2 summarizes the substrate triggering data presented here. The triggering ratio provides insight into the efficiency of product formation. For substrate triggered enzymes, the productive triggering ratio was expected to be 1. A productive triggering ratio of less than 1 suggests the enzyme can react with O₂ without primary substrate. As seen in Table 4.2, the productive triggering ratio is about 1 for WT FIH and almost 10-fold lower for D201E and D201G. The unproductive triggering ratio is extremely high for substrate triggered O₂ reactions, such as WT FIH which has a triggering ratio of 156, and low (0.14 and 2.2) for constitutively triggered reactions, seen with the D201X variants.

<table>
<thead>
<tr>
<th>Variant</th>
<th>$k_{\text{cat(CTAD)}}$ (min⁻¹) d[CTAD⁰⁸H]/dt</th>
<th>$k_{\text{cat(CTAD)}}$ (min⁻¹) d[O₂]/dt</th>
<th>Productive triggering ratio</th>
<th>O₂ uptake -CTAD (min⁻¹)</th>
<th>Unproductive triggering ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>28 ± 3</td>
<td>33 ± 4</td>
<td>0.85</td>
<td>-0.18 ± 0.002</td>
<td>156</td>
</tr>
<tr>
<td>D201A</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>D201E</td>
<td>0.08 ± 0.63</td>
<td>3.6 ± 0.2</td>
<td>0.02</td>
<td>-0.59 ± 0.002</td>
<td>0.14</td>
</tr>
<tr>
<td>D201G</td>
<td>0.58 ± 0.02</td>
<td>6.1 ± 0.7</td>
<td>0.10</td>
<td>-0.26 ± 0.001</td>
<td>2.2</td>
</tr>
</tbody>
</table>
Substrate triggered activation of the Fe(II) cofactor for reaction with O₂ is a hallmark trait of the αKG dependent oxygenases²¹,²³,²⁵ and is thought to act as a defense mechanism against deleterious auto-oxidation reactions and improper substrate binding.⁴,⁵ Reported reactions for this class of enzymes with O₂ in the absence of substrate were sluggish, for example TauD reacted 1,000 times faster with O₂ in the presence of substrate.²⁶ However, the results presented here suggest this is not always the case as D201E reacts with O₂ as well with substrate as it does without, leading to the notion that this variant is constitutively triggered. Previous studies suggest untriggered enzymes may proceed through a different mechanism as there is no evidence of accumulated Fe(IV)=O intermediate under these conditions, therefore we sought to track the fate of O₂ in this untriggered pathway.⁵,²⁶

4.3.2 Triggering efficiency

The O₂ reactivity studies described above indicated that the D201X variants react with O₂ without substrate present, leading to uncoupled turnover. In order to track the fate of O₂ in this untriggered mechanism, autohydroxylation and ROS production studies were conducted. Autohydroxylation has been reported for other αKG dependent oxygenases including TfdA and Jmjd6, as well as WT FIH.¹⁴,¹⁵,²⁰,²⁷ Previous studies revealed that the D201X variants autohydroxylated faster than WT FIH, however rates of autohydroxylation were not stated.¹² Autohydroxylation is evidence by the accumulation of a pink chromophore (λₘₐₓ= 515 nm, ε₅₁₅= 2500 M⁻¹cm⁻¹) in D201A and D201G and a purple Fe(III)-O-Trp296 chromophore for D201E and WT FIH (λₘₐₓ= 583 nm, ε₅₈₃= 3000 M⁻¹cm⁻¹).¹² Herein we report on the initial rate of Trp⁴⁰H and quantification of autohydroxylated product formation in the (Fe⁺αKG)FIH samples upon exposure to air. As seen in Figure 4.3, D201A produced the most Trp⁴⁰H product at the fastest rate, compared the WT FIH and
the other D201X variants. D201A autohydroxylated about 50% of the available enzyme whereas D201E, D201G, and WT FIH autohydroxylated 10-20%. The initial rates of Trp$^\text{OH}$ product formation were reported (Table 4.3). Results suggest the concentration of Trp$^\text{OH}$ is high when the variants have poor triggering.

**Figure 4.3** Formation of autohydroxylated product, evidenced by the appearance of a chromophore at 583 nm for WT and D201E and at 515 nm for D201G and D201A. D201E (triangle), D201A (diamond), WT (square), and D201G (circle).

Autohydroxylation assays were conducted using αKG (500 µM), FeSO$_4$ (100 µM) and FIH (100 µM) in 50 mM HEPES pH 7.00. 100 mM NaCl was added to the assay buffer for D201G.

4.3.3 ROS production

In addition, some uncoupled αKG dependent oxygenases have been reported to produce ROS as a byproduct.$^{17,28}$ Possible ROS include superoxide (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), and hydroxyl radicals (OH$^\cdot$) based on the number of electrons transferred to O$_2$.$^4,29$ To probe if ROS were formed by the D201X variants as a product of untriggered reactions with O$_2$, a UV-Vis spectroscopy based assay was conducted. A peroxidase/ABTS assay was utilized to detect H$_2$O$_2$ formation by monitoring the accumulation of ABTS$^+$ at
405 nm in the absence of CTAD (Fig. 4.4). The 1:2 stoichiometry of H$_2$O$_2$ to ABTS\(^+\) allowed for the concentration of H$_2$O$_2$ to be easily determined. Without CTAD, very low amounts of H$_2$O$_2$ were produced. D201E quickly reacted and was inactivated, whereas WT FIH, D201G and D201A showed a linear increase in product formation over time. The autohydroxylation and peroxidase assays in the absence of CTAD provided insight into the fate of O$_2$ without substrate triggering, however there was still unaccounted for O$_2$ following substrate triggering.

![Figure 4.4](image)

**Figure 4.4** H$_2$O$_2$ formation detected by oxidation of ABTS in the absence of CTAD. D201E (triangle), D201A (diamond), WT (square), and D201G (circle). H$_2$O$_2$ assays contained αKG (500 µM), FeSO$_4$ (50 µM), CTAD (80 µM), ABTS (150 µM), and FIH (20 µM) in 50 mM HEPES pH 7.00. 100 mM NaCl was added to the D201G assay buffer.

<table>
<thead>
<tr>
<th></th>
<th>$^3$Trp$^{OH}$ ($k_{obs}$) min$^{-1}$</th>
<th>$^4$H$_2$O$_2$ ($v_e/[E]$) min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>0.008 ± 0.001</td>
<td>0.002 ± 0.30</td>
</tr>
<tr>
<td>D201A</td>
<td>0.056 ± 0.002</td>
<td>0.008 ± 0.03</td>
</tr>
<tr>
<td>D201E</td>
<td>0.029 ± 0.003</td>
<td>0.015</td>
</tr>
<tr>
<td>D201G</td>
<td>0.027 ± 0.001</td>
<td>0.003 ± 0.12</td>
</tr>
</tbody>
</table>

$^3$ Trp$^{OH}$ data was fit to a single exponential equation.

$^4$ H$_2$O$_2$ data was fit to a linear equation. The slope of the data was divided by the enzyme concentration (20 µM) to obtain the initial rate.
Based on the O₂ kinetics and spectroscopic data presented here, we propose the following branched chemical mechanism of untriggered reactivity with O₂ for the D201X variants in the absence of CTAD. As shown in Scheme 4.2, these variants proceed through the same states as WT FIH through the formation of the Fe(IV)=O intermediate. From this state, there were two potential pathways: hydrogen atom transfer to form a Trp²⁹⁶ radical, resulting in autohydroxylation or hydrolysis to form H₂O₂. As a result of the data presented here, we propose that a combination of kinetic and structural factors regulate the fate of O₂ from the Fe(IV)=O intermediate.

Scheme 4.2 Proposed branched mechanism of untriggered O₂ reactivity in αKG dependent oxygenases in the absence of primary substrate (R).

### 4.3.4 Ferryl geometry at branch point

In order to test our hypothesis that untriggered reactions with O₂ deviate from the consensus chemical mechanism following formation of the Fe(IV)=O intermediate, EPR studies were conducted. Recently, vanadyl was shown to be a suitable Fe(IV)=O structural mimic to facilitate EPR and crystallographic studies of this crucial intermediate state in TauD.³⁰ We adopted this technique to gain insight into the structure of the metal center of the D201X variants and WT FIH. Samples consisting of (VO+succinate)FIH with and without CTAD were prepared, loaded into quartz capillary tubes and flash frozen. All
samples were run at liquid nitrogen temperatures to ensure powder pattern spectra free of molecular tumbling.

Vanadyl EPR spectra typically exhibit axial ($A_z > A_x = A_y$) or near-axial symmetry due to the dominance of the vanadium-oxygen interaction in defining the d-orbital splitting. Q-Band EPR simulations of the experimental data shown in Figure 4.5 revealed all D201X variants and WT FIH displayed axial symmetry with and without CTAD, which was expected for the (VO+succinate)FIH sample composition. Additional analysis of the hyperfine splitting revealed a geometric shift in the Fe(IV)=O intermediate upon addition of CTAD to WT FIH and D201G as evidenced by a 4-5 MHz change in $A_\perp$ (Table 4.4). Interestingly, $A_\perp$ increased in the presence of CTAD for WT FIH and decrease for D201G. These geometric differences may explain the coupled versus uncoupled nature of WT and D201G respectively following CTAD binding. The D201E and D201A variants did not exhibit any geometric shift upon addition of CTAD, suggesting these variants are poised to react with $O_2$ without substrate triggering.

Taking a closer look at the WT FIH EPR spectra (Figure 4.5), we see that the spectra without CTAD yielded simulation parameters of $g = [1.943, 1.979, 1.979] \pm 0.001$ and $A = [515, 195, 180] \pm 3$ MHz. Upon addition of CTAD, $A_\perp$ (derived from the average of $A_x$ and $A_y$) was increased, yielding simulation parameters of $A = [525, 195, 190] \pm 3$ MHz. CTAD addition also increased $A_z$ by 10 MHz suggesting weakening of the V=O bond. The small shifts in $g$ values +/- CTAD are still being investigated at the time of this writing. The $A_\parallel$ hyperfine simulation value provides insight into the identity of the ligands in the equatorial positions of the vanadyl. For WT FIH, the observed $A_\parallel = 512$ MHz (170 x $10^{-4}$ cm$^{-1}$) correlates to the expected value of 170.9 x $10^{-4}$ cm$^{-1}$ for two carboxylate, one
perpendicular, and one parallel nitrogen ligand. While $A_\parallel$ values may deviate from the theoretical value due to changes in symmetry, it provides a reasonable reference to establish the ligand coordination environment of the Fe(IV)=O intermediate. This data suggested that these variants uncouple after the formation of the Fe(IV)=O intermediate as all the variants formed the Fe(IV)=O based on $A_\parallel$ values for the V=O mimic.

Figure 4.5 Vanadyl Q-Band EPR of WT FIH and D201X variants in the presence of succinate, with or without CTAD. WT FIH (black), D201A (green), D201E (blue), and D201G (red). Samples were composed of 1 mM FIH, 0.9 mM VOSO$_4$, 0.9 mM succinate,
and 0.9 mM CTAD (when noted) in 50 mM HEPES pH 7.00. The D201G sample was prepared in 50 mM HEPES/100 mM NaCl buffer pH 7.00.

Table 4.4 (VO+succ)FIH Q-Band EPR Simulation Parameters.

<table>
<thead>
<tr>
<th></th>
<th>5$A_{\perp}$ (MHz)</th>
<th>$A_{\parallel}$ (MHz)</th>
<th>6$g_{\perp}$</th>
<th>$g_{\parallel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT FIH</td>
<td>188</td>
<td>515</td>
<td>1.979</td>
<td>1.943</td>
</tr>
<tr>
<td>WT CTAD</td>
<td>193</td>
<td>525</td>
<td>1.980</td>
<td>1.943</td>
</tr>
<tr>
<td>D201A</td>
<td>181</td>
<td>504</td>
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<td>1.943</td>
</tr>
<tr>
<td>D201A CTAD</td>
<td>181</td>
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<td>1.943</td>
</tr>
<tr>
<td>D201E</td>
<td>181</td>
<td>509</td>
<td>1.978</td>
<td>1.943</td>
</tr>
<tr>
<td>D201E CTAD</td>
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<td>509</td>
<td>1.978</td>
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<tr>
<td>D201G CTAD</td>
<td>185</td>
<td>510</td>
<td>1.979</td>
<td>1.943</td>
</tr>
</tbody>
</table>

The change in $A_{\perp}$ for the D201X variants that productively hydroxylate substrate indicate that the metal centers of WT FIH and chloride-saturated D201G are triggered by substrate, leading to $O_2$ activation. In contrast, the geometry of the metal centers in D201E and D201A were insensitive to CTAD and therefore were constitutively triggered. The lack of sensitivity of D201E and D201A supported their increased rates of autohydroxylation.

---

5 Hyperfine uncertainty values: $A \pm 3$ MHz
6 $g$ values uncertainty: $g \pm 0.002$
4.4 Conclusion

This study provides further insight into the kinetics of uncoupling for the D201X variants of FIH, offering a basis for reactivity of constitutively triggered enzymes in the αKG dependent oxygenase family of enzymes. It was previously established that variants at the 201 site could hydroxylate peptide substrate, given the facial triad remained intact with either a carboxylate or an anion to coordinate the metal (Chapter 2). Kinetic studies varying O₂ revealed that the variants reacted with O₂ much faster than they produced CTAD⁰H, suggesting a branched catalytic mechanism. The facial triad variants retained the ability to activate O₂ but lost the ability to trigger upon substrate binding. The formation of Trp⁰H and H₂O₂ furnished a pathway for this unreacted oxygen. This is the first reported example of ROS formation in FIH. This work directly implicates the native aspartate residue in substrate triggering. EPR provided structural data to demonstrate that the metal geometry changed in the presence of CTAD, consistent with changes in coordination reported by Solomon et al.24 Based on the evidence provided here, we conclude that the facial triad is essential for coupling hydroxylation to O₂ activation in FIH.

4.5 Supplemental Information

4.5.1 O₂ consumption

To confirm the D201X variants consumed O₂ in the presence of CTAD, additional assays were conducted. As shown in Fig 4.6, WT, D201G, and D201E all rapidly consume O₂ with CTAD present. D201A slowly reacts with O₂.
Figure 4.6. O₂ consumption of the D201X variants and WT FIH with CTAD present.

The different enzyme variants are represented as follows: WT FIH (black), D201A (green), D201E (blue), D201G (red). Assays were conducted at 37 °C and contained ascorbate (50 µM), αKG (100 µM), FeSO₄ (50 µM), CTAD (80 µM- when noted) and FIH (10 µM) in 50 mM HEPES pH 7.00. D201G assay buffer contained 100 mM NaCl.

4.5.2 Steady-state kinetics

Table 4.5 summarizes the steady-state kinetic data for the D201X variants and WT FIH, which was presented in various parts of the results and discussion section of this chapter.
4.5.3 ROS production

Additional H$_2$O$_2$ assays were conducted in the presence of CTAD. Figure 4.7 shows the formation of H$_2$O$_2$ under the given conditions, suggesting that H$_2$O$_2$ formation was an alternate pathway for O$_2$ consumption both in the presence and absence of CTAD.

---

7 Assays were conducted at 37 °C and contained ascorbate (50 µM), αKG (100 µM), FeSO$_4$ (50 µM), CTAD (80 µM) and FIH (1-5 µM) in 50 mM HEPES pH 7.00. D201G assay buffer contained 100 mM NaCl.
Figure 4.7 H$_2$O$_2$ formation detected by oxidation of ABTS in the presence of CTAD. D201E (triangle), D201A (diamond), WT (square), and D201G (circle). H$_2$O$_2$ assays contained αKG (500 µM), FeSO$_4$ (50 µM), CTAD (80 µM), ABTS (150 µM), and FIH (20 µM) in 50 mM HEPES pH 7.00. 100 mM NaCl was added to the D201G assay buffer.
4.6 References


APPENDIX A

MODULATING HIF-1α TRANSCRIPTIONAL RESPONSE THROUGH ENZYME DELIVERY

A.1 Introduction

Due to the plethora of genes regulated by Hypoxia Inducible Factor (HIF)-1, control over this transcription factor is of high therapeutic interest. In this research, we aimed to manipulate the catalytic activity of the HIF hydroxylases to control the hypoxic response through enzyme delivery. Overexpressed HIF-1α levels are found in numerous disease states such as tumor growth and hypertension, highlighting the need for delivery of active enzyme to downregulate genes involved in these processes.¹ We hypothesized that in vivo HIF-1α levels could be controlled through targeted delivery of FIH using nanomaterials. Purified protein was delivered to normoxic HeLa cells. Delivery efficiency was monitored through gene expression analysis both at the mRNA and protein levels as outlined in Scheme A.1. Development of an efficient delivery system provides a broad range of control over genes regulated by HIF-1α.
A.2 Methods

A.2.1 Delivery of FITC-FIH polymer emulsion

FIH was labeled using fluorescein isothiocyanate (FITC) to quickly confirm enzyme delivery into cells using a polymer stabilized nanoparticle delivery system.(xx ref) A 1 mg stock of His tagged FIH in 50 mM HEPES pH 7.00 was thawed on ice. 150 µg of solid FITC were dissolved in 50 µL DMSO for protein labeling. A 6:1 ratio of FIH:FITC was then prepared in 100 mM NaHCO₃ pH 8.5 and incubated overnight at 4 °C. The FIH:FITC solution was loaded on to a prepacked gravity PD-10 desalting column (GE Healthcare) to remove unbound FITC. Following FITC labeling of FIH, a 1:1 ratio of FITC-FIH and polymer emulsion was prepared. The emulsion was formed by dissolving the polymer in a mixture of linoleic acid and decanoic acid. 50 µL of the FITC-FIH-polymer emulsion was diluted in 500 µL serum free DMEM (GenClone) containing 1% pen/strep antibiotic; this mixture was added to HeLa cells plated at 60,000 cells per well.
and incubated for 1 hour at 37 °C and 5% CO₂. Following incubation, the DMEM media was rinsed off and replaced with 1X PBS. Cells were visualized using fluorescent microscopy (Nikon A1 Spectral Confocal) to confirm enzyme delivery.

A.2.2 RT-qPCR primer design and validation

Housekeeping and target gene primers were designed using Primer Blast software and ordered from IDT (25 nmol DNA oligio standard desalting). Tₘ and length of housekeeping primer (beta actin) were designed to be as close to those of the target gene primers as possible. 10 µM working stocks of primers were prepared in nuclease free H₂O and validated. Assays containing 2X SYBR Green Master Mix and 0.4 µM primer were prepared, varying concentrations of cDNA (1:1, 1:5, 1:25, 1:125) to determine optimal annealing temperature and measureable Cₜ range for the template DNA. An annealing temperature gradient was run on the BioRad CFX Connect Real-Time PCR Detection System instrument followed by a thermal melt program to verify Tₘ of each primer in the presence of template DNA. Products were then run on an agarose gel to identify possible primer-dimers. A standard curve of Cₜs was constructed from the serial dilutions of template DNA. The optimized annealing temperatures were used to conduct the following RT-qPCR experiments.

A.2.3 RT-qPCR

HeLa cells were treated for 24 hours at 37 °C and 5% CO₂ with either no CoCl₂, 100 µM CoCl₂ or 250 µM CoCl₂, then were trypsinized and pelleted. Cells were lysed with sonication (2 seconds on/1 minute off for three cycles at 50 amplitude) followed by mRNA
isolation using a Pure Link mRNA Isolation Kit (Life Technologies). The mRNA was then converted to cDNA using an iScript cDNA Synthesis Kit (BioRad). The concentration of cDNA (A_{260}) was verified using a nanodrop. Samples were prepared for RT-qPCR in 96-well PCR plates and sealed to prevent evaporation. Samples contained 2X SYBR Green Master Mix (ThermoFisher), 0.4 μM forward primer, 0.4 μM reverse primer, 500 ng template cDNA, and nuclease free H_{2}O. Primers were designed and validated as described above. Plates were run on a BioRad CFX Connect Real-Time PCR Detection System instrument with the following program:

\[
\begin{align*}
\text{Initial Denaturation} & \quad 95^\circ \text{C} & \quad 10 \text{ min} & \quad 1 \text{ cycle} \\
\text{Denaturation} & \quad 95^\circ \text{C} & \quad 15 \text{ s} & \quad 40 \text{ cycles} \\
\text{Annealing/Extension} & \quad 60^\circ \text{C}* & \quad 1 \text{ min} & \\
\end{align*}
\]

*annealing/extension temperature was optimized for each set of primers

Expression of target genes was normalized to expression of beta actin housekeeping gene and quantitated using the comparative \( C_{T} \) method (\( 2^{-\Delta\Delta C_{T}} \)).

\( A.2.4 \) Western Blotting

Western blotting procedures required substantial optimization to visualized HIF-1α protein levels in CoCl_{2} treated mammalian cells. HeLa cells were treated for 24 hours at 37 °C and 5% CO_{2} with either no CoCl_{2}, 100 μM CoCl_{2} or 250 μM CoCl_{2}. HeLa cells were then extracted from the cell culture plate using RIPA buffer (Fisher) containing 10 μL/mL protease cocktail inhibitor (Sigma #P8340). Cells were scraped into a sterile falcon tube and incubated on ice for 15 minutes. Next, cells were lysed with sonication (2 second on/1 minute off for three cycles at 50 amplitude), incubated on ice for 15 minutes, and
centrifuged for 5 minutes at 12,500 rpm (Eppendorf, FA-45-18-11 rotor) at 4 °C. The supernatant containing nuclear extract was then harvested and the concentration of total protein concentration was verified using a bicinchoninic acid (BCA) assay. Protein was aliquotted and stored at -80 °C for future use.

10% acrylamide SDS PAGE gels were prepared for protein separation. 50 µg of denatured nuclear protein were loaded in each well. 15 µL of a prestained protein marker (Fisher) was also loaded on to the gel as a molecular weight control. The SDS PAGE gel was run at 120 V until the dye front reached the bottom of the gel. Separated proteins were then transferred on to a nitrocellulose membrane at 100 v for 1 hour and 45 minutes at 4 °C. The membrane was then blocked in 5% dry milk in TBST for 1 hour while shaking at room temperature. Blocking buffer was removed and replaced with HIF-1α primary antibody (Cell Signaling) solution (1:1,000 5% BSA in TBST). The membrane was then incubated in primary antibody solution overnight at 4 °C while shaking. Following primary antibody incubation, the membrane was rinsed three times with TBST to remove primary antibody solution. The membrane was then incubated in secondary antibody (Novus anti rabbit) solution (1:20,000 5% BSA in TBST) for 1 hour while shaking at room temperature. The membrane was again rinsed three times with TBST prior to adding Pierce ECL Western Blotting Substrate (Fisher). This chemiluminescent substrate was incubated on the membrane for 1 minute at room temperature, then visualized with UV light on BioRad ChemiDoc system.
A.2.5 Proliferation assays

Cell proliferation was monitored using a Cell Titer 96 Aqueous One solution (Promega) assay. Cells were plated at various densities in a 96 well plate containing 100 µL of media containing no CoCl$_2$, 50 µM CoCl$_2$, 100 µM CoCl$_2$, 250 µM CoCl$_2$, 500 µM CoCl$_2$, or 1 mM CoCl$_2$ and incubated for 24 hours at 37 ºC and 5% CO$_2$. Following incubation, 20 µL of Promega Cell Titer 96 reagent was added directly to the cell media. The 96 well plate was then incubated for 1 hour at 37 ºC and 5% CO$_2$. Absorbance at 490 nm was recorded for each well to assess cell viability during hypoxic treatment.

A.2.6 FIH-polymer emulsion delivery optimization

HeLa cells were plated at a density of 3 x 10$^5$ cells per well and left to adhere overnight. A solution of unlabeled His-FIH-polymer emulsion was prepared in 1X PBS containing 2.4 µM His-FIH, 36 µM polymer, and 10% linoleic/decanoic acid emulsion. This His-FIH-polymer emulsion was added to DMEM media containing 10% FBS and 1% pen/strep. The DMEM-His-FIH-polymer emulsion solution was added to the HeLa cells and incubated for 24 hours at 37 ºC and 5% CO$_2$. Following incubation, cells were trypsinized and lysed for RT-qPCR and western blot analysis as described above.

A.3 Results and Discussion

A.3.1 FITC-FIH polymer emulsion controls

In collaboration with the Rotello group at UMass Amherst, a polymer-stabilized nanocapsule delivery system (Scheme A.2) was optimized for delivery of His tagged FIH into mammalian cells.
To determine if this polymer emulsion system would be amenable for FIH delivery, initial controls were conducted with FITC labeled FIH. FIH was labeled with FITC and incubated with the polymer to form the nanocapsule emulsion, which was then added to the HeLa cell growth medium. As HeLa cells are well characterized and known for their robust nature, initial delivery attempts were conducted with this cell line. As seen in Figure A.1, preliminary data suggested this polymer stabilized nanocapsule system delivered FIH into the cells. Delivered FIH appeared in green in Figure A.1D whereas there was no green signal in the cells without FITC labeled FIH (Fig. A.1C) as expected. The brightfield images (Fig. A.1A-B) suggested the cells were still healthy and adherent following incubation with the polymer emulsion. The areas of punctate FITC signal seen in Figure A.1D suggest there was some protein aggregation upon delivery.
Figure A.1 FIH delivery in normoxic HeLa cells. Confocal microscopy images collected by the Rotello group. (A) Bright field image of HeLa cells without delivery. (B) Bright field image of HeLa cells with delivered FITC labeled FIH in polymer stabilized nanocapsules. (C) Fluorescence image of HeLa cells without delivery. (D) Fluorescence image of HeLa cells with delivered FITC labeled FIH in polymer stabilized nanocapsules.

FITC-labeled FIH was distributed throughout the cell upon delivery. FITC provided a simple and efficient readout to confirm enzyme delivery into the cells using the polymer stabilized nanocapsule system. However, FITC labeling killed the catalytic activity of FIH, therefore a new fluorescent method of detection was sought to visualize enzyme delivery and retain activity.
Figure A.2 Activity of FIH in the presence of the polymer emulsion. Initial rate assays were conducted at 2 mM ascorbate, 200 µM aKG, 50 µM FeSO₄, 80 µM CTAD, 10 µM polymer, and 0.7 µM FIH in 50 mM HEPES pH 7.00 at 37 °C. Time points were quenched every 15 seconds for 2 minutes and analyzed using MALDI-TOF MS to measure the rate of hydroxylation.

Upon discovering that FITC-labeled His-FIH was inactive, additional activity assays were conducted in the presence of polymer. His-FIH maintains catalytic activity when incubated with the polymer on the bench top (Fig. A.2) which provided justification to test the activity of His-FIH following delivery into HeLa cells. His-FIH was used for delivery because it has a shorter purification, yields more protein than cleaved purifications, and provides a fluorescent readout for delivery using a His antibody instead of FITC.

A.3.2 Optimization of hypoxic cell treatment and delivery conditions

Once it was established that FIH could be delivered into mammalian cells using the Rotello polymer stabilized nanocapsule system, controls were conducted to mimic hypoxia in cells. Delivery of FIH to hypoxic cells was of interest as FIH is normally inhibited at low pO₂. Activating FIH in hypoxia through enzyme delivery could regulate HIF-1α levels,
which are normally overexpressed in hypoxia. As an oxygen-regulated cell culture incubator was not readily available for use, hypoxia was mimicked using CoCl₂. CoCl₂ is a well-known to replace iron in the active site of the HIF hydroxylases, rendering them active as seen in hypoxia.⁴

Controls were conducted to optimize the amount CoCl₂ and length of CoCl₂ treatment required to induce hypoxic conditions in mammalian cells. Establishing methods for efficient detection of HIF-1α mRNA and protein levels following inhibition of the HIF hydroxylases by CoCl₂ was necessary before assessing the effect of delivered FIH. Initial RT-qPCR controls were conducted by incubating HeLa cells with either 100 µM of 250 µM CoCl₂ for 24 hours. Cells were lysed and prepared for RT-qPCR as described in the methods section (A.2.3).
Figure A.3 Quantitative RT-PCR following mRNA isolation from HeLa cells. Expression of target genes was normalized to expression of beta actin and quantitated using the comparative $C_T$ method ($2^{-\Delta\Delta C_T}$). mRNA levels of HIF-1α and related genes are reported following 24 hour incubation with CoCl₂.

Incubation with CoCl₂ had minimal effect on gene expression of HIF-1α and related genes in HeLa cells. The most significant result was an almost 2-fold increase in the mRNA levels of GAPDH following 100 µM CoCl₂ treatment. Similar fold changes were expected for HIF-1α and VEGFA, however lesser increases in mRNA were seen. The increase in FIH mRNA levels with increasing CoCl₂ was surprising as hypoxia renders FIH transcriptionally silent, but it should not affect mRNA levels. These controls provided a baseline for comparison of mRNA levels following FIH delivery to CoCl₂ treatment cells.
After optimizing initial controls of mRNA quantitation following CoCl$_2$ treatment, western blot assays were conducted to assess the effect of CoCl$_2$ treatment on protein levels. CoCl$_2$ concentrations, incubation time, and number of cells plated were varied to visualize protein levels using western blot. Several attempts at western blot on CoCl$_2$ treated protein extracts failed to detect an accumulation of HIF-1$\alpha$ as would be expected in hypoxia. Modifying antibody dilutions and blocking conditions did not improve results.

![Western blot optimization to detect HIF-1$\alpha$ protein levels in CoCl$_2$ treated HeLa cells and untreated MCF-7 cells.](image)

**Figure A.4** Western blot optimization to detect HIF-1$\alpha$ protein levels in CoCl$_2$ treated HeLa cells and untreated MCF-7 cells.

As a control for increased HIF-1$\alpha$ protein, an aliquot of MCF-7 (a breast cancer cell line) nuclear lysate was obtained from the Hardy lab. MCF-7 HIF-1$\alpha$ protein levels were visualized using western blot (Fig. A.4), however HIF-1$\alpha$ remained elusive in CoCl$_2$ treated HeLa cells. This blot confirmed that our antibody recognized the correct target and suggested an increase in HIF1$\alpha$ protein levels following CoCl$_2$ levels might be more apparent in MCF-7 cells.
Figure A.5 Proliferation assays to assess cell viability following 24 hour treatment with 100 µM CoCl₂.

Following the observation that HIF-1α protein levels were more easily detected by western blot in MCF-7 cells, proliferation assays were conducted with both HeLa and MCF-7 cells treated with 100 µM CoCl₂. Results confirmed that cell viability was not affected by mimicking hypoxia at various cell densities (Fig. A.5).

A.4 Conclusion and Future Directions

From the data presented here, we conclude FIH was delivered successfully into HeLa cells using the polymer-stabilized nanoparticle emulsion system developed by the Rotello lab at UMass Amherst. Attempts at mimicking hypoxia in CoCl₂ treated mammalian cells proved inconsistent, both in HeLa and MCF-7 cells. Additional data from optimization experiments in both cell lines can be found in notebooks VCIV and VCVI.

Delivery attempts of His-FIH-polymer emulsions were halted until baseline hypoxic levels of mRNA and protein for HIF-1α and related genes could be reproducibility established. Cell culture and hypoxia controls were taken over by another graduate student with more mammalian cell culture experience.
Once hypoxia can be maintained reliably in mammalian cells, delivery attempts can be continued to investigate the effect of addition of active FIH on HIF-1α both in hypoxia and normoxia. Upon establishing the effect of delivered FIH on HIF-1α mRNA and protein levels, the activity of the delivered FIH must be confirmed. Additionally, siRNA knockdowns of the HIF hydroxylase genes in the cell line of interest should be conducted in order to ensure that the changes in gene expression/protein levels following delivery are indeed due to the delivered protein and not endogenous protein.

A.5 Supplemental Information

A.5.1 RT-qPCR primers

HIF-1α forward
5’-TGGTGTTACTCAGCACCTTTTAG-3’

HIF-1α reverse
5’- AATCTCCGTCCTCAACCTCT-3’

GAPDH forward
5’- ACCCTGGCTCCATGAACATTT-3’

GAPDH reverse
5’- TGGTCAACCCTTCCACG-3’

VEGFA forward
5’-GAGCCCAGAGTGGCGA-3’

VEGFA reverse
5’-GATGATTCTGCCCTCCTCC-3’

FIH forward
5’-CTTATTGAGAATGAGGAGCCTGT-3’

FIH reverse
5’-TCTTGCAGGTATTCAAGGTC-3’

Beta Actin forward

161
5’- CCTCGCCTTTGCCGAT-3’

Beta Actin reverse
5’-GCGGCGATATCATCATCCA-3’
A.6 References


APPENDIX B

COMPOUND SCREENING TO IDENTIFY SELECTIVE INHIBITORS OF HIF HYDROXYLASES

B.1 Introduction

The HIF hydroxylases are often the subject of inhibition studies as they regulate key physiological processes. Of this class of enzymes, our lab specifically focuses on Factor Inhibiting HIF (FIH) and Prolyl Hydroxylase Domain 2 (PHD2). Inhibitors of FIH and PHD2 are attractive targets to mediate conditions which result from upregulation of HIF-1α, such as in wound healing and tissue repair by reducing cellular apoptosis, promoting release of growth and angiogenic factors, and intensifying the formation of extracellular matrix.\textsuperscript{1–3} The high degree of similarity between the active sites of FIH and PHD2 presents a challenge of inhibitor selectivity as both enzymes coordinate a Fe(II) metal center with a His\textsubscript{2}Asp facial triad.\textsuperscript{4} However, selective inhibition has been demonstrated with other metalloenzyme families such as lipoxygenases\textsuperscript{5} and matrixmetalloproteinases,\textsuperscript{6} suggesting that differences in active site size and shape can impart selectivity.

Previous inhibition studies have identified some classes of compounds known to inhibit PHD2 such as pyrones, pyridinones, and catechols.\textsuperscript{7} The goal of this project was to expand knowledge of structural requirements for FIH and PHD2 inhibition by screening small libraries of compounds to identify structurally diverse inhibitors. The types of molecules and/or functional groups that inhibit activity of the HIF hydroxylases were evaluated. This was accomplished through small compound library screens, defining the
extent of inhibition for those compounds, and determining their mechanism of action. This work contributed new knowledge to understanding the types of molecules and interactions required for HIF hydroxylase inhibition.

B.2 Methods

All reagents were purchased from commercial vendors and used as received, with the exception of recombinant enzyme and inhibitor compounds.

B.2.1 PHD2 protein purification

Recombinant human PHD2 was expressed and purified in truncated form containing only the catalytic domain (residues 177-426). PHD2 was overexpressed with an N-terminal GST fusion tag in E. coli BL21(DE3) cells using a pGEX-4T-1 vector (Stratagene). The GST-PHD2 construct was purified using affinity chromatography (GE Bioscience GStrap), followed by thrombin cleavage to remove the GST tag. Thrombin was removed with a HiTrap Benzamidine column (GE Healthcare). Protein was incubated with 50 mM EDTA to remove exogenous metals then buffer exchanged into 50 mM HEPES/150 mM NaCl pH 7.00 for storage. Protein purity was evaluated using SDS-PAGE and mass spectrometry.

B.2.2 Compound solubility

Inhibitor compounds were synthesized by the Xiao lab at UMass Amherst and received in solid form. 50 mM stock solutions of each compound were prepared in 100%
DMSO and stored at -20 °C. Compounds were diluted to 5 mM working stocks using 50 mM CAPS pH 12.05.

**B.2.3 Initial rate assays**

Each assay was conducted using 1 mM ascorbate, 7.5 µM αKG, 20 µM (NH₄)₂Fe(SO₄)₂, 20 µM CODD, 100 µM inhibitor compound, and 1 µM PHD2 in 50 mM HEPES pH 7.00 at 37°C with 50 uL total reaction volume. All reagents except substrate were mixed and incubated at room temperature for 2.5 minutes followed by a 30 second incubation at 37°C. The reaction was then initiated with CODD and conducted at 37°C. 5 uL aliquots were quenched in 20 uL of MALDI matrix (4-α-cyanohydroxycinnamic acid in 66% acetonitrile and 0.2% trifluoroacetic acid). Quenching occurred at 30 second, 1 minute, 1.5 minute, and 2 minute time points. PHD2 hydroxylation of the CODD substrate was monitored by comparing the relative intensities of the CODD peak (2134 m/z) to the hydroxylated CODD peak (2150 m/z) using a Bruker Microflex MALDI TOF.

**B.2.4 IC₅₀ assays**

Time-dependent initial rate assays varying the concentration of inhibitor established the IC₅₀ for each compound. Assays contained 1 mM ascorbate, 7.5 or 50 µM αKG, 20 µM (NH₄)₂Fe(SO₄)₂, 20 µM CODD, and 1 µM PHD2 in 50 mM HEPES pH 7.00 at 37°C with 50 uL total reaction volume. Assays were conducted in the same manner as the initial rate studies described above. The initial rate of enzyme activity at each inhibitor concentration was plotted in Origin and fit to a dose response function.
B.2.5 Thermal shift assays

Thermal shift assay experiments were conducted using a BioRad CFX Connect Real-Time PCR Detection System instrument. Protein-inhibitor solutions were dispensed in 50 µL aliquots into clear 96-well PCR plates and sealed to prevent evaporation. The PCR plates were placed in the thermal block of the PCR instrument and heated at a rate of 1 °C/20 s. Fluorescence was measured and an image was collected every 0.5 °C. Reference wells contained protein without inhibitor. Relative fluorescence intensity data was obtained from the instrument, plotted for comparison to control samples, and the sigmoidal portion of the data was fit using the Boltzman equation to obtain the $T_M$.

\[
y = A_1 - A_2 e^{\frac{(x - x_0)}{dX}} + A_2^1
\]

All $\Delta T_M$ values are in reference to apo PHD2. Assays were conducted using 5 µM WT PHD2, 5 µM MnSO$_4$, 100 µM ligand, and 5 x Sypro Orange dye in 50 mM HEPES pH 7.00. $\Delta T_M$ was calculated by subtracting the $T_M$ of apo PHD2 from the $T_M$ of PHD2+ligand samples.

B.3 Example Screening Study

To efficiently assess the potency of inhibitor compound libraries, a screening procedure was developed and is described below:

1. Initial rate determination of enzyme activity in the presence of 100 µM inhibitor compound.

---

1 $A_1$ = initial temperature  
   $A_2$ = final temperature  
   $x_0$ = midpoint ($T_M$)  
   $dX$ = time constant
2. IC₅₀ assays on inhibitor compounds with greater than 50% initial rate inhibition in step 1.

3. Thermal shift assay to evaluate impact of compound on enzyme stability.

4. Competition assays to determine mechanism of action for inhibitor compound.

![Scheme B.1 Nobiletin-derivative compound structures.](image)

A selection of nine nobiletin-derivative compounds were synthesized by the Xiao lab at UMass Amherst (Scheme B.1). Nobiletin is a type of o-methylated flavonoid isolated from the tiansheng plant with documented anti-tumor activity through inhibition of angiogenic mediators such as HIF-1α, VEGF, and NF-κB.⁸ These compounds were attractive as potential inhibitors of the HIF hydroxylases as they could compete with αKG for binding to the active site metal. Compounds were dissolved in 100% DMSO to a concentration of 50 mM for solubility, then diluted to 5 mM working stocks in 50 mM CAPS pH 12.05 for initial activity screens as described above. As seen in Figure B.1, compounds 5, 7, and 9 had the greatest impact on enzyme activity, inhibiting the initial rate of PHD2 hydroxylation of CODD by more than 50%. Structural analysis of these
compounds suggested that the ortho and meta hydroxyl groups on the benzene ring of compounds 5 and 9 conferred selectivity over the other compounds.

**Figure B.1** Initial rate of PHD2 hydroxylation of CODD in the presence of Xiao lab compounds. Assays were conducted with 1 mM ascorbate, 7.5 µM aKG, 20 µM (NH₄)₂Fe(SO₄)₂, 100 µM compound, 1 µM PHD2, and 20 µM CODD in 50 mM HEPES pH 7.00. * denotes most potent inhibitors.

The Thermal Shift Assay is a label-free technique which monitors interactions between a target molecule and its binding ligands. Ligand-binding affinity was assessed from the shift in the unfolding temperature (ΔT_M) obtained in the presence of ligands relative to that obtained in the unbound form of the molecule. In this assay, the thermocycler function on an RT-PCR instrument was used to vary the melting temperature while Sypro Orange dye provided the fluorescent signal. Destabilization of the target by the ligand results in a lower melting temperature, whereas an increase in melting temperature in the presence of ligand suggests stabilization. As seen in Figure B.2, all of the compounds stabilized the (Mn)PHD2 enzyme form, inferring that the compounds were
mimicking αKG binding. N-oxalylglycine (NOG) acted as a positive control and provided a reference Δ T_M for a known strong inhibitor of PHD2.\textsuperscript{10}

![Graph showing thermal shift assay results](image)

**Figure B.2** Thermal shift assay to determine stability of PHD2 in the presence of the Xiao lab compounds. Assays were conducted using 5 µM WT PHD2, 5 µM MnSO\textsubscript{4}, 100 µM ligand, and 5 x Sypro Orange dye in 50 mM HEPES pH 7.00. * denotes most potent inhibitors from initial rate screen (Fig. B1).

<table>
<thead>
<tr>
<th>Sample</th>
<th>T_M (°C)</th>
<th>ΔT_M (°C) from PHD2+Mn</th>
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<tr>
<td>Apo PHD2</td>
<td>44.1 ± 0.05</td>
<td>--</td>
</tr>
<tr>
<td>Compound</td>
<td>ΔTm</td>
<td>IC50</td>
</tr>
<tr>
<td>-------------------</td>
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</tr>
<tr>
<td>(Mn)PHD2</td>
<td>45.1 ± 0.04</td>
<td>--</td>
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</tr>
<tr>
<td>(Mn+C2)PHD2</td>
<td>47.6 ± 0.07</td>
<td>2.5</td>
</tr>
<tr>
<td>(Mn+C3)PHD2</td>
<td>48.2 ± 0.06</td>
<td>3.1</td>
</tr>
<tr>
<td>(Mn+C4)PHD2</td>
<td>47.9 ± 0.06</td>
<td>2.8</td>
</tr>
<tr>
<td>(Mn+C5)PHD2</td>
<td>47.1 ± 0.09</td>
<td>2</td>
</tr>
<tr>
<td>(Mn+C6)PHD2</td>
<td>47.9 ± 0.06</td>
<td>2.8</td>
</tr>
<tr>
<td>(Mn+C7)PHD2</td>
<td>48.0 ± 0.06</td>
<td>2.9</td>
</tr>
<tr>
<td>(Mn+C8)PHD2</td>
<td>48.4 ± 0.04</td>
<td>3.3</td>
</tr>
<tr>
<td>(Mn+C9)PHD2</td>
<td>48.2 ± 0.05</td>
<td>3.1</td>
</tr>
</tbody>
</table>

Unfortunately, none of the compounds stabilized PHD2 to the extent of NOG (Table B.1). The ΔTm of 11 °C for NOG was 4-5 fold greater than that of all the nobiletin derivatives, suggesting these compounds would have high IC₅₀’s against PHD2. To test this theory, IC₅₀ studies were carried out with compounds 5 and 9 as they had the largest impact on the initial rate of PHD2 activity in Figure B2. Figure B.3 reveals the moderate IC₅₀’s for these compounds under low and saturating αKG conditions to test for competition with αKG binding.
Figure B.3 IC50 studies monitoring the initial rate of CODD hydroxylation by PHD2 varying the concentration of compounds 5 (A) and 9 (B) at low (black) and high (red) concentrations of αKG. Assays were conducted in the presence of 1 mM ascorbate, 7.5/50 μM αKG, 20 μM (NH4)2Fe(SO4)2, 100 μM compound, 1 μM PHD2, and 20 μM CODD in 50 mM HEPES pH 7.0

B.4 Conclusion and Future Directions

Based on the lackluster binding and activity results presented here, studies of the nobiletin derivative compounds were halted. Activity screens with FIH produced similar
results. In a continued effort to identify structural requirements for selective inhibition of the HIF hydroxylases, additional compounds were screened through various collaborations. A ~40 compound library from the Olsen lab at Midwestern University also failed to inhibit the HIF hydroxylases to a significant extent. Structures of the Olsen lab compounds are described below (Scheme B.2). An ideal inhibitor would selectively inhibit either FIH or PHD2 (but not both) with an IC$_{50}$ of $< 1 \, \mu$M by competing with αKG for active site binding. Another method of action for HIF hydroxylases is Fe(II) chelation, however this could have detrimental effects on other pathways in vivo. In order to increase chances of successful identification of selective inhibitors, larger libraries of αKG mimic compounds should be screened.
B.5 Supplemental Information

Scheme B.2 Structures of compounds from Olsen lab.
B.6 References


Huisgen, R. 1,3-Dipolar Cycloaddition Chemistry. (Wiley, 1984).


Mills, S. A.; Marletta, M. A., Metal binding characteristics and role of iron oxidation in the ferric uptake regulator from Escherichia coli. *Biochemistry* 2005, 44 (41), 13553-13559.


