Selective Inhibition and Mechanistic Studies of the Human O2 Sensor, Prolyl Hydroxylase Domain 2 (PHD2)

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SELECTIVE INHIBITION AND MECHANISTIC STUDIES OF THE HUMAN O$_2$
SENSOR, PROLYL HYDROXYLASE DOMAIN 2 (PHD2)

A Dissertation Presented

by

SHANNON COATES FLAGG

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 2011

Department of Chemistry
DEDICATION

For my husband Robert M. Flagg, for the opportunity to attend and the support to complete graduate school, this would not have been possible without him.

For my brother Dr. Jay E. Coates, Jr. who provided me with the inspiration.

For my father Jay E. Coates for instilling in me to always finish that which you begin.

For my mother who taught me all things are possible with perseverance and belief.

For those people who are a special part of my life and who have played key roles in some of life’s most challenging times and in no small part have contributed to my success, Teresa Coates, Holly Walters and Kristen Huber.
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Breanne Holmes for the inhibitor analysis on FIH
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ABSTRACT

SELECTIVE INHIBITION AND MECHANISTIC STUDIES OF THE HUMAN O₂ SENSOR, PROLYL HYDROXYLASE DOMAIN 2 (PHD2)

SEPTEMBER 2011

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Directed by: Professor Michael J. Knapp

Prolyl Hydroxylase Domain 2 (PHD2) has been identified as a key oxygen sensor in humans along with Factor Inhibiting Hypoxia Inducible Factor (FIH). As such PHD2 and FIH play critical roles in myriad pathways of medical relevance by hydroxylation of their target substrate hypoxia inducible factor (HIF), a transcription factor responsible for the regulation of over 100+ genes. With such critical roles in human physiology the ability to selectively regulate these two enzymes could potentially lead the way for novel therapeutic treatments of a vast array of disease states from cancer to myocardial infarction.

We report on three classes of iron chelators which show promise for independent regulation of the HIF hydroxylases. Compounds representing the pyrones/pyridinones, pyridines and catechols were tested and found to have differential impacts on PHD2 and FIH under the same experimental conditions. The mode of inhibition is the result of binding to the active site iron and is supported by UV-visible and electroparamagnetic resonance spectroscopy.

PHD2 at the current time does not have a well resolved mechanistic understanding regarding its catalytic cycle and subsequent rate determining steps. I have employed pH,
solvent isotope, and X-ray absorption studies in an effort to gain further understanding regarding PHD2’s overall mechanism. Our data support that dissociation of an iron(II)-OH$_2$ bond centered about the active site contributes to a portion of the overall rate determining steps in the catalytic reaction of PHD2 that activates oxygen and ends with the production of hydroxylated substrate.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGMENTS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTER

1. OXYGEN SENSING

   Introduction                                                            1
   Hypoxia Inducible Factor (HIF)                                          2
   HIF Hydroxylases
     Prolyl Hydroxylase Domain 2 (PHD2)                                     5
     Factor Inhibiting Hypoxia Inducible Factor (FIH)                      6
     The Oxygenase Family of Enzymes                                       8
     Related Oxygenase Enzymes of Interest                                 9
     α-Ketoglutarate, Iron (II) Dependent Oxygenases                       12
     Proposed Mechanism for the HIF Hydroxylases                          16

2. SELECTIVE INHIBITION OF THE HIF HYDROXYLASES PHD2 AND FIH

   Introduction                                                            18
   Comparisons of PHD2 and FIH Active Sites                               18
   Known Inhibitors                                                      20
   Inhibitor Classes Tested for Selective Inhibition                      21
     Pyrones/Pyridinones                                                   22
     Pyridines                                                            23
     Catechols                                                           24
   Materials and Methods
     Protein Expression and Purification                                  24
     The Oxygen Dependent Degradation Domain (ODDD) Substrate             30
     FIH Purification and Peptide Substrate                               31
     Activity and Inhibition Assays                                       31
     UV-Vis Spectroscopy                                                33
     Electroparamagnetic Resonance Spectroscopy (EPR)                     33
   Results & Discussion
     Dose Response to Inhibitors                                          34
     UV-Visible Spectroscopy of Inhibitor Binding                         39
     Electroparamagnetic Resonance Spectroscopy (EPR)                     41
   Conclusions                                                          44
3. MECHANISTIC STUDIES INVOLVING pH AND SOLVENT ISOTOPE EFFECTS ON PHD2 .............................................................47

Introduction .............................................................................................................47
Methods and Materials ..........................................................................................49
  Mixed Buffer Preparation of MES-PIPES-HEPES (MPH) ...................................49
  Protein and Solutions Preparation for pH and SIE Experiments ......................50
  UV-Visible Spectroscopy ....................................................................................51
  X-Ray Absortion Spectroscopy .........................................................................51
  Viscosity Test ......................................................................................................52
  Stability Assays ..................................................................................................53
  Incubation Assays ...............................................................................................53
  Data Fitting ..........................................................................................................54
Results & Discussion ............................................................................................54
  pH Dependence of PHD2 Catalyzed Hydroxylation of HIF-1α .......................54
  Stability versus Activity of PHD2 .......................................................................61
  Incubation Assays: Determining if Full Activity of PHD2 is Recoverable ..........63
  Acid and Base Forms of PHD2 ...........................................................................65
  X-Ray Absorption Spectroscopy .......................................................................66
  Solvent Isotope Effects on PHD2 .......................................................................72
  Viscosity Assays ..................................................................................................77
Conclusions ............................................................................................................79

APPENDIX: SUPPLEMENTARY DATA ..................................................................84

BIBLIOGRAPHY ....................................................................................................90
LIST OF TABLES

Table 2.1 IC50 values for PHD2 and FIH. *For Hop-Me the concentration to achieve exactly half the maximal rate is 16 µM, for BuCat it is 1.0 µM, the values in the table are reflective of the second inhibitory event as a two site binding mechanism was witnessed in PHD2. .................................................................37

Table 2.2 EPR Sectral parameters for (Cu)FIH and (Cu)PHD2. Parameters were determined using Spincount. ........................................................................................................42

Table 3.1 Kinetic parameters for PHD2 under different pH values ........................................57

Table 3.2 Estimated $k_{cat}/K_M$ after statistical analysis on the range of $K_M$'s from original data set, final estimated $K_M = 1.67 \pm 0.32$ µM. The estimated $K_M$ was then used with the experimentally determined $k_{cat}$ to generate the kinetic parameter $k_{cat}/K_M$. ......................................................................................59

Table 3.3 XANES and EXAFS analysis of Fe$^{2+}$αKG:PHD2 at pH 6.5 and 8.5. $^a$r (Å) is the radial distance between metal and ligand. $^b$σ2 is the root mean square disorder in the metal-ligand distance. $^c$R is the goodness of fit. Numbers in parentheses represent standard deviation for least square fits. $^d$Distances in [ ] correspond to atoms in a O-C-C-O chelate ring and were constrained to vary with a single value of Δr for the chelate ring. .........................69

Table 3.4 Kinetic parameters for PHD2 under different pD values. All data was obtained by linear regression of the initial rates collected under varied ODDD concentration (1-50 µM), and then fitted to the Michaelis-Menten equation to obtain $k_{cat}$ and $K_M$.................................................................75

Table 3.5 Observed kinetic parameters of PHD2 in water and deturium, $pK_a$ and observed SIE.........................................................................................................................79
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>The domains of HIF1-α/β showing the sites of proline and asparagine hydroxylation that regulate HIF.</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>The consensus mechanism for the HIF hydroxylases.</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>The pathway of HIF-1α as directed by the HIF hydroxylases PHD2 and FIH. In the presence of O₂ FIH hydroxylates HIF-1α on Asn803 blocking interaction with the transcription factor P300, halting all P300 dependent transcription. PHD2 hydroxylates either Pro402 or Pro 564, or both leading to proteosomal degradation by signaling the binding of the von Hippal Lindau protein (pVHL) part of ubiquitin ligase complex. In the absence of O₂ HIF-1α is able to dimerize with the β-subunit triggering transcriptional activity. Figure courtesy of Saban, E.</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td>Catalytic mechanism of extradiol catechols dioxygenase.</td>
</tr>
<tr>
<td>Figure 1.5</td>
<td>Aromatic amino acid hydroxylase mechanism.</td>
</tr>
<tr>
<td>Figure 1.6</td>
<td>The hydroxylation reaction of TauD.</td>
</tr>
<tr>
<td>Figure 1.7</td>
<td>Reaction catalyzed by P4H.</td>
</tr>
<tr>
<td>Figure 1.8</td>
<td>Example of the β-barrel jelly roll fold for the prolyl hydroxylases FIH and PHD2 and including a zoomed view of their iron(II) facial triad (PDB 1H2M, 2G19). Figure created with PyMol.</td>
</tr>
<tr>
<td>Figure 1.9</td>
<td>Proposed mechanism of the HIF hydroxylases PHD2 and FIH. Mechanism begins with 6 coordinate iron, αKG displaces two H₂O molecules, substrate binding triggers last H₂O molecule to exit opening a site for O₂ activation. The subsequent steps are proposed and have yet to be supported until the release of hydroxylated substrate and a regeneration of the resting state enzyme.</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Inhibitors tested. (1) CNP, 3-cyano-6-methyl-2(H) pyridinone(2) NOP, hydroxypyridine 1-oxide, (3) DHP, 2,3-dihydroxypyridine, (4) Hop-OH, 5-hydroxy-2-hydroxymethyl-4-pyrone, (5) Hop-COOH, 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid, (6) Hop-Me, 3-hydroxy-2-methyl-4-pyrone, (7) HOPO, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone, (8) 4NCat, 4-nitrocatechol, (9) MeCat, 4-methyl catechol, (10) BuCat, 4-tert-butylecatechol.</td>
</tr>
</tbody>
</table>
Figure 2.2 Stratagene pGEX-4T-1 Vector used for expression and purification of PHD2 catalytic domain consisting of residues 177-426. Figure from Stratagene protocol.

Figure 2.3 MALDI spectra displaying the peptide (2133 Da) plus a sodium adduct, followed by additive sodium products. The product peak is labeled as MO+Na, a typical linear regression resulting from analysis is shown as an inset.

Figure 2.4 Initial inhibitor screening. PHD2 assay conditions, 1.5 μM PHD2, 10 or 200 μM αKG, 20 μM ferrous ammonium sulfate, 2 mM ascorbate, 60 μM ODDD and 100 μM of inhibitor. FIH assay conditions, 0.5 μM FIH, 10 or 500 μM αKG, 500 μM FeCl₂, 2mM ascorbate, 66 μM CTAD and 100 μM inhibitor. Assays were at 37 °C and time points extracted and quenched followed by analysis on MALDI for PHD2 and ESI-MS for FIH. Product formation was calculated by the equation \[ [\text{Sub}^{\text{OH}}] = \frac{[\text{Sub}^{\text{OH}}]}{[\text{Sub}^0]} \times [\text{Sub}^0], \] where Sub is CTAD or ODDD respectively. (1) CNP, (2), NOP, (3) DHP, (4) Hop-OH, (5) Hop-COOH, (6) Hop-Me, (7) HOPO, (8) 4NCat, (9) MeCat, (10) BuCat. FIH collection by B. Holmes.

Figure 2.5 Dose response curves for all inhibitory. Reaction conditions are 1.5 μM PHD2, 10 μM αKG, 20 μM ferrous ammonium sulfate, 2 mM ascorbate, 60 μM ODDD and 0-1 mM for each inhibitor.

Figure 2.6 PHD2 and FIH in the presence of iron(II) and 4NCat, pH 7.0. Samples were prepared anaerobically and sealed in cuvettes for data collection.

Figure 2.7 Control spectra for 4-Nitrocatechol. Samples were prepared anaerobically at pH 7 and 12, 4NCat with iron (II) at pH 7, PHD2 at pH 7.

Figure 2.8 X-Band EPR spectral changes upon Hopo binding to FIH and PHD2. FIH (0.65mM), CuSO₄ (0.60mM), αKG (0.60mM), HOPO (0.60mM) in 50mM HEPES pH 7.50. 9.615 GHz frequency, 5 mW power, 5G modulation amplitude, 100 GHz modulation frequency, 20ms time constant at 100 K. PHD2 (0.65mM), CuSO₄ (0.60mM), αKG (0.60mM), HOPO (0.60mM) in 50mM HEPES pH 7.50. 9.598 GHz frequency, 5 mW power, 5G modulation amplitude, 100 GHz modulation frequency, 20ms time constant at 100 K.

Figure 3.1 Catalytic steps involved in PHD2 catalysis. Focus is upon the first irreversible step when oxygen is activated denoted by the \( k_{\text{cat}} \) arrow.

Figure 3.2 Kinetic fits for PHD2 at varied pH. All assays include 0.3 μM PHD2, 15 μM ferrous ammonium sulfate, 200 μM αKG, 2 mM ascorbic acid and...
ODDD concentrations from 0-80 µM. Product formation was calculated using the equation [ODDD\textsuperscript{OH}] = \gamma_{\text{ODDD-OH}} \times \text{[ODDD]}_0. All data was fit using the Michaelis-Menten equation.

Figure 3.3 The kinetic parameters \( k_{\text{cat}}/K_M \) and \( K_M \) for PHD2.

Figure 3.4 The PHD2 \( k_{\text{cat}} \) data fit to determine the pH of 7.32 ± 0.01.

Figure 3.5 Revised \( k_{\text{cat}}/K_M \) after application of an averaged \( K_M \) value indicating a decreasing specificity constant with increasing pH.

Figure 3.6 Effect of preincubation on the activity of PHD2. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2mM ascorbate and 15 µM ODDD. For stability curve PHD2 was incubated at pH 8.5 and assayed at pH 7.0. For activity curve PHD2 was incubated at each individual pH and assayed at the same pH as for incubation. Both curves have been normalized to activity at pH 6.5.

Figure 3.7 PHD2 with either water or hydroxyl coordinated at the sixth position just prior to ODDD binding.

Figure 3.8 PHD2 Incubation assay to determine activity recovery. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2mM ascorbate and 15 µM ODDD. Assay time range, 1.4-30.0 minutes. PHD2 was incubated at pH 8.5 and assays were conducted at pH 7.0.

Figure 3.9 pH 8.5 and pH 6.5 forms of PHD2. Samples include 50 µM PHD2, 45 µM ferrous ammonium sulfate and 50 µM αKG. Samples were prepared anaerobically in a Coy chamber to maintain the Fe(II) state. The resulting spectra is obtained as a difference spectra, (pH 8.5-pH6.5).

Figure 3.10 XAS regions for determining local geometric and/or electronic environments about a metal ion. Figure courtesy of N. Giri.

Figure 3.11 Iron K-Edge XANES spectra; (Fe\textsuperscript{2+}αKG:PHD2) at pH 6.5 (red), and pH 8.5 (black).

Figure 3.12 EXAFS analysis. Left: Unfiltered, \( k^3 \)-weighted EXAFS spectra (Fe(PHD)-αKG at pH 6.5 (top) and at pH 8.5 (bottom)) and fits (black lines). Right: Fourier-transformed EXAFS data and fits.

Figure 3.13 Individual steps believed to be involved in the catalysis of PHD2. Based upon pH and SIE studies focus is on \( k_5 \) where the release of water is thought to occur opening an open coordination site for \( O_2 \) activation with the iron(II)state maintained.
Figure 3.14 Kinetic fits for PHD2 at varied pD. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2 mM ascorbic acid and ODDD concentrations from 0-50 µM. Product formation was calculated using the equation \[ [\text{ODDD}^{\text{OH}}] = \chi_{(\text{ODDD-OH})} \times [\text{ODDD}]_0 \] for all initial rates which comprise the kinetic curves.

Figure 3.15 Solvent isotope effects, (D₂O, triangles, H₂O, squares) pKₐ fits for PHD2. Data is comprised of full Michaelis-Menten curves for each data point. Fully protonated form, 2.99 ± 0.08, pKₐ 7.22 ± 0.03, deprotonated 0.31 ± 0.02. Fully deuterated form 3.3 ± 0.06, pKₐ 7.89 ± 0.03, dedeuterated form 0.34 ± 0.04. The observed solvent isotope effect on \( k_{\text{cat}} \) is 0.91 ± 0.03.

Figure 3.16 \( K_M \) under various pD’s. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2 mM ascorbic acid and ODDD concentrations from 0-50 µM. Product formation was calculated using the equation \[ [\text{ODDD}^{\text{OH}}] = \chi_{(\text{ODDD-OH})} \times [\text{ODDD}]_0 \]. The \( K_M \) was derived from fitting the data to the Michaelis-Menten equation.

Figure 3.17 \( k_{\text{cat}}/K_M \) in D₂O, A) \( k_{\text{cat}}/K_M \) using \( K_M \) generated from Michaelis-Menten fits. B) \( k_{\text{cat}}/K_M \) using an averaged \( K_M \) (1.73 ± 0.57) which assumes little if any fluxuation in the value as the actual \( K_M \) is below the detection limits of data collection.

Figure 3.18 The solvent viscosity impact on PHD2. Assay includes 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 1 mM ascorbate and 10 µM ODDD. Activity is measured as previously disclosed. A maximal velocity of 2.07 ± 0.05 min⁻¹ was obtained for the control assay, and in the same buffer with 10% sucrose the observed rate is 2.04 ± 0.02 min⁻¹.

Figure 3.19 The catalytic cycle of PHD2.

Figure A1 Succinate production as compared to ODDD-OH production to determine coupling ratio. PHD2 1.0 µM, ferrous ammonium sulfate 20 µM, αKG 500 µM, 2 mM ascorbate, 80 µM ODDD.
CHAPTER 1

OXYGEN SENSING

Introduction

Our atmosphere is 21% oxygen and by mass oxygen is the second most abundant element of earth at 30% second only to iron at 35%. Iron and oxygen have been incorporated into the most fundamental workings of life throughout the evolution of the planet including the enzymes covered by this research. While oxygen is essential for aerobic life it can also damage it through oxidation and therefore it would seem plausible that there exist a process which regulates O\(_2\) homeostasis. Globally the need for oxygen to sustain the majority of life on earth should be self evident. Yet beyond inhalation of oxygen into the lungs lies a complex pathway of oxygen regulation and distribution that is still under discovery. The lack of sustained oxygen regulation within any physiological system can lead to disastrous effects from cellular oxidative damage due to hyperoxia or elevated O\(_2\) levels to cellular and tissue death from hypoxia, defined as a critically low O\(_2\) concentration. Our energy needs are regulated via oxygen as it is the final electron acceptor in aerobic respiration leading to the production of ATP (adenosine-5’-triphospate), deemed the energy currency of the body. Aerobic respiration generates 29-30 ATP, whereas anaerobic respiration or glycolysis only generates a net of 2ATP’s, making aerobic respiration 19 times more efficient\(^1\). Normally throughout human physiology oxygen concentrations vary widely from ~21% in the lungs to as low as ~1% at the corticomedullary junction of the kidneys. Despite this wide range in which oxygen must be regulated within any given location of the body, there exists a tightly controlled
regulatory system to keep this vital element in check. Leading the regulation of this pathway is the transcriptional regulator Hypoxia Inducible Factor (HIF) discovered in 1992 as being required for the transcription of erythropoietin (EPO) in humans, which leads to red blood cell production and subsequently oxygen delivery\(^2\). To stress the significance of this pathway, consider a tumor which by its very nature is hypoxic and requires \(O_2\) via the vascular system to sustain its growth and existence; regulate the system and just possibly, regulate the tumor. There are many other instances in which hypoxia is a major contributing factor to the pathophysiology of the underlying problem aside from cancer like stroke and myocardial infarction\(^3\). While HIF is the main transcription factor regulating genes involved in oxygen homeostasis it has its own set of master controllers which are iron(II), \(\alpha\)-ketoglutarate dependent hydroxylases, Prolyl Hydroxylase 2 (PHD2) and Factoring Inhibiting Hypoxia Inducible Factor (FIH). These two enzymes ultimately determine whether or not HIF is able to transcribe genes in response to \(O_2\) changes in localized environments. Acquiring knowledge of the oxygen delivery pathway and its regulators may possibly lead to extensive new ways in which to combat some of the most prevalent health disorders that plague our existence.

**Hypoxia Inducible Factor (HIF)**

Variation in oxygen availability requires an adaptive response to cope with these changes. The variation in oxygen availability may be due to something as simple as a change in altitude to a disease state within the body. Whatever the underlying cause, HIF is the key to balancing the system. HIF is comprised of two subunits, the \(\alpha\) and the \(\beta\). HIF-1\(\beta\) is constitutively expressed while the HIF-1\(\alpha\) subunit is tightly regulated through
prevalent oxygen concentration\textsuperscript{4,5}. It is important to note that there are three isoforms of HIF, HIF-1, HIF-2 and HIF-3 and it has been determined that the primary oxygen regulator of the isoforms is HIF-1 and therefore focused upon in this research\textsuperscript{6-8}. The individual domains of the $\alpha$ and $\beta$ subunits contain a basic-Helix-Loop-Helix and Per-ARNT-Sim (bHLH-PAS) regions necessary for heterodimerization and DNA binding, and are located at the amino terminal half of the construct. HIF-1$\beta$ initially was identified as the Aryl Hydrocarbon Receptor Nuclear Translocator protein (ARNT), as it was found to dimerize with the aryl hydrocarbon receptor upon binding of aryl hydrocarbons as is the case with dioxin\textsuperscript{9}. ARNT domains are typical amongst a vast family of bHLH-PAS heterodimeric transcription factors. At the carboxy terminus of HIF-1$\alpha$ lies the C-Terminal Transactivation domain (CTAD) as well as the Oxygen Dependent Degradation Domain (ODDD)\textsuperscript{10,11}. It is through the CTAD and the ODDD domains that transcription leading to over hundred plus genes are regulated via available oxygen concentrations (Figure 1.1). Some of the key genes regulated via HIF-1 are responsible for angiogenesis, erythropoiesis, iron regulation, anemia, and energy metabolism to mention a few.

\begin{figure}[h]
\centering
\includegraphics[width=0.8\textwidth]{domains.png}
\caption{The domains of HIF1-$\alpha/\beta$ showing the sites of proline and asparagine hydroxylation that regulate HIF.}
\end{figure}

During conditions of normoxia or the state in which a particular tissue, organ, or cell has adequate $O_2$ available as deemed normal for the local environment, HIF-1$\alpha$ is rapidly
degraded with a half-life of less than five minutes\(^4\). If the oxygen requirements of the local environment are not satisfied, hypoxia ensues and HIF-1\(\alpha\) is stabilized. As the oxygen concentration drops a direct relationship with increased HIF-1\(\alpha\) levels is witnessed\(^{12, 13}\). In the presence of adequate oxygen two enzymes, prolyl-hydroxylase domain 2 (PHD2) and factor inhibiting hypoxia inducible factor (FIH) hydroxylate HIF-1\(\alpha\) at specific residues, Pro\(^{402}\), Pro\(^{564}\) or Asn\(^{803}\) respectively. The \(\alpha\)-subunit is then either rapidly degraded through a ubiquitin-proteasome pathway in the case of PHD2 or is blocked from interaction with specific transcription factors necessary for gene expression in the case of FIH\(^{12-19}\).

**HIF Hydroxylases**

The HIF hydroxylases are the major regulators of the hypoxic response in humans by acting upon HIF-1\(\alpha\) ultimately determining its ability to regulate genes crucial in the hypoxia pathway. There are three isoforms of PHD found in humans, (PHD 1,2,3) and a asparaginyl hydroxylase, FIH. PHD2 and FIH are Fe(II) \(\alpha\)KG dependent dioxygenases that utilize \(O_2\), and couple the hydroxylation of the substrate to the oxidative decarboxylation of \(\alpha\)KG to succinate and \(CO_2\) (Figure 1.2)\(^{20, 21}\).

![Figure 1.2 The consensus mechanism for the HIF hydroxylases](image-url)
The overall reaction for the HIF hydroxylases involves a shift from a six coordinate resting enzyme to a five coordinate state that is primed for reaction with oxygen. Once αKG binds, two water molecules are displaced and as substrate docks it is thought that this triggers release of the last water molecule opening a coordination site available for oxygen chemistry to occur. Once active the enzyme couples the hydroxylation of substrate where one of the oxygen atoms is inserted into succinate, resulting from oxidative decarboxylation of αKG, and the second oxygen atom is incorporated into the product, i.e. substrate. Some studies report a lower $K_M$ for O$_2$ with respect to PHD2 than for FIH, but in general the $K_M$ for O$_2$ for both enzymes is higher than ambient oxygen concentrations. This high level of $K_M$ indicates that relatively small changes in O$_2$ concentrations can have a significant impact on PHD2 and FIH activity$^{22, 23}$. This difference in $K_M$ for the HIF hydroxylases also suggests that once PHD2 has been suppressed by insufficient O$_2$ HIF-1α is able to escape degradation, however FIH still remains active altering its transcriptional activity$^{24, 25}$. For example, in 1% O$_2$ FIH is still active while PHD is fully suppressed, this seemingly supports that FIH is the primary O$_2$ sensor under hypoxic conditions$^{26}$. Another factor regarding the true $K_M$ of oxygen for these enzymes is that it ranges with varied substrate length and at the current time success has not been achieved in expressing full length HIF-1α and so synthetic peptides are employed for substrate-enzyme interaction and kinetic studies$^{23}$.

**Prolyl Hydroxylase Domain 2 (PHD2)**

Initially discovered in *C. elegans* the prolyl hydroxylases (PHD1, PHD2, PHD3) have been determined to be the key oxygen regulatory enzymes along with an asparaginyl
hydroxylase, factor-inhibiting HIF (FIH) in humans. Each of the PHD’s differs in the amount of their mRNA but all have a ubiquitous mode of expression\(^ \text{27} \). Despite there being three isoforms each of which must have specific roles it was determined by siRNA research that the silencing of PHD2 was enough to fully stabilize HIF1\( \alpha \) and thereby was deemed the primary O\(_2\) sensor of the PHD’s\(^ \text{28} \). PHD2 is an iron(II), \( \alpha \)-ketoglutarate dependent enzyme belonging to the dioxygenase family which utilize molecular oxygen in the oxidative decarboxylation of \( \alpha \)-ketoglutarate to succinate and CO\(_2\) release coupled to hydroxylation of their target substrate. PHD2 targets two proline residues within the HIF-1\( \alpha \) domain, Pro\(^ {402} \) in the NODD (N-terminal Oxygen-Dependent Degradation Domain) and Pro\(^ {564} \) in the ODDD (Oxygen Dependent Degradation Domain). The hydroxylation of either or both triggers the degradation of HIF-1\( \alpha \) via a ubiquitin ligase pathway. It should also be noted that prolyl hydroxylation is substantially more sensitive than asparaginyl hydroxylation to inhibition by iron chelators and transition metal ions and that the order of suppression of hydroxylation follows as Pro\(^ {402} \) > Pro\(^ {564} \) > Asn\(^ {803} \)\(^ \text{(29)} \). When PHD2 hydroxylates HIF-1\( \alpha \) the end result is proteosomal degradation, which is vastly different than the result of asparaginyl hydroxylation which alters transcriptional activity. PHD2 is commonly located in the cytoplasm and is suggested to be the dominant isoform of the three under normoxic conditions with the highest level of expression occurring across a range of cell types\(^ \text{30-32} \).

**Factor Inhibiting Hypoxia Inducible Factor (FIH)**

HIF-1\( \alpha \)’s second major regulator is FIH. FIH hydroxylates Asn\(^ {803} \) of the CTAD domain on the \( \beta \)-carbon which is independent of the prolyl hydroxylase modifications in the
ODDD\textsuperscript{33-36}. This event prevents the interaction of HIF-1α with the transcriptional co-activator p300 also known as CREB-binding protein. The end result of this hydroxylation is the halting of all p300 dependent transcription, but not the degradation of HIF-1α as is the case with prolyl hydroxylation. FIH like PHD2 is also commonly found in the cytoplasm. It should be noted that the distribution of PHD2 and FIH does differ depending on the mitigating circumstances of the local biology. For example, it has been demonstrated that the PHD’s are strongly expressed in the cytoplasm of normal bronchial epithelium and weakly in fetal lung bronchial epithelium, while expression is nuclear in the chondrocytes\textsuperscript{37}. FIH expression is strongly expressed in the cytoplasm of normal bronchial epithelium and nuclear expression exists in the bronchial cartilage. FIH in sharp contrast to PHD displays a strong expression in the fetal bronchial epithelium highlighting the differential regulation upon the same substrate between the two enzymes\textsuperscript{37}. The contrasting outcomes on HIF-1α regulation by the HIF hydroxylases is demonstrated in Figure 1.3.
Figure 1.3 The pathway of HIF-1α as directed by the HIF hydroxylases PHD2 and FIH. In the presence of O₂ FIH hydroxylates HIF-1α on Asn803 blocking interaction with the transcription factor P300, halting all P300 dependent transcription. PHD2 hydroxylates either Pro402 or Pro 564, or both leading to proteosomal degradation by signaling the binding of the von Hippal Lindau protein (pVHL) part of ubiquitin ligase complex. In the absence of O₂ HIF-1α is able to dimerize with the β-subunit triggering transcriptional activity. Figure courtesy of Saban, E\textsuperscript{38}.

The Oxygenase Family of Enzymes

Members of the oxygenase super-family of enzymes are found throughout prokaryotes and eukaryotes and execute a variety of chemistry. The reactions catalyzed in this family include ring opening/closing, desaturations, eliminations, halogenations, epoxidations and focal to this research, hydroxylation reactions\textsuperscript{39-42}. These reactions have significant roles in the biosynthesis of collagen, antibiotics, alkylated DNA repair, secondary metabolites generated from plants and microbial sources, and key to this work, cellular oxygen sensing\textsuperscript{40,42-45}. By studying related enzymes in this family it may be possible to gain further insight and knowledge about the less well understood mechanisms of other oxygenase enzymes like PHD2 and FIH. A simplified version of the hydroxylation reaction executed by members of this family including FIH and PHD2 is depicted below
in which αKG and O\(_2\) are utilized in conjunction with substrate generating succinate, CO\(_2\) and product.

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

The αKG dependent enzymes inclusive of PHD2 and FIH are part of a subfamily of the non-heme iron(II) containing superfamily of enzymes. These enzymes possess a His\(_2\)Asp/Glu facial triad about their iron core and catalyze a vast array of reactions. Heme containing enzymes possess four coordination sites occupied by the porphyrin of heme and a fifth coordination site occupied by one proximal residue thereby leaving only one available site for the binding of O\(_2\). In contrast, the non-heme class involves an iron coordination environment formed from three endogenous protein ligands leaving three coordination sites available for cofactors and substrates. This non-heme superfamily can be subdivided into multiple classes which include the extradiol cleaving catechols dioxygenases, \(\alpha\)-ketoglutarate dependent dioxygenases, tetrahydropterin dependent hydroxylases, bacterial prolyl hydroxylases and biosynthetic oxidases\(^{39, 46-49}\). Examination of these enzymes may provide insightful information regarding the HIF hydroxylases as key intermediates for FIH and PHD2 have yet to be isolated.

**Related Oxygenase Enzymes of Interest**

**Extradiol Cleaving Catechol Dioxygenases**

The extradiol cleaving catechol dioxygenases perform ring cleavage reactions of aromatic compounds\(^{50-52}\). The best understood of these enzymes is homoprotocatechuate 2,3-dioxygenase (HPCD). In an effort to gain mechanistic insights to this class of enzymes
an alternative and slow reacting substrate was employed for HPCD, 4-nitrocatechol, a compound also employed for purposes of the body of work contained within this thesis. The knowledge gained from this work was the revealing of three distinct intermediates generated during the catalytic cycle (Figure 1.4).

![Figure 1.4 Catalytic mechanism of extradiol catechols dioxygenase](image)

Intermediate one is a Fe(II)-O₂ complex that determined the Fe-O bond length to be 2.4 Å and that the 4-nitrocatechol ring is not planar, suggesting that iron is maintained in the Fe(II) state and that substrate gives one electron to activate oxygen. Intermediate two is a Fe(II)-peroxo-substrate complex whereby oxygen attacks the C2 of 4-nitrocatechol generating a peroxo bridge. The third intermediate found is the Fe(II)-product complex. The resulting data from these crystallized intermediates provides evidence regarding key residues with the active site which form hydrogen bonds that aid in the stabilization of the deprotonated substrate and in promoting heterolytic dioxygen bond cleavage⁴⁶, ⁵³-⁵⁸.
**Tetrahydropterin Dependent Hydroxylases**

The tetrahydropterin dependent hydroxylases generate hydroxylated aromatic amino acid residues through the donation of two-electrons from tetrahydropterin. In this example which is believed to be similar to the HIF hydroxylases, the binding of substrate and cofactor at iron generates an open coordination site for the binding and subsequent reaction of oxygen. Once bound, oxygen forms a peroxo-bridge from the Fe(II) to the cofactor. Heterolytic O₂ bond cleavage generates a 4a-hydroxypterin and a Fe(IV)=O intermediate species⁵⁹, ⁶⁰. Upon the formation of the highly reactive Fe(IV)=O species it is then able to abstract a hydrogen from the nearby substrate, as is believed to be the case with the HIF hydroxylases. This hydroxyl group proceeds through a rebound mechanism to a radical substrate and the completion of the cycle ending in the hydroxylated product (Figure 1.5)⁶¹.

![Figure 1.5 Aromatic amino acid hydroxylase mechanism](image-url)
α-Ketoglutarate, Iron (II) Dependent Oxygenases

**Taurine:αKG dioxygenase (TauD)**

TauD is a taurine (2-Aminoethanesulfonic acid), αKG dependent dioxygenase from *E. coli* that catalyzes the oxygenolytic release of sulfite from taurine utilizing αKG, molecular oxygen and Fe(II), producing succinate, CO₂ and sulfite as by-products of the reaction. In a reaction quite similar to that of PHD2, studies of TauD have provided great insight into the potential workings of the HIF hydroxylases as they are in the same sub-family of enzymes. In particular, TauD revealed a highly reactive Fe(IV)=O intermediate and provided evidence for the hydrogen abstraction by a non-heme ferryl complex. TauD also revealed a secondary oxygen activation mechanism. In the absence of prime substrate TauD performs a self-hydroxylation reaction which renders the enzyme dead. This auto-hydroxylation reaction has also been witnessed in FIH and questions whether or not this is a safety-net mechanism guarding from oxidative damage by the oxygen sensing HIF hydroxylases. This mechanism has not been supported in PHD2 but it is suspected.
Figure 1.6 The hydroxylation reaction of TauD

Collagen Prolyl-4-Hydroxylase (P4H)

Collagen Prolyl-4-hydroxylase is also a αKG, iron(II)-dependent dioxygenase that cross-links human collagen helices in the connective tissues by hydroxylating proline generating the formation of 4-hydroxyproline residues. Without this vital enzyme collagen is unable to fold into its natural triple helical molecules. A direct result from lack of P4H activity is the development of scurvy, a disease associated with lack of vitamin C (ascorbate) that cumulates in weak connective tissues leading to open lesions. Interestingly enough, ascorbate is believed to help maintain the Fe(II) state in these enzymes as oxidation to Fe(III) renders the enzyme inactive. PHD2 like P4H also requires ascorbic acid for repetitive turnover. The reaction catalyzed by P4H is depicted in Figure 1.7.
The prolyl hydroxylase enzymes catalyze the most prevalent posttranslational modifications in humans by the formation of 4-hydroxyproline, this is in no small part because collagen is the most abundant protein found in the body.

**The Bacterial Proline/Prolyl 4-Hydroxylases (P4H)**

A key difference in the bacterial prolyl 4-hydroxylases is that hydroxy-proline is generated from a free proline versus a peptidyl proline. As these enzymes catalyze the hydroxylation of free proline they are deemed proline hydroxylases instead of prolyl.

There are prolyl hydroxylases found in bacteria like anthrax-P4H that binds to a (GPP) collagen-like peptide. These enzymes lend themselves as informative to the overall mechanism underlying their close relatives, the prolyl hydroxylases. They are members of the non-heme iron(II), αKG, dioxygenase family that require molecular oxygen but have less stringent requirements for substrate than the prolyl version. The structural analysis of the bacterial P4H’s have yielded information about charged residues that play integral roles in the binding of substrate, providing a framework for similar studies on PHD2 and FIH.

All the prolyl and proline hydroxylases utilize O₂, αKG and iron(II). The activity of these enzymes is known to increase in magnitude with the addition of ascorbate, presumably acting as a reducing agent maintaining iron in the 2⁺ state. They all contain a
highly conserved iron binding motif with rare exceptions. Sequence homology amongst these enzymes is almost nonexistent and yet they share a similar three-dimensional structure classified as the β-barrel jelly roll fold (Figure 1.8).

![Figure 1.8 Example of the β-barrel jelly roll fold for the prolyl hydroxylases FIH and PHD2 and including a zoomed view of their iron(II) facial triad (PDB 1H2M, 2G19). Figure created with PyMol.](image)

Based upon knowledge gained from the aforementioned enzymes and their isolated intermediates a mechanism for the HIF hydroxylases is proposed in Figure 1.9. In PHD2 only the accumulation of an Fe(II) species has been witnessed and precisely which Fe(II) form that is has not been resolved.

69
Proposed Mechanism for the HIF Hydroxylases

Figure 1.9 Proposed mechanism of the HIF hydroxylases PHD2 and FIH. Mechanism begins with 6 coordinate iron, αKG displaces two H₂O molecules, substrate binding triggers last H₂O molecule to exit opening a site for O₂ activation. The subsequent steps are proposed and have yet to be supported until the release of hydroxylated substrate and a regeneration of the resting state enzyme.

Of interest in this mechanism are the steps that involve αKG binding and the docking of substrate. When substrate docks it is proposed to release the last H₂O molecule opening a site for oxygen activation. At this point in the reaction there exists an opportunity to halt activity by the introduction of inhibitors which could bind similarly to αKG. Excluding the ability of αKG from binding by mimicking it in the form of an inhibitor may lead to
novel therapeutic treatments in the future. This possibility is currently under exploration and had yielded informative information regarding the potential to selectively inhibit PHD2 and/or FIH which will be a topic of discussion within this thesis. A secondary topic up for discussion regarding the mechanism of PHD2 is to gain a more robust picture of the individual steps involved in the mechanism, specifically rate-limiting steps that are essential to catalytic turnover. Exploration of this latter topic can in turn be used to further the first aim of selective inhibition of the HIF hydroxylases.
CHAPTER 2

SELECTIVE INHIBITION OF THE HIF HYDROXYLASES PHD2 AND FIH

Introduction

The potential for treatment of various disease states via the inhibition of the HIF hydroxylases is a current goal amongst researchers. The ability to reduce sensitivity to hypoxia through possible preconditioning towards hypoxia may provide novel therapies for the treatment of ischemic disorders such as stroke and heart attacks. These enzymes play pivotal roles in the inflammatory response, anemia, metabolism, red blood cell production and vascularization to mention a few and are prime for therapeutic targeting in which myriad diseases could be ameliorated. PHD2 and FIH share a similar structure and utilize the same cofactors, even acting upon the same substrate. They do however have their differences with respect to the docking of co-factors and the coordination environment surrounding the active sites of each that should allow for selective inhibition.

Comparisons of PHD2 and FIH Active Sites

The target for selective inhibition of these enzymes is their iron-centered active site which does pose some challenges as these sites are highly conserved in PHD2 and FIH\textsuperscript{21, 70-72}. Both active sites possess a 2-His/1-Asp facial triad about the iron center and both enzymes are dependent on the same cofactors for activity and target the same substrate, HIF1-\(\alpha\), albeit at different locations. Key differences between PHD2 and FIH are with respect to their Fe(II) and \(\alpha\)KG binding sites. While both possess the HXD/E..H facial triad the binding in PHD2 is tighter, with affinities for the binding of iron, (~0.01-0.03
µM) for PHD2 and (~0.5 µM) for FIH. This fact may partially be explained by the smaller active site of PHD2. The orientation of the aspartate ligand in FIH is different than PHD2, it coordinates to the iron and to the backbone of the substrate while in PHD2 the aspartate ligand coordinates to the iron and a well resolved water molecule in the crystal structure. Measurement of the active site pockets reveals that for the non-substrate bound form of PHD2 the pocket size ranges between 319-355Å³ (PDB’s 2G1M, 2G19, 3OUJ, 2HBT, 2HBU) and that of FIH 1005-1222 Å³ (PDB’s 1H2N, 1MZE, 1MZF, 2CGN). In the presence of substrate the active site pocket shrinks in both enzymes but far more drastically in FIH, 242-497 Å³ (PDB’s 1H2M, 1H2L, 1H2K) versus PHD2 167-344 Å³ (PDB’s 3HQR, 3HQU). Thus each enzyme in theory should produce a differential response to an identical inhibitor. This difference coupled with the fact that and they share virtually no sequence homology despite sharing similar structures lends itself to the possibility of selective inhibition. Another key difference is that FIH requires a dimeric state for catalysis while PHD2 functions in a monomeric state. FIH can also be catalytically active with only the two histidines bound to the iron. The PHD2 pocket is actually deeper than that of FIH and predominantly hydrophobic in nature consisting of residues Ile, Met, Ala, Tyr, Tyr, Thr, Ile, Tyr, Tyr, Ile, Tyr, Phe, Val, Ala, Thr, Trp. This hydrophobicity may function to help guard PHD2 from possible oxidative damage that could arise from Fenton chemistry about the iron center. FIH also exhibits a similar hydrophobicity in its shallower active site pocket, the contributing residues are from Ile, Met, Ala, Tyr, Tyr, Thr, Thr, Ile, Tyr, Tyr, Ile, Tyr, Leu, Phe, Val, Ala, Thr, and Trp. PHD2 appears to involve an induced fit mechanism that results in almost completely blocking the active site as a
loop closes over the entrance upon substrate binding. Other key differences in the enzymes have to do with specific residues that make up the second coordination sphere about the iron core and contribute to hydrogen bonding networks about the iron and $\alpha$KG binding sites. Of particular interest is Gln$^{147}$ in FIH which has its mirror in PHD2’s Ala$^{301}$. The longer side chain in FIH is in position to clash with certain aromatic inhibitors like \{[(4-hydroxy-8-iodoisoquinolin-3-yl) carbonyl] amino\} acetic acid which was employed for the crystallization of PHD2. This compound has proven a viable inhibitor of PHD2 while not impacting the activity of FIH, presumably due to an inability to gain access to the iron due to steric clash. A residue of interest in PHD2 is Val$^{376}$ which forms a steric clash with the $d$-enantiomer of $N$-oxylylglycine (NOG). PHD2 and FIH are also members of separate subfamilies of the $\alpha$-KG oxygenases dictated by the location and function of their C-terminal helices relative to their active sites. In FIH these helices are required for dimerization, while PHD2 does not dimerize for catalytic activity. This coupled with the abovementioned differences between these enzymes lays the framework for ferreting out selective inhibitors of each.

**Known Inhibitors**

It has been found that $N$-oxylylglycine (NOG) and dimethyl $N$-oxalylglycine (DMOG) have the capability to inhibit both enzymes. This inhibition is achieved through bidentate binding to the iron, as both of these compounds are structurally similar to $\alpha$-ketoglutarate. While these inhibitors halt activity in other oxygenases and are therefore not selective, derivatization which resulted in $N$-oxalyl amino acid inhibitors did support that as the side chain increased the level of inhibition decreased for PHD2 and a
preference for the L-N-oxalyl derivatives over the D-N-oxalyl forms was noted\textsuperscript{78}. The effects on FIH supported a preference for the D-enantiomer forms with the N-oxalylphenylalanine derivative showing selective inhibition for FIH over PHD2, suggesting that bulk and hydrophobicity could serve to distinguish between the enzymes\textsuperscript{78}. Selective inhibition has also been explored with elaborated isoquinolines\textsuperscript{79, 80} and imidazo[1,2-a]pyridines\textsuperscript{81} which have been reported as potent inhibitors of PHD2 (IC\textsubscript{50}~1 \textmu M). 4-Oxo-1,4-dihydro-1,10-phenanthroline-3-carboxylic acid was found to inhibit FIH and PHD, with an IC\textsubscript{50} of 60 \textmu M for FIH and a K\textsubscript{i} of 10 \mu M for PHD2. Even among non-specific iron chelators research supports that N-oxalyl-D-phenylalanine (NOG) and desferrioxamine (DFO) both of which are strong chelators display differential inhibition between FIH and PHD2\textsuperscript{22, 70, 78}. Another comparison involving DFO and DIP (dipyridyl), another strong metal chelator, noted that DIP halted FIH associated hydroxylation at high doses while DFO did not. This is indicative that more interactions are at play than just simple chelation\textsuperscript{26}.

**Inhibitor Classes Tested for Selective Inhibition**

Selection of inhibitors was based upon the structural ability to chelate iron in a bidentate manner like \(\alpha\)-ketoglutarate. The compounds tested come from the pyridine, pyrone/pyranone and catechol chemical classes (Figure 2.1). The goal is to start with basic chelating groups to exploit differences between the HIF hydroxylases and selectively inhibit each, with the future goal of designing more selective inhibitors based on the results of this work. Within each class of compounds are variations that introduce different substituents on the base compound that may potentially enhance or disrupt
hydrogen bonding networks within the αKG binding pocket, and that explore the volume difference of the active sites by the introduction of bulky side groups.

Figure 2.1 Inhibitors tested. (1)CNP, 3-cyano-6-methyl-2(H) pyridinone(2)NOP, hydroxypyridine 1-oxide , (3) DHP, 2,3-dihydroxypyridine, (4) Hop-OH, 5-hydroxy-2-hydroxymethyl-4-pyrene, (5) Hop-COOH, 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid, (6) Hop-Me, 3-hydroxy-2-methyl-4-pyrene, (7) HOPO, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone, (8) 4NCat, 4-nitrocatechol, (9) MeCat, 4-methylcatechol, (10) BuCat, 4-tert-butylcatechol.

Pyrones/Pyridinones

Pyrones are a class of compounds containing an unsaturated six-membered ring inclusive of one oxygen atom and a ketone functional group. The compounds selected for this body of work are (4) Hop-OH, 5-hydroxy-2-hydroxymethyl-4-pyrene, (5) Hop-COOH, 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid, (6) Hop-Me, 3-hydroxy-2-methyl-4-
pyrone, (7) HOPO, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone. Coordination to the iron is likely to occur via the hydroxyl and keto functional groups as is seen with other metalloproteins\textsuperscript{82}. These compounds are viable building blocks for selective inhibition as they are known to be safe for human consumption and they chelate metals. For instance, (4) Hop-OH, 5-hydroxy-2-hydroxymethyl-4-pyrone, aka kojic acid is commonly found throughout the food and cosmetic industries, (5) Hop-COOH, 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid, aka comenic acid is deemed safe and is under study for a potential anti-inflammatory and neuroprotection use\textsuperscript{83}. (6) Hop-Me, 3-hydroxy-2-methyl-4-pyrone, aka maltol, is a naturally occurring compound found in the food chain and is prominently used as a flavor enhancer, like kojic acid it can chelate metals. (7) HOPO, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone, aka deferiprone, is currently employed as an oral drug that chelates iron\textsuperscript{84}.

**Pyridines**

The pyridines are heterocyclic organic compounds containing a saturated six-membered ring with nitrogen. The compounds selected for testing include, (1) CNP, 3-cyano-6-methyl-2(H)-pyridinone, (2) NOP, hydroxypyridine-1-oxide and, (3) DHP, 2,3-dihydroxypyridine. Pyridine derivates are known to chelate metal and the cyano group of 3-cyano-6-methyl-2(H) pyridinone is a known inhibitor of cytochrome C oxidase\textsuperscript{85}. Hydroxypyridine 1-oxide is not a naturally compound but is rapidly degraded with a half-life of less than a week in soil microorganisms\textsuperscript{86}. Also making these compounds of interest is that pyridines and dihydroxypyridines can proceed through a degradation pathway that is mediated by oxygenases\textsuperscript{87}.
Catechols

Catechols and their derivatives are known to chelate metals very tightly. They are used in a variety of flavors and fragrances and also as precursors to pesticides with small amounts occurring naturally in fruits and vegetables. The catechols under investigation for this study are, (8) 4NCat, 4-nitrocatechol, (9) MeCat, 4-methylcatechol, and (10) BuCat, 4-tert-butylcatechol. Each compound provides a basis for exploiting the volume of the active site by varying the side group. Also, as the side group varies so does the hydrogen bonding network within the αKG binding site. 4-nitrocatechol has been found to function as a secondary substrate for a Mn(II)-dependent extradiol-cleaving dioxygenase via bidentate binding at the iron active site, making this compound particularly interesting.\(^8\)

Materials and Methods

Protein Expression and Purification

Full length PHD2 has proven troublesome to express and purify in its full length form. Hence, using the catalytic domain of this protein which includes residues 177-426 lessens the challenge of expression and purification while still supporting an active enzyme. To aid in successful purification constructing the gene within a plasmid that contains a tag is desirable. The Stratagene pGEX-4T-1 plasmid provides the necessary solution (Figure 2.2). The vector map shows the insertion region for the chosen gene lies between a glutathione S-transferase tag and an ampicillin resistance gene. The initial region of the insertion area contains a thrombin cleavage site for removal of the GST tag from the recombinant protein when required. The insertion region contains six restriction enzyme sites where the target gene may be inserted. Starting with full length human PHD2
contained within pcDNA3.1 plasmid for eukaryotic protein expression primers are designed to clone out the region of interest. Cloning out the gene of interest allows for insertion into a bacterial vector expression system. The 5’ primer (CGGATCCGGCCCAACGGGCAGAC) anneals on the gene at residue 177 and contains a BamHI restriction enzyme site within the primer to allow insertion into the pGEX-4T-1 vector. The 3’ primer is designed in the same fashion (CATTTCAGAGATCTAAGG) and contains an EcoRI restriction enzyme site. The restriction enzymes sites within the primers initially produce flaps on either end of the target which after several rounds of PCR will become

Figure 2.2  Stratagene pGEX-4T-1 Vector used for expression and purification of PHD2 catalytic domain consisting of residues 177-426. Figure from Stratagene protocol.

part of the target gene sequence with two restriction enzyme cut sites allowing the gene to be inserted into the pGEX vector between the BamHI and EcorRI sites. The PCR program cycle for this protocol which promotes the annealing of the PHD2 gene into the pGEX vector is as follows, 90 °C for 30 seconds, 50°C for 30 seconds, 72 °C for 1 minute, this cycle is repeated for 30 times followed by a 5 minute cycle of 72°C. To
prepare both the pGEX vector and the newly amplified gene for fusion both require treatment with the BamHI and EcoRI restriction enzymes which target the sequences G/GATCC and G/AATTC respectively. Both of these restriction enzymes produce ‘sticky ends’ producing the cuts for the insertion of PHD2 into the vector. After individual digestion of both the vector and the PHD2 gene the final products will be linear DNA molecules. It is then necessary to treat each product with a PCR purification kit (QIAquick PCR Purification Kit by Qiagen) to remove all restriction enzymes and buffer components of the reaction except for the dsDNA. At this point there are still two individual products, linear pGEX and linear PHD2 gene. The two products are mixed in a PCR reaction tube at a ratio of one vector to three gene products as this is considered the industry standard based on published protocols. To achieve the proper ratio it is necessary to obtain the concentrations of each product by measuring the absorbance at 260nm. Although it is possible to roughly determine concentrations by running a DNA agarose gel using a known amount of an appropriate marker the more accurate result will be achieved from absorbance data. Once mixed the sticky ends produced by the restriction enzymes will anneal and ligase is added to the mixture to seal the gaps. This is achieved by mixing the vector and inserting in a 1:3 ratio respectively, adding ligase and allowing to sit at room temperature for about five minutes. The ligation product is now ready for transformation into a competent cell line. The competent cell line used is Stratagene XL1-blue as this particular line of cells is designed for the uptake and copying of DNA versus a cell line that is designed for protein expression. Competent cells can be purchased or prepared based upon several published protocols. Competent cells must be stored at the bottom or a -80°C freezer as they are extremely sensitive to temperature
fluctuations and may lose their ability to take up plasmids if the temperatures are varied too much or too often. Aliquots of 100µL should be removed from -80°C and immediately placed on ice to thaw. This process takes roughly five to eight minutes at which point the cells are ready for use. Using the Stratagene protocol for the cell line, dsDNA amounts of 0.1-50ng can be used per aliquot of cells. This number must be experimentally determined for each plasmid and several different amounts should be tested to ascertain the optimal amount. Approximately 30-40ng of dsDNA pGEX-4T-1-PHD2 is mixed with the XL1-Blue cells and Stratagene’s transformation protocol for this cell line is then followed. In brief, mix dsDNA with cells, incubate on ice for 30 minutes, heat shock for 45 seconds, allow recovery on ice for 10 minutes, add LB growth media and incubate with vigorous shaking for one hour. Fresh LB agar plates containing 0.1mg/ml of ampicillin should already be prepared and pre-warmed at 37°C prior to use. After the one hour of incubation, plate the transformed cells on the agar plates and incubate overnight at 37°C or at least 16 hours. If transformation is successful colonies should be visible on the plate, if not then troubleshooting of the transformation process will be required. Selection of multiple colonies are extracted from the plates and prepared 5mL mini cultures containing 0.1 mg/ml of ampicillin are inoculated for the cultures to grow overnight, approximately 16 hours. Following growth the plasmid is isolated using Qiagen Mini-prep Kit. At this point it is necessary to check the contents of the plasmid as there are two possibilities that exist for what may be present based on the colonies selected, an empty vector, or the vector with the inserted gene. E. coli is known to take up only one type of plasmid so in theory each colony should be representative of only one type of the possible products referenced above. To determine the contents of
the colonies selected, the plasmids are isolated once again using the Qiagen mini-prep kit and protocol. Using 5μL of the PCR product, add 1 μl of 20 U/ml of both BamH1 and EcoR1, incubate at 37°C for one hour. Also prepared is a singly cut plasmid using only BamH1 which produces a single linear stretch of DNA. A 0.8% agarose gel is then prepared by adding 0.8g of agarose to 100mL of 1X TAE buffer. When the restriction enzyme reaction is complete, extract 5μL of the product to 1μL of 6X DNA loading dye by NE Biolabs and load into the gel. The gel power box is set to run at a constant 95 mA and run until the dye front reaches to approximately 75% of the length of the gel. A 1 kilobase marker (New England Biolabs) is used as the pGEX vector is 4900 base pairs and the PHD2 insert is 740 base pairs for a total of 5640 base pairs. If only one band is present this is representative of only the original plasmid, if two bands are present this is the plasmid plus the gene. The singly cut plasmid produces a band which aligns between the markers for 5000 and 6000 base pairs suggesting a product with the correct size for the plasmid plus the gene. A doubly cut plasmid produces two bands also suggesting the desired target. With these results the samples require sequencing to confirm the actual content of the insert in the plasmid. The samples are prepared for sequencing by adhering to the protocol listed on the Genewiz.com web site for sample submission. Once positive sequence results are obtained the process or PHD2 expression is initiated. PHD2 expression is achieved by growing the transformed PHD2-pGEX in E. coli BL-21 cells that are grown in 2X YTA media (16 g L⁻¹ Tryptone, 10g L⁻¹ yeast extract, 5g L⁻¹ NaCl). Culture volumes of 1.5L of sterilized 2X YTA media containing 100μg ml⁻¹ of ampicillin are seeded from a 300mL overnight culture with a OD₆₀₀ of approximately 1.4 at 1:50 seeding volume and grown at 37°C until a OD₆₀₀ of .8-1.0 is achieved. Once the
desired OD reading is achieved the cells are induced with 1mM IPTG (Isopropyl β-D-1-thiogalactopyranoside) and the temperature is dropped to 25°C and grown overnight. The lower temperature facilitates the solubility of GST fusion proteins by slowing down the folding process allowing more time for confirmation sampling of the lowest energy confirmations of the protein. The cultures are then centrifuged and the cell paste collected for purification. The cell paste is generally frozen for future use and freezing prior to lysis is required to increase lysis efficiency. The cell paste is lysed with 2mL of lysis buffer per gram of cell paste. The lysis buffer is the binding/wash buffer of the MagneGST purification system with EDTA added to a final concentration of 0.5mM. Lysing in this buffer eliminates the need for a dialysis step after lysis. The lysis buffer also contains 1mM AEBSF (4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride), a protease inhibitor, 1mM DTT, 0.5% Triton-X, and 300 µg/mL lysozyme. Lysis is achieved through sonication until the lysate is non-viscous. The lysate is then centrifuged at 18,000 G for 30 minutes to remove cellular debris. The lysate is then cleared using a 0.45µm syringe filter in final preparation for loading onto the Promega MagneGST magnetic purification resin. Once the binding step is completed the resin is washed three times with wash buffer to remove any non-specifically bound proteins. The protein is then eluted using 25mM glutathione in 25mM Tris-HCL pH 8. After all protein has been processed it is loaded into a 10kDa dialysis tube for buffer exchange with PBS pH 7.0. At this point the solution now contains purified GST-PHD2 fusion protein as confirmed by SDS-PAGE analysis with bands appearing at approximately 54kDa. To this solution restriction grade thrombin is added at a final concentration of 0.5 units per mg of fusion
protein. The volume of the fusion protein solution should be 2 mL for every milligram of protein requiring GST tag cleavage. 

Upon completion of GST tag cleavage which takes 16 hours at 4°C the resulting solution is passed back through the MagneGST resin to remove the GST tag, followed by a HiTrap Benzamidine FF column (GE Healthcare) to remove the thrombin as per the manufacturer’s protocol. The resulting solution is then transferred to a Vivaspin centrifugal concentrator to achieve a concentrated solution of pure PHD2, the final concentration of protein is dependent upon the type of experiments to be performed but may be concentrated up to 1.5 mM without any aggregation or precipitation. At the same time as concentration the buffer is exchanged into 50 mM HEPES pH 7.0. Protein purity is assessed by SDS-PAGE gel and mass spectrometry.

The Oxygen Dependent Degradation Domain (ODDD) Substrate

A synthetic peptide representing a segment of the oxygen dependent degradation domain (ODDD) of HIF-1α consisting of residues ODDD556-574 DLDLEALAPYIPADDDFQL was used for all assays, the proline that is hydroxylated is underlined. The ODDD sequence is slightly modified from the natural sequence of DLDLEMLAPYIPMDDFQL in which the underlined methionine residues were replaced with alanine to eliminate possible oxidation products upon mass spectrometry analysis. These modification ease analysis while not impacting the recognition sequence of LXXLAP deemed necessary for PHD2 hydroxylation. The substrate is received as a lyophilized powder and is re-suspended in 50 mM HEPES pH 7.0 or the buffer required for experimental purposes. Determination of the resulting concentration of peptide is
achieved by dilution into 0.1 M NaOH and placed in a quartz cuvette for a UV-Vis reading. In 0.1 M NaOH the singular tyrosine is deprotonated producing a peak at 293 nM. The molar absorbency for deprotonated Tyr is 2400 M$^{-1}$ cm$^{-1}$. From this value and by taking at least five independent aliquots the concentration of substrate is calculated.

**FIH Purification and Peptide Substrate**

FIH is isolated and purified as previously described$^{89,90}$. FIH utilizes a peptide fragment of the CTAD domain consisting of 39 residues with the following sequence and containing Asn$^{803}$, DESGLPQLTSLTSYDAEVNAPIQSRNLQGEELLRALDQVN. This peptide is purchased from EZBiolab’s as a lyophilized powder.

**Activity and Inhibition Assays**

Activity assays were conducted using 1.5 µM PHD, 2mM ascorbic acid (Acros), 0-500 µM α-ketoglutarate (Acros) and 20µM ammonium iron (II) sulfate (Acros) in HEPES buffer pH 7.0 (Fisher). A peptide fragment of HIF1-α (ODDD) consisting of residues 556-574 with the sequence of DLDLEALAPYIPADDFLQ and containing the target proline residue (P564) purchased from GL Biochem (Shanghai) LTD was used for determination of hydroxylation by MALDI. Assays were conducted at 37°C and time points were extracted and quenched in a matrix consisting of 4-α-cyano hydroxycinnamic acid with a 2:1 ration of acetonitrile and 0.2% trifluoroacetic acid. Samples were then analyzed on a Bruker Daltonics Omniflex MALDI-TOF and the results were interpreted as a ratio of the parental peak to the hydroxylated peak which exhibits a mass shift of 16 from the parental. A typical raw MALDI-TOF spectra is depicted and labelled below.
Figure 2.3 MALDI spectra displaying the peptide (2133 Da) plus a sodium adduct, followed by additive sodium products. The product peak is labeled as MO+Na, a typical linear regression resulting from analysis is shown as an inset.

The peptide shows up as the peptide mass (2133 Da) plus a sodium adduct and subsequent salt peaks representing plus 2Na⁺, 3Na⁺ etc. are also detected. Regardless of which peaks are analyzed the resulting ratios are the same for any given set of peaks. A typical linear regression from analyzing the MALDI data is show as an inset. Inhibitors were dissolved in 50 mM HEPES pH 7.0 as were α-ketoglutarate and ascorbic acid. The inhibitors tested were 3-cyano-6-methyl-2(H) pyridinone (1), 2-hydroxypyridine 1-oxide (2), 2,3-dihydroxypyridine (3), 5-hydroxy-2-hydroxymethyl-4-pyrone (4), 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid (5), 3-hydroxy-2-methyl-4-pyrone (6), 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone (7), 4-nitrocatechol (8), 4-methylcatechol (9), and 4-tert-
butylcatechol (10). Initial screening of PHD2 was conducted as for activity assays listed above with the exception of using either 10 μM or 200 μM α-ketoglutarate. Dose response curves also followed the same guidelines as for activity assays with the concentration of inhibitor used ranging from 0-1 mM.

**UV-Vis Spectroscopy**

Solutions of enzyme, iron, buffer and inhibitor were prepared by mixing 30μM PHD2, 25 μM ferrous ammonium iron (II) sulphate and 25 μM inhibitor under anaerobic conditions in a coy chamber. All mixtures were in 50 mM HEPES buffer at pH 7.0. Data collection was performed on an Agilent HP8453 in septa sealed cuvettes.

**Electroparamagnetic Resonance Spectroscopy (EPR)**

X-Band EPR spectra were recorded on a Bruker Elexsys E-500 ESR Spectrometer equipped with DM4116 cavity, with samples placed in a liquid-nitrogen finger dewar. Samples were prepared by combining each enzyme with CuSO₄ in ratio of 1:0.9 then adding αKG or inhibitor as indicated. In each of the samples, the CuSO₄ solution was slowly added in 0.5 μL increments to prevent protein precipitation.
Results & Discussion

Dose Response to Inhibitors

An initial screen of the inhibitors was tested against PHD2 and FIH to isolate if the compounds displayed a differential inhibition profile. Each enzyme was screened using $\alpha$-ketoglutarate concentrations approximately at the $K_M$ which was determined to be, 7.0 $\pm$ 2.5 $\mu$M for PHD2 and $K_M = 22 \pm 6$ $\mu$M for FIH. A secondary screen was assayed at saturating $\alpha$-KG concentrations as less effective inhibition under these conditions is a signature for competitive inhibition. The screen indicated that from each chemical class multiple compounds were able to decrease enzyme activity by 50% or more (Figure 2.4). Two compounds from the pyrones/pyridinone class and two catechols were effective inhibitors of PHD2 at sub-saturating $\alpha$-ketoglutarate concentrations ([$\alpha$KG] ~ $K_m$). A similar variety of compounds were effective inhibitors of FIH at sub-saturating $\alpha$KG. The non-overlap of inhibitors for FIH and PHD2 strongly suggested that one or more of these compounds would be selective toward these enzymes.
Figure 2.4 Initial inhibitor screening. PHD2 assay conditions, 1.5 μM PHD2, 10 or 200 μM αKG, 20 μM ferrous ammonium sulfate, 2 mM ascorbate, 60 μM ODDD and 100 μM of inhibitor. FIH assay conditions, 0.5 μM FIH, 10 or 500 μM αKG, 500 μM FeCl₂, 2mM ascorbate, 66 μM CTAD and 100 μM inhibitor. Assays were at 37 °C and time points extracted and quenched followed by analysis on MALDI for PHD2 and ESI-MS for FIH. Product formation was calculated by the equation $[\text{Sub}^{\text{OH}}] = \chi_{\text{(Sub-OH)}} \times [\text{Sub}]$, where Sub is CTAD or ODDD respectively. (1) CNP, (2), NOP, (3) DHP, (4) Hop-OH, (5) Hop-COOH, (6) Hop-Me, (7) HOPO, (8) 4NCat, (9) MeCat, (10) BuCat. FIH collection by B. Holmes.

It was determined that based upon the initial screening that full dose response curves for each enzyme would be conducted to isolate their respective IC₅₀ values, dose response curves for FIH are not shown but a summary of data is included in Table 2.1 (Figure 2.5). PHD2 exhibited typical single site binding dose response curves to the majority of the inhibitors tested with the exception of HOPO (6) and BuCat (10). PHD2 binding to these compounds resulted in an apparent two-site binding dose response with inflections at 16 nM and 25 μM for HOPO and 50 nM and 50 μM for BuCat. While it cannot be definitively stated why this result is achieved it is plausible that the inhibitor is binding at a second metal binding site discovered in PHD2 with an as of yet unknown function. The second plausible possibility is that two molecules of inhibitor are binding at the
active site which has been witnessed in crystallographic studies of FIH in which two molecules of a hydroxyamic acid analogue bind to the iron, partially displacing the Asp$^{201}$ from coordinating the iron$^{93}$. It is worth recalling at this point that FIH only requires coordination of the two histidines in order to maintain catalytic activity and therefore displacement of the aspartate in these two enzymes is a further source for selective inhibition$^{74}$.

Figure 2.5 Dose response curves for all inhibitors. Reaction conditions are 1.5 μM PHD2, 10 μM αKG, 20 μM ferrous ammonium sulfate, 2 mM ascorbate, 60 μM ODDD and 0-1 mM for each inhibitor.
The dose-response curves were in agreement with the initial screens and indicated that compounds from each structural class inhibited FIH and PHD2 with IC$_{50}$ < 500 μM (Table 2.1).

<table>
<thead>
<tr>
<th>Compound</th>
<th>PHD2 IC$_{50}$ (μM)</th>
<th>FIH IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 CNP</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>2 NOP</td>
<td>3</td>
<td>300</td>
</tr>
<tr>
<td>3 DHP</td>
<td>5</td>
<td>200</td>
</tr>
<tr>
<td>4 Hop-OH</td>
<td>400</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>5 Hop-COOH</td>
<td>30</td>
<td>400</td>
</tr>
<tr>
<td>6 Hop-Me</td>
<td>1000</td>
<td>800</td>
</tr>
<tr>
<td>7 Hopo</td>
<td>40*</td>
<td>400</td>
</tr>
<tr>
<td>8 4NCat</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>9 MeCat</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>10 BuCat</td>
<td>30*</td>
<td>20</td>
</tr>
</tbody>
</table>

Table 2.1 IC$_{50}$ values for PHD2 and FIH. *For Hop-Me the concentration to achieve exactly half the maximal rate is 16 μM, for BuCat it is 1.0 μM, the values in the table are reflective of the second inhibitory event as a two site binding mechanism was witnessed in PHD2. FIH experiments performed by B. Holmes.

Each of the compounds tested was a more effective inhibitor of PHD2 than of FIH, reflective of the differences between the two enzymes. This result may also feed back into the fact that PHD2 exhibits tighter binding constants for iron and αKG$^{73}$. In the pyridine class the trend for both enzymes is the same, NOP≈DHP>CNP. CNP had no inhibition effect on either Hydroxylase up to 1 mM even though the cyano functional group is a known metal chelating inhibitor of cytochrome C oxidase. Despite seeing a similar trend there is an approximate 100-fold preference of NOP toward PHD2 and similar results are observed for DHP. In the pyrones/pyridinones different trends do exist. For PHD2 regarding the functional groups of the base compound the preference is
2Me>COOH>OH>Me while for FIH it is COOH>2Me ≈Me>OH. In this case there is additional bulk and hydrophobicity provided by HOPO that is clearly favored in PHD2 over the single methyl moiety in Hop-Me while FIH does not distinguish between the two. The trends in FIH favor the COOH moiety indicating that the functionality of the carboxy group may provide hydrogen bonding contacts within the active site that are similar with those of αKG. The conformation of the hydroxyl group of Hop-OH also poses interesting results as the hydroxy group is able to function as a hydrogen bond donor/acceptor within the pocket if the fit is acceptable. The IC$_{50}$ value for PHD2 is half that of FIH signaling the position and conformation of this moiety is a framework to build upon. Also of interest in this class of compounds is that the singular methyl moiety of HOPO generated an IC$_{50}$ value twice that for PHD2 as for FIH and is the only example within the compounds where FIH is inhibited over PHD2. HOPO demonstrated a two-site binding mechanism when bound to PHD2 resulting in two inflection points at 21 nM and 43 µM. The initial response on PHD2 at 21 nM decreases the enzymes activity by approximately 30% from an activity of 1.0 min$^{-1}$ to 0.7 min$^{-1}$. The second inhibitory event occurs at approximately 43µM with a reduction in rate to 0.3 min$^{-1}$ equivalent to a 70% overall reduction of rate. The concentration to achieve exactly half the maximal activity occurs at approximately 16 µM.

This further supports that the second coordination sphere about the iron active site is vital for purposes of gaining selective inhibition. There is also a much wider range in IC$_{50}$ values for this class of compounds by more than 400-fold. The IC$_{50}$ values of the catechol class support their strong chelating abilities on both enzymes. The relative trend is that FIH appears to be more tolerant of sterically bulky inhibitors than PHD2 which
may in part be attributed to their relative pocket sizes mentioned previously. Of interest is that FIH did not discriminate BuCat from MeCat while PHD2 showed roughly a ten-fold difference between the two compounds.

**UV-Visible Spectroscopy of Inhibitor Binding**

Integral to determining the mode of inhibition is support for the binding of the selected compounds to the metal center. Inhibitory effects can be the result of multiple reasons ranging from denaturation, nonspecific binding, precipitation, or aggregation\textsuperscript{94}. The use of spectroscopy with metalloenzymes can provide evidence to support inhibition is the result of chelation because of changes in the electronic environment that occur at a metal center when different species are bound which shift charge transfer bands. For purposes of this study 4NCat was employed for UV-Vis studies as it exhibits visible absorption bands and displays shifts in response to deprotonation. 4-Nitrocatechol has also provided insight into substrate binding and p\textsubscript{Ka} shifts in the catechol dioxygenase family\textsuperscript{95-97}. The \(\pi-\pi^*\) transition for 4NCat (p\textsubscript{Ka}=6.6, 10.7) is found at 410 nM in Homoprotocorchnaechuate 2,3-dioxygenase, a related enzyme in function possessing an extinction coefficient of 10,800 M\(^{-1}\) cm\(^{-1}\) at pH 7.5\textsuperscript{97}. 4NCat in H\(_2\)O exhibits wavelength and extinction coefficient shifts representative of its protonation state as the pH is varied. At pH 7.25 \(\lambda_{\text{max}} = 426 (\varepsilon=11,000), \) pH 8 \(\lambda_{\text{max}} = 428 (\varepsilon=14,700)\) at pH 12.5 \(\lambda_{\text{max}} = 383\) and 511 (\(\varepsilon=6,300\) and 10,400)\textsuperscript{95}. We find that PHD2 and FIH in solution with iron and 4NCat exhibit results consistent with the binding of 4NCat in the 4NC\(^{-1}\) charge state (Figure 2.6).
Figure 2.6  PHD2 and FIH in the presence of iron(II) and 4NCat, pH 7.0. Samples were prepared anaerobically and sealed in cuvettes for data collection.

Control spectra were also collected for 4NCat in the presence and absence of FeSO₄ at pH 7.00 (λₘₐₓ = 427 nM) and pH 12.00 (λₘₐₓ = 515 nM). The (Fe+4NCat)PHD2 spectra exhibited a λₘₐₓ of 420 nM and FIH at 428 nM both consistent with a singly deprotonated 4NCat bound to the metal center, for control spectra see Figure 2.7.
Figure 2.7 Control spectra for 4-Nitrocatechol. Samples were prepared anaerobically at pH 7 and 12, 4NCat with iron (II) at pH 7, PHD2 at pH 7.

**Electroparamagnetic Resonance Spectroscopy (EPR)**

Since 4NCat is the only inhibitor which exhibits strong visible bands for the detection of metal binding, EPR was employed as a secondary measure to support the model of the inhibitors binding to the iron core. HOPO was chosen for EPR experiments as it displayed an inhibitory effect on both enzymes as well as displaying a two-site binding mechanism towards PHD2. This result in and of itself warrants further investigation of the compound to support chelation as the mode of binding. Copper (II) was employed as a probe for both PHD2 and FIH as iron(II) is ERP silent due to the lack of an unpaired electron. The results are indicative of HOPO binding the iron in both enzymes, and not simply a result of binding free metal in solution as can be observed from the spectral changes (Table 2.2, Figure 2.8). For each enzyme in the presence of copper the magnitude of $g_{ll}$ and $A_{ll}$ provide significant information regarding the electronic changes centered about the metal as they are dependent upon the ligand environment. In essence
if the ligand is changed from αKG to HOPO and no changes are detected in $g_{II}$ and $A_{II}$, after factoring in all the controls, then the inhibitor is not binding to the metal. Detectable changes in these parameters upon ligand substitution assure that binding is occurring at the metal active site.

<table>
<thead>
<tr>
<th>EPR Parameters</th>
<th>$g_{II}$</th>
<th>$g_{\perp}$</th>
<th>$A_{II}$ (G)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(CuSO₄)</td>
<td>2.28</td>
<td>2.07</td>
<td>161.3</td>
</tr>
<tr>
<td>(Cu+αKG)</td>
<td>2.28</td>
<td>2.08</td>
<td>143.4</td>
</tr>
<tr>
<td>(Cu+HOPO)</td>
<td>2.28</td>
<td>2.06</td>
<td>164.1</td>
</tr>
<tr>
<td>(Cu+HOPO)PHD</td>
<td>2.32</td>
<td>2.07</td>
<td>145.1</td>
</tr>
<tr>
<td>(Cu+αKG)PHD</td>
<td>2.35</td>
<td>2.07</td>
<td>141.6</td>
</tr>
<tr>
<td>(Cu)PHD</td>
<td>2.29</td>
<td>2.06</td>
<td>147.9</td>
</tr>
<tr>
<td>(Cu+HOPO)FIH</td>
<td>2.33</td>
<td>2.07</td>
<td>136.7</td>
</tr>
<tr>
<td>(Cu+αKG)FIH</td>
<td>2.35</td>
<td>2.07</td>
<td>138.1</td>
</tr>
<tr>
<td>(Cu)FIH</td>
<td>2.28</td>
<td>2.06</td>
<td>152.1</td>
</tr>
</tbody>
</table>

Table 2.2 EPR Spectral parameters for (Cu)FIH and (Cu)PHD2. Parameters were determined using Spincount.

The spectral features of (Cu)PHD2 and (Cu)FIH share many similarities as can be expected based on their 2-His1-Asp facial triads. There is also no witnessing of any observable fine structure detail that would be consistent with splitting from the protein derived ligands. Both the (Cu)FIH and (Cu)PHD2 display four relatively weak hyperfine splitting features as a result of binding to Cu(II). However, the addition of the anionic αKG ligand in conjunction with Cu(II)PHD2 caused these hyperfine features to become
well resolved (Figure 2.8). The addition of this ligand generates increases in the $g_{II}$ and decreases in $A_{II}$ values as would be expected upon metal binding. The values for FIH are in agreement with previously reported values. To determine the mode of binding and determine if the inhibitors were binding at the active site HOPO was employed for the EPR studies. The (Cu + HOPO) spectra exhibited hyperfine splitting that was similar to the (Cu)FIH and (Cu)PHD spectra with $g_{\text{eff}}$ ($g_{\perp} = 2.06$, and $g_{II} = 2.28$) and a slightly larger hyperfine coupling value ($A_{II} = 164.1G$). However, there is additional resolvable hyperfine coupling in the $g_{\perp}$ region of the (Cu + HOPO) spectra. These additional resolvable hyperfine features are diminished in the (Cu + HOPO)FIH and (Cu + HOPO)PHD2 spectra, indicating that there is a difference in ligand coordination and possibly geometry in the presence of enzyme. There is also an increase in anisotropy
similar to that observed for the addition of αKG with $g_{\text{eff}} \ (g_\perp = 2.07, \text{ and } g_\parallel = 2.33)$ for 
(Cu + HOPO)FIH and $g_{\text{eff}} \ (g_\perp = 2.07, \text{ and } g_\parallel = 2.32)$ for (Cu + HOPO)PHD2. Additionally both (Cu + HOPO)FIH and (Cu + HOPO)PHD2 show decreased hyperfine coupling compared to the (Cu + HOPO) spectra, suggesting the presence of an anionic ligand interaction with Cu(II). The changes in electronic structure indicate that Cu(II) is being incorporated at the active sites of both PHD2 and FIH, and that HOPO is binding to the copper at the active site. This strongly denotes that the main mechanism of inhibition is through chelation of inhibitors at the active site to the Fe(II) rather than sequestering it from the enzyme through chelation in solution.

**Conclusions**

The catalytic activity of the HIF hydroxylases in this study is perturbed by the chelating ability of the selected inhibitors. While these compounds do not exhibit a definitive selectivity between PHD2 and FIH, they do hint at the possibility that the internal binding pocket of each enzyme which has marked variation can be exploited to further this cause. Both FIH and PHD2 share a similar active site with a 2His-1Asp facial triad and but they do exhibit differences in the hydrogen bonding networks within the enzyme core. The contacts made between active site residues and α-KG within both enzymes provide key contacts in which it may be possible to build selectivity. Exploration of the key residues within the active site of each enzyme reveal that PHD2 utilizes Arg$^{383}$ and Tyr$^{329}$ from a core DSBH to bind to the 5-carboxylate of α-ketoglutarate while FIH employs Lys$^{214}$ and Tyr$^{145}$ which does not stem from a DSBH. These residues interactions with the inhibitors may be a contributing factor to the differences seen in the IC$_{50}$ values especially amongst
the inhibitors of the pyrones/pyridinones class which displayed differences in the overall trends of binding with PHD2 showing preference towards the dual methyl moiety versus FIH’s preference toward the carboxylate moiety. In lieu of the results that most of the inhibitors preferentially inhibited PHD2 over FIH with the exception of HOPO it is not unreasonable to assume that much of this difference can be attributed to potential steric clash from specific FIH residues. For example, FIH Gln$^{147}$ projects into the active site pocket to within 5 Å of the αKG 5’carboxylate position, the equivalent residue in PHD2 is Ala$^{301}$, a far less obtrusive residue (PDB 1H2M, 3HQU respectively). Overall the pyridine/pyridinone class of inhibitors show promise for further elaboration in the development of selectivity toward the HIF hydroxylases. These compounds displayed substantial variation in IC$_{50}$ values reflective of changing functional groups and bulk. The catechols as a whole proved to be potent inhibitors exemplative of the strong chelating ability associated with this class and their subsequent derivatives. Of notable interest in this class is BuCat which displayed a two-site binding mechanism with PHD2 with the initial inhibitory event occurring at 41 nM equating to a 20% reduction in rate and a secondary inhibitory event at 32 µM and a 80% reduction in the rate with the actual IC$_{50}$ occurring at approximately 1 µM. FIH showed a standard one-site binding mode with an IC$_{50}$ of 20-fold to that of PHD2. This may partially be explained by the size of the binding pockets of each enzyme, while FIH does possess and overall larger pocket it is shallower in comparison with that of PHD2 and the bulky tert group cannot be accommodated to the same extent, providing further scaffolding for future design of inhibitors. While the pyridine class of compounds tested did not display any relative
differences in trends amongst them it is notable to mention that there is an approximate 100-fold preference of NOP toward PHD2 and similar results are observed for DHP.

In short we find that the pyrone/pyridinone class of chelates to be promising for further development of selective inhibitors for the HIF hydroxylases. The formation constants for this class towards $2^+$ transition metals are modest and it is very likely that the $IC_{50}$ values reflect structural differences between the two enzymes. As HOP-Me, aka maltol has been used as a foundation for inhibitors of matrix metalloproteases, basing further inhibitors on this framework may lead to increasingly potent selective compounds for PHD2 and/or FIH.
MECHANISTIC STUDIES INVOLVING pH AND SOLVENT ISOTOPE EFFECTS ON PHD2

Introduction

The study of enzyme-catalyzed reactions under the influence of pH can provide information regarding the underlying kinetic and chemical mechanisms. With the introduction of solvent isotope effects (SIE), even greater information about the chemical mechanism via identifying isotope-sensitive steps may be obtained. Solvent isotope effects can be observed when enzyme catalyzed reactions are measured in equivalent buffers based in either H$_2$O or D$_2$O. The SIE arises as a result of the different physical properties of H$_2$O and D$_2$O, which include greater density and viscosity as well as stronger hydrogen bonds, all of which can impart changes in protein-substrate interactions as hydrogen is exchanged for deuterium. The impacts of D$_2$O have the potential to affect enzyme stability, conformational change, and substrate binding as well as the kinetic and equilibrium constants associated with the overall reaction and care needs to be taken to execute proper controls before final analysis is complete. The SIE is deemed as a global effect since substitution occurs at multiple sites versus substrate isotope effects in which one or only a few positions are substituted. Ultimately the SIE is measured as a ratio of the reaction in water to the reaction in deuterium$^{100,101}$. When the kinetic parameters $k_H/k_D$, $V_H/V_D$ or $(V/K)_H/(V/K)_D$ are larger than unity (larger in H$_2$O) a normal solvent isotope effect is observed. When the parameters are less than unity (larger in D$_2$O), it is called an inverse solvent isotope effect. Inverse solvent isotope effects only arise under certain circumstances and are rarely reported and therefore they can be highly diagnostic of underlying chemical mechanisms.
An inverse SIE can be attributed to the acid dissociation constant of a thiol, a diffusion-sensitive conformational change arising from solvent viscosity effects, or the dissociation of a metal-chelated water$^{102-105}$.

While it is noted that studying enzyme-substrate reactions at arbitrary substrate concentrations may prove useful they also can have decreased value. This is the result of the fact that the steady-state rate of the reaction encompasses all of the $pK_a$ values of the entire kinetic system which includes free enzyme, substrate, intermediates and complexes formed. However, what can be gained from these studies is the pH dependence of the catalytic constant $k_{cat}$, which unveils $pK_a$ values for certain enzyme-substrate complexes or intermediates and provides the specificity constant $k_{cat}/K_M$. When pH studies are combined with SIE a broader spectrum of information may be obtained about the overall system under investigation.

When considering the complete catalysis of PHD2 involving $k_{cat}$ and $K_M$ the focal point are the steps after $\alpha$KG and substrate have bound and the enzyme is primed for oxygen activation. At this junction the enzyme should be in a five coordinate state with one water ligand still in place, if only briefly. A general scheme for the kinetic parameters believed to be involved in the mechanism of PHD2 is shown in (Figure 3.1).
Figure 3.1 Catalytic steps involved in PHD2 catalysis. Focus is upon the first irreversible step when oxygen is activated denoted by the $k_{\text{cat}}$ arrow.

**Methods and Materials**

**Mixed Buffer Preparation of MES-PIPES-HEPES (MPH)**

A mixed buffer solution of MES hydrate (2-(N-morpholino)ethanesulfonic Acid) from Sigma, PIPES (1,4 – Piperazine Diethane Sulfonic Acid from Fisher Biotech, and HEPES (4-(2-Hydroxyethyl)-1-Piperazineethanesulfonic Acid from Fisher Scientific (MPH) was prepared consisting of 20 mM of each buffer to avoid changing buffers to study the full range of pH thereby eliminating any effects from the buffer alone. A 200 mL solution of 60mM MPH buffer, consisting of 20 mM MES, 20 mM PIPES and 20 mM HEPES was made and divided in two. One half of the buffer was brought up to a pH of 8.88 by the addition of 1M NaOH and the ionic strength was calculated, this buffer is deemed the base form. The ionic strength at 37°C was calculated for each buffers contribution by using a ‘buffer calculator’ (www.biomol.net/en/tools/buffercalculator.htm), the ionic strength of the NaOH that was added to produce the base form of the buffer was then...
calculated based upon the equation, \( I = \frac{1}{2} \sum c_i z_i^2 \). The final ionic strength was determined to be 130 mM for the base form. To the acid form of the MPH buffer which has a pH of 5.8, NaCl was added as a solid to match the ionic strength of the base form at 130 mM. These two solutions of buffer of identical ionic strength were then mixed at various ratios to achieve a range of pH without any variation of the ionic strength. MPH buffer was also prepared exactly as listed above except D\(_2\)O was substituted for H\(_2\)O to dissolve all buffer components and NaOH was dissolved in 99.9\% D\(_2\)O to pD the base from of the buffer. These buffers were used for all solvent isotope experiments. For all D\(_2\)O buffer preparation, the pH electrode was equilibrated in D\(_2\)O for 30 minutes prior to measuring the actual pD of the full range of prepared buffers, and applying the equation of pD = pH + 0.4.

**Protein and Solutions Preparation for pH and SIE Experiments**

Aliquots of PHD2 were diluted to a final concentration of 7.5 µM in either the appropriate 60 mM MPH buffer of H\(_2\)O or D\(_2\)O as dictated by the current experiment. The pD of deuterated buffers was calculated by the following equation, pD=pH + 0.4. The resulting dilutions of PHD2 in D\(_2\)O buffer contain a final concentration of 97.87\% D\(_2\)O, 1 µL of this stock is placed in 24 µL of a 99.9\% D\(_2\)O component mixture for all reactions resulting in a 97.82\% deuterated buffer. All stock solutions of ascorbic acid, α-ketoglutarate, iron and ODDD were prepared in either H\(_2\)O or D\(_2\)O as dictated by the experiment. All assays for both H\(_2\)O and D\(_2\)O work were conducted using 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 1 mM ascorbic acid and 200 µM α-ketoglutarate. For all kinetics experiments, the substrate, ODDD was varied between 1-50
µM. All components of the reaction were mixed and initiation of the experiment was induced by the addition of ODDD. Time points were extracted and quenched in a MALDI matrix consisting of 4-α-cyano hydroxycinnamic acid with a 2:1 ration of acetonitrile and 0.2% trifluoroacetic acid. Samples were then analyzed on a Bruker Daltonics Omniflex MALDI-TOF and the results were interpreted as a ratio of the parental peak to the hydroxylated peak which exhibits a mass shift of 16 from the parental peak.

**UV-Visible Spectroscopy**

Acid and base forms of PHD2 were prepared anaerobically in a coy chamber by mixing 50 µM PHD2 with 45 µM ferrous ammonium sulfate and 50 µM αKG into fully degassed MPH buffer at pH 6.5 or pH 8.5 respectively. The cuvettes were sealed with septa and analyzed on a Agilent HP8453 to determine if any spectral changes between the two samples could be elucidated. The spectra were then evaluated by subtraction of the pH 6.5 from pH 8.5.

**X-Ray Absorbtion Spectroscopy**

Sample preparation for analysis by XAS was prepared anaerobically in a Coy chamber by mixing 1 mM PHD2, 0.9 mM ferrous ammonium iron (II) sulphate and 0.9 mM αKG in 60 mM MPH buffer at pH 6.5 for the acid form of the enzyme and pH 8.5 for the base form. Both samples were initially brought up to a volume of approximately 500 µL with their respective buffers for the addition of iron and αKG and subsequent incubation for 15 minutes. Samples were then treated with CheleX® to remove all unbound metal from the samples for 30 minutes. After the incubation time to remove excess unbound iron the
samples were then concentrated to a final volume between 40-50 µL resulting in the final concentrations listed above. Samples were then loaded into a XAS sample holder and immediately submerged in liquid N\textsubscript{2} in the evacuated anti-chamber of the Coy chamber and transferred to -80°C until sample analysis could be performed. XAS data collection and analysis were performed as reported previously\textsuperscript{106}. X-ray absorption near edge spectroscopy (XANES) data was collected from -200 eV to +200 eV with respect to the Iron edge energy (7111.2 eV). Extended X-ray absorption fine structure (EXAFS) data was collected to $k = 14$ Å\textsuperscript{-1} above the edge energy. Data was fit using FEFF software which provides for the calculation of phase shifts and effective scattering amplitudes of polarization dependent single and multiple scattering X-ray Absorption Fine Structure (XAFS) and X-ray Absorption Near-Edge Structure (XANES) spectra for clusters of atoms.

**Viscosity Test**

PHD2 was assayed in 50 mM HEPES D\textsubscript{2}O buffer at pH 7.0 by mixing 0.3 µM PHD2, 1 mM ascorbate, 15 µM ferrous ammonium sulfate and 200 µM αKG all prepared in D\textsubscript{2}O and initiating the reaction with 15 µM ODDD. The identical assay was also performed by adding 10% sucrose to a non-deuterated 50 mM HEPES pH 7.0 buffer to mimic the viscosity imposed by D\textsubscript{2}O to exclude viscosity as the underlying factor observed in the rate differences. The relative viscosity ($\eta/\eta_0$) of D\textsubscript{2}O at 37°C is 1.31 mPa*\textperiodcentered s. The relative viscosity of a 10% sucrose solution at 20°C is 1.333 and at 37°C it is approximately 1.3 mPa*\textperiodcentered s and therefore approximates the viscosity of D\textsubscript{2}O\textsuperscript{107}. Assays were conducted at 37°C and time points were extracted and quenched in a matrix consisting of 4-α-cyano
hydroxycinnamic acid with a 2:1 ration of acetonitrile and 0.2% trifluoroacetic acid. Samples were then analyzed on a Bruker Daltonics Omniflex MALDI-TOF and the results were interpreted as a ratio of the parental peak to the hydroxylated peak which exhibits a mass shift of 16 from the parental.

**Stability Assays**

PHD2 stability was assessed by incubating the enzyme at various pH’s for 15 minutes prior to the initiation of the assay. As standard linear rates are collected within five minutes a fifteen minute incubation was more than appropriate. After the incubation time the enzyme was mixed as 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 1.0 mM ascorbic acid and the assay was initiated with 15 µM ODDD in a three component buffer of 60 mM MPH at pH 7.0 and the collection of linear data was complete within approximately four minutes for all assays. Assays were conducted at 37°C and time points were extracted and quenched in a matrix consisting of 4-α-cyano hydroxycinnamic acid with a 2:1 ration of acetonitrile and 0.2% trifluoroacetic acid. Samples were then analyzed on a Bruker Daltonics Omniflex MALDI-TOF and the results were interpreted as a ratio of the parental peak to the hydroxylated peak which exhibits a mass shift of 16 from the parental.

**Incubation Assays**

Incubation assays were conducted to determine if PHD2 was able to regain full activity after exposure to a pH which resulted in the reduction of initial rate. PHD2 was exposed to 60 mM MES,PIPES, HEPES buffer at pH 8.5 which is out of the stability range for 15
minutes. The enzyme was then reintroduced to a 60 mM MPH buffer at pH 7.0 and the initial rate data collected via time point quench aliquots. Final assay concentrations are as follows, 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM aKG, 1.0 mM ascorbic acid and the assay was initiated with 15 µM ODDD. Assays were conducted at 37°C and time points were extracted and quenched in a matrix consisting of 4-α-cyano hydroxycinnamic acid with a 2:1 ration of acetonitrile and 0.2% trifluoroacetic acid. Samples were then analyzed on a Bruker Daltonics Omniflex MALDI-TOF and the results were interpreted as a ratio of the parental peak to the hydroxylated peak which exhibits a mass shift of 16 from the parental.

**Data Fitting**

All data was fit with OriginPro software using either a simple liner regression for initial rate data or fitting to the Michaelis-Menten equation. H₂O and D₂O data was fit to the following equation to ascertain the actual pKₐ for each data set where enzH is the fully protonated form of the enzyme and enz is the deprotonated form

\[
k_{cat} = \frac{enzH \cdot 10^{pK_a}}{10^{H^+} + 10^{-pK_a}}.
\]

**Results & Discussion**

**pH Dependence of PHD2 Catalyzed Hydroxylation of HIF-1α**

In an effort to identify catalytically relevant steps and gain a better understanding of the hydroxylation mechanism of PHD2 we measured the kinetic parameters \(k_{cat}\), \(K_M\) and \(k_{cat}/K_M\) as a function of pH over the pH scale from 5.80 to 8.88. It was determined that
activity and potential stability issues arise under pH 6.30 and therefore data that falls under this pH was not considered in the final analysis. These experiments were conducted in a three component buffer of 60 mM MES, PIPES and HEPES to avoid buffer dependent results and also as a method of controlling the ionic strength by mixing acid and base forms of the buffer to obtain a full range of pH for H2O buffers. All data was fit to the Michaelis-Menten equation, after initial rate data was calculated from MALDI analysis of the modified substrate (Figure 3.2). Product formation was calculated using the equation \[ [\text{ODD}^{\text{OH}}] = \chi_{(\text{ODD-OH})} \times [\text{ODD}]_0 \] and subsequently fit using a linear regression to obtain all initial rate information. All data was linear over the allotted collection time.
Figure 3.2 Kinetic fits for PHD2 at varied pH. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2 mM ascorbic acid and ODDD concentrations from 0-80 µM. Product formation was calculated using the equation $[\text{ODDD}^{\text{OH}}] = \chi_{(\text{ODDD-OH})} \times [\text{ODDD}]_0$. All data was fit using the Michaelis-Menten equation.

The collection of the kinetic parameter $k_{\text{cat}}/K_M$ proved to be troublesome due to the very low $K_M$ value for the ODDD (Table 3.1). This value is in the approximate range of 1.0-2.0 µM in the biologically relevant pH scale with a decline in the $K_M$ detected beginning at pH 8.03. Current detection methods for the substrate do not allow for substrate concentrations that fall below 1.0 µM in the reaction vessel as extracted aliquots are minimally diluted and quench into a MALDI matrix for data collection. The
concentration of substrate as the instrument detects it is 0.5 µM, this low concentration is difficult to detect and while peaks are visible the signal to noise ratio tends to be high. This fact excludes the ability at the current time to reduce ODDD concentrations which would allow for a more accurate measurement of the true $K_M$. In lieu of this the $k_{cat}/K_M$ appears somewhat random and values above pH 8.03 are unreliable as all data as analyzed produce initial rates which were equivalent at all substrate concentrations.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{cat}, \text{min}^{-1}$</th>
<th>$K_M, \mu\text{M}$</th>
<th>$k_{cat}/K_M, \mu\text{M}^{-1}\text{min}^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.32</td>
<td>2.64 ± 0.09</td>
<td>0.60 ± 0.28</td>
<td>4.40 ± 2.06</td>
</tr>
<tr>
<td>pH 6.45</td>
<td>2.61 ± 0.09</td>
<td>3.13 ± 0.69</td>
<td>0.79 ± 0.17</td>
</tr>
<tr>
<td>pH 6.62</td>
<td>2.37 ± 0.11</td>
<td>1.79 ± 0.69</td>
<td>1.32 ± 0.51</td>
</tr>
<tr>
<td>pH 6.95</td>
<td>2.05 ± 0.08</td>
<td>1.77 ± 0.72</td>
<td>1.16 ± 0.47</td>
</tr>
<tr>
<td>pH 7.20</td>
<td>1.87 ± 0.09</td>
<td>0.61 ± 0.46</td>
<td>2.75 ± 2.08</td>
</tr>
<tr>
<td>pH 7.40</td>
<td>1.41 ± 0.08</td>
<td>0.73 ± 0.46</td>
<td>1.93 ± 1.22</td>
</tr>
<tr>
<td>pH 7.64</td>
<td>1.04 ± 0.02</td>
<td>1.57 ± 0.03</td>
<td>0.68 ± 0.06</td>
</tr>
<tr>
<td>pH 7.85</td>
<td>0.98 ± 0.04</td>
<td>0.04 ± 0.09</td>
<td>24.3 ± 54.6</td>
</tr>
<tr>
<td>pH 8.03</td>
<td>0.60 ± 0.01</td>
<td>1.53 ± 0.21</td>
<td>0.39 ± 0.05</td>
</tr>
<tr>
<td>pH 8.25</td>
<td>0.51 ± 0.02</td>
<td>1.23 ± 0.31</td>
<td>0.42 ± 0.11</td>
</tr>
<tr>
<td>pH 8.5</td>
<td>0.57 ± 0.02</td>
<td>0.65 ± 0.17</td>
<td>0.87 ± 0.23</td>
</tr>
<tr>
<td>pH 8.88</td>
<td>0.46 ± 0.02</td>
<td>0.18 ± 1.12</td>
<td>2.56 ± 15.9</td>
</tr>
</tbody>
</table>

Table 3.1 Kinetic parameters for PHD2 under different pH values
Figure 3.3 The kinetic parameters $k_{\text{cat}}/K_M$ and $K_M$ for PHD2.

Using the observed $k_{\text{cat}}$ values the data was then plotted and fit to determine the $pK_a$ according to the equation, $k_{\text{cat}} = \frac{\text{enzH} \times 10^{[H^+]} + \text{enz} \times 10^{-pK_a}}{10^{[H^+]} + 10^{-pK_a}}$ (Figure 3.3).

Figure 3.4 The PHD2 $k_{\text{cat}}$ data fit to determine the $pK_a$ of 7.22 ± 0.03.

The $k_{\text{cat}}$ parameter unlike that of $K_M$ displayed a more systematic decline with increasing pH generating viable kinetic data. However in the pH range of 6.32 to 8.03 the $K_M$
remains fairly constant (Figure 3.3) and in an effort to obtain a more viable value for $k_{\text{cat}}/K_M$ the average was taken and used to calculate a secondary approximation of $k_{\text{cat}}/K_M$. This secondary approximation was achieved by the application of statistics to the observed $K_M$ values to obtain a singular value with propagated errors (Table 3.2).

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_{\text{cat}}/K_M$, $\mu$M$^{-1}$min$^{-1}$ Using Averaged $K_M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.32</td>
<td>2.06 ± 1.48</td>
</tr>
<tr>
<td>6.45</td>
<td>2.04 ± 1.47</td>
</tr>
<tr>
<td>6.62</td>
<td>1.85 ± 1.33</td>
</tr>
<tr>
<td>6.95</td>
<td>1.63 ± 1.17</td>
</tr>
<tr>
<td>7.20</td>
<td>1.46 ± 1.05</td>
</tr>
<tr>
<td>7.40</td>
<td>1.10 ± 0.79</td>
</tr>
<tr>
<td>7.64</td>
<td>0.81 ± 0.58</td>
</tr>
<tr>
<td>7.85</td>
<td>0.76 ± 0.55</td>
</tr>
<tr>
<td>8.03</td>
<td>0.46 ± 0.33</td>
</tr>
</tbody>
</table>

Table 3.2 Estimated $k_{\text{cat}}/K_M$ after statistical analysis on the range of $K_M$’s from original data set, final estimated $K_M = 1.28 \pm 0.92 \mu$M. The estimated $K_M$ was then used with the experimentally determined $k_{\text{cat}}$ to generate the kinetic parameter $k_{\text{cat}}/K_M$.  

59
While it is herein acknowledged that it would be preferable to have more sensitive detection methods in which to accurately pin-point the $K_M$ values at each particular pH, the original data set is highly suggestive of the trend observable in the revised $k_{cat}/K_M$ data, as the $K_M$ does not significantly change across a range of pH. The lack of changing $K_M$ values is also suggestive that substrate binding may be factored out of the equation with reference to the observable differences for $k_{cat}$.

The overall observed pH dependence of PHD2 also has comparisons in other related enzymes like TauD. TauD is an $\alpha$-KG, Fe(II) dependant dioxygenase enzyme that when tested across a pH range of 4.5-10.8 was found to have a maximal activity at pH 6.9, similar to that found for PHD2\textsuperscript{108}. Procollagen-proline dioxygenase, an enzyme very closely related to PHD2 exhibits a maximal activity at pH 7.8 for the coupled reaction of $\alpha$-KG decarboxylation to peptidyl hydroxylation, but no pH dependence for the
uncoupled reaction\textsuperscript{109}. These examples provide support that the witnessed pH dependence of PHD2 is not an anomalous event.

### Stability versus Activity of PHD2

The optimal pH for PHD2 activity was found to be within 6.5-7.0. The stability of PHD2 was assayed and found to be stable in the range of 6.30 to 7.60 with a sharp drop in stability occurring between 7.60 and 7.80. However, when stability is compared against activity it appears that the drop in activity which is far more steep of a decline may not necessarily be associated with a loss of structure (Figure 3.6).

![Relative Stability vs. Activity](image)

Figure 3.6 Effect of preincubation on the activity of PHD2. All assays include 0.3 \( \mu \)M PHD2, 15 \( \mu \)M ferrous ammonium sulfate, 200 \( \mu \)M \( \alpha \)KG, 2mM ascorbate and 15 \( \mu \)M ODDD. For stability curve PHD2 was incubated at pH 8.5 and assayed at pH 7.0. For activity curve PHD2 was incubated at each individual pH and assayed at the same pH as for incubation. Both curves have been normalized to activity at pH 6.5.

It is plausible that the stability results depicted in figure 3.5 arise from the H\( _2 \)O ligand in the sixth coordination site being replaced by OH as the pH increases. The hydroxyl bond
is shorter and stronger than the iron(II)-\(\text{H}_2\text{O}\) bond and may not displace as easily on substrate binding as does the water-ligand (Figure 3.7).

![Diagram of PHD2 with either water or hydroxyl coordinated at the sixth position just prior to ODDD binding.]

Figure 3.7 PHD2 with either water or hydroxyl coordinated at the sixth position just prior to ODDD binding.

The iron-water bond length in PHD2 based on PDB 2G19 without substrate bound is 2.09 Å, as a comparative example for the change in bond lengths associated with the exchange of water for a hydroxyl we can look to a related enzyme system, SyrB2, a non-heme, αKG-dependent halogenase. The underlying chemistry of this related enzyme employs virtually the same consensus mechanism as PHD2 except the end result is a halogenation reaction and not a hydroxylation. The measured bond lengths of the iron-water bond in SyrB2 is 2.10 Å with a bond dissociation energy of 6.0 Kcal/mol, and the bond length for the hydroxyl form is 1.88 Å with a dissociation energy of 52 Kcal/mol\textsuperscript{110,111}. It should be noted that the hydroxyl form of SyrB2 if for the Fe(III) form, not Fe(II) and relevant Fe(II)-OH bond length information for similar enzymes could not be obtained. It is not unreasonable based on the similarity of the iron-water bond lengths in both enzymes that the shift to iron-OH form in PHD2 would result in a similar shortened bond length and greater dissociation energy, thereby making less readily available the empty coordination site necessary for oxygen activation to occur. Stability assays were conducted by incubating PHD2 across the pH spectrum and assaying at pH 7.0, while activity assays
were conducted in each individual pH of the selected range. Since the stability and activity assays are conducted in a similar manner the fact that the activity falls far below the estimated stability gives this notion further validity along with the results that support activity begins to sharply drop off at pH 7.2, well within the stability range which does not decline until at least the pH 7.6 mark is reached.

**Incubation Assays: Determining if Full Activity of PHD2 is Recoverable**

To determine if the activity loss of PHD2 which begins to occur at approximately pH 7.2 is recoverable, incubation assays were conducted. PHD2 was incubated in 60 mM MPH buffer at pH 8.5 and then reintroduced into pH 7.0 buffer followed by initial rate experiments. PHD2 regains full activity upon reintroduction to pH 7.0 buffer within minutes of the exchange (Figure 3.8). This strongly suggests that the resulting loss of enzymatic activity is not due to a complete obliteration of structure and is more likely an effect attributable to the changing pH and iron-ligand environment especially since activity begins to drop within the pH range deemed stable. Considering the observed $pK_a$ of 7.32 ± 0.01 is in the expected range for a metal-OH being protonated to form a metal-OH$_2$, this is further support for iron-aquo ligand being responsible for the sharp decrease in activity versus loss of catalytic ability due to enzyme stability$^{112}$.  

Figure 3.8 PHD2 Incubation assay to determine activity recovery. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2mM ascorbate and 15 µM ODDD. Assay time range, 1.4-30.0 minutes. PHD2 was incubated at pH 8.5 and assays were conducted at pH 7.0.

Following all control experiments with regards to the results of determining the kinetic parameters for PHD2 which indicated a titratable pKₐ at 7.32 ± 0.02 determined by fitting the data to the following equation:

\[
k_{\text{cat}} = \frac{\text{enzH} \times 10^{pK_a}}{10^{pK_a} + \text{enz} \times 10^{-pK_a}}
\]

Equation 1 Where enzH is the fully protonated form of PHD2, and enz is the deprotonated form.

The fully protonated form of PHD2 displayed at \( k_{\text{cat}} \) of 2.72 ± 0.01 while the fully deprotonated from has a \( k_{\text{cat}} \) of 0.30 ± 0.02. To further investigate these results particularly with regards to the iron-ligand environment of PHD2 at a higher pH range UV-visible spectroscopy experiments were initiated to explore if any difference in their spectra at different pH’s could be detected.
Acid and Base Forms of PHD2

Two samples of PHD2 were prepared identically with the exception of the chosen pH of the solution and prepared anaerobically in a Coy chamber in an effort to maintain the Fe(II) state. Many related αKG-dependent dioxygenases display changes to their coordination environment about the iron in approximately the 500 nM range upon binding of varying cofactors attributable to a ligand-to-metal charge transfer (MLCT). These transitions are witnessed in FIH(Fe^{II}+ αKG) at 500 nM, Taurine Dioxygenase (TauD) at 530 nM and in Clavaminate Synthase 2 (CS2) at 476 nM. In the PHD2 difference spectrum (pH 8.5-pH6.5), we see a broad absorption feature with at $\lambda_{\text{max}}$ at 485 nM, comparable to CS2 and another prominent feature at 342 nM which likely reflects a shift in the charge transfer bands (Figure 3.9).

Figure 3.9  pH 8.5 and pH 6.5 forms of PHD2. Samples include 50 µM PHD2, 45 µM ferrous ammonium sulfate and 50 µM αKG. Samples were prepared anaerobically in a Coy chamber to maintain the Fe(II) state. The resulting spectra is obtained as a difference spectra, (pH 8.5-pH6.5).
The two samples at pH 6.5 and pH 8.5 exhibit differences with respect to the coordination environment of the iron and are likely to be the result of deprotonation of the iron-OH$_2$ ligand as there are no other variances between the two samples. The red shift observed at 350 nM may be attributed to the removal of a proton from the pH 8.5 sample which will result in electron destabilization and a shift to higher energy. All controls were prepared as the samples and do not give rise to the features exhibited when PHD2 is present.

**X-Ray Absorption Spectroscopy**

X-Ray absorption spectroscopy can provide several viable pieces of information regarding the ligand environment as it pertains to the local electronic and or geometric structure about a metal ion. From the x-ray absorption near edge region (XANES) of the spectra the coordination number of the metal can be ascertained. From the extended x-ray absorption fine structure (EXAFS) radial distances of the ligands and the type of ligands, specifically nitrogen, oxygen and sulfur can be determined (Figure 3.10).

![XAS regions for determining local geometric and/or electronic environments about a metal ion. Figure courtesy of N. Giri.](image)

Figure 3.10  XAS regions for determining local geometric and/or electronic environments about a metal ion. Figure courtesy of N. Giri.
Upon analysis of the PHD2 (Fe$^{II}$ + αKG) form of the enzyme it should be possible to distinguish the nitrogen atoms from the coordinating histadines, the αKG oxygens, the aspartate oxygen, and if present the aquo-ligand and/or the hydroxyl ligand which would be distinguished by its shorter radial distance from the iron. The actual assignment of the ligands whether they are imidizole nitrogens or oxygens comes from data fitting analysis which will disallow an improper assignment that will be obvious via skyrocketing reduced chi-square values ($\chi^2$). Ideally, when assigning ligands there should be at least a 30% reduction in the reduced $\chi^2$ from the original data to support proper ligand assignment$^{115}$.

Two samples were prepared for X-ray absorption spectroscopy (XAS) as previously prepared for UV-Vis experiments of the acid and base forms of PHD2 with respect to the pH of the solution.

![Figure 3.11 Iron K-Edge XANES spectra; (Fe$^{2+}$:αKG:PHD2) at pH 6.5 (red), and pH 8.5 (black)](image)

For the data collection shown in figure 3.11, eight scans were averaged for the Fe$^{2+}$:αKG:PHD2 at pH 6.5 and twelve scans were averaged for Fe$^{2+}$:αKG:PHD2 at pH 8.5. The intensity of the peak associated with a 1s $\rightarrow$ 3d electronic transition was then
used to indicate the coordination number/geometry of metal sites$^{116, 117}$. For EXAFS analysis of the data collected at the iron $K$-edge, a limit of $2 - 12$ Å$^{-1}$ was used. Structural models of the metal sites involving coordination numbers from $2 - 7$ were systematically evaluated for all possible combinations of N/O- and S-donors by holding the number of scattering atoms in each shell to integer values. The number of histidine imidazole ligands involved in the coordination sphere was estimated by multiple-scattering analysis as previously described$^{118-120}$. The amplitudes and phase shifts for multiple scattering paths of the iron-imidazole ligands were generated using FEFF (v. 8.0) with the coordinates of an iron-imidazole ligand obtained from the crystal structure of human PHD2 with Fe(II) and αKG (3OUJ). Scattering paths of similar length were combined in one shell, as described by Costello et al.$^{119, 120}$. During the fitting process, coordination numbers were constrained to be integer values and a scaling factor of 0.9 was used. Bond lengths, $\sigma^2$ and a single value of $\Delta E_0$ were allowed to vary in each fit. The best iron-imidazole models were further examined using multiple-scattering analysis derived from the rigid O-C-C-O five-membered chelate rings with parameters obtained using FEFF (v. 8.0). In this analysis, distances in the chelate ring were constrained to vary with a single value of $\Delta r$.

To compare different models (fits) the $R$-factor and reduced $\chi^2$ parameters can be assessed, in which case both parameters should be minimized. Although $R$ will always improve with an increasing number of shells (adjustable parameters), reduced $\chi^2$ will go through a minimum and then increase, indicating that the model is over fitting the data. Best fits were judged by using two goodness of fit parameters, reduced $\chi^2$ and $R$, and the deviation of $\sigma^2$ from typical values (table 3.3).
### Table 3.3 XANES and EXAFS analysis of Fe^{2+}-αKG-PHD2 at pH 6.5 and 8.5.

<table>
<thead>
<tr>
<th>Sample</th>
<th>XANES Analysis</th>
<th>EXAFS Analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Edge E(eV)</td>
<td>1s→3d peak area (x10^{-2} eV)</td>
</tr>
<tr>
<td>Fe(PHD)-αKG at pH 6.5</td>
<td>7121.2(2)</td>
<td>8.7(8)</td>
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<td></td>
</tr>
<tr>
<td>Fe(PHD)-αKG at pH 8.5</td>
<td>7120.9(2)</td>
<td>7.9(4)</td>
</tr>
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<td></td>
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* ^a r (Å) is the radial distance between metal and ligand.*

* ^b σ_2^2 is the root mean square disorder in the metal-ligand distance.*

* ^c R is the goodness of fit. Numbers in parentheses represent standard deviation for least square fits.*

* ^d Distances in [ ] correspond to atoms in a O-C-C-O chelate ring and were constrained to vary with a single value of Δr for the chelate ring.*
**XANES Analysis**

XAS experiments of PHD2 bound to iron and α-ketoglutarate at pH 6.5 and 8.5 were investigated to determine data about the coordination number and geometry of the metal center. XANES data indicates that Fe(II) has vacancies in the 3d manifold that give rise to peaks associated with 1s → 3d electronic transitions that are observed in the pre-edge XANES region of the K-edge spectra in both the samples (Figure 3.11). The peak area intensities of the 1s → 3d transitions depend on the coordination number and geometry of the metal sites\(^{116, 117}\). By comparing the 1s → 3d transition peak areas of the PHD2 samples with typical values for known coordination numbers/geometries we were able to determine the coordination numbers of both the PHD2 samples from the XANES data as being five-coordinate.

Comparisons of XANES data (Table 3.3, Figure 3.11) for the two PHD2 samples show that each spectrum is distinct. Thus, the structure of the iron site is sensitive to pH. The lowering of K-edge energy from pH 6.5 to pH 8.5 indicates an increase in electron density at the iron site. The edge energies observed for Fe\(^{2+}\):αKG:PHD2 at pH 6.5 and at pH 8.5 are 7121.2 eV and 7120.9 eV respectively. No changes in the coordination number of the Fe(II) center are apparent at pH 6.5 or at pH 8.5. In the PHD sample at pH 6.5 the 1s → 3d peak area (~ 8.7 x 10\(^{-2}\) eV) is indicative of a five-coordinate complex (typical values are ~8 – 13 x 10\(^{-2}\) eV). The complex remains five-coordinate with a 1s → 3d peak area of ~7.9 x 10\(^{-2}\) eV upon increasing the pH to 8.5.
**EXAFS Analysis**

The analysis of EXAFS provides information about the number and types of ligands bound to a metal, and metric details of the metal site structure. This information is obtained from the best fits of the data and is summarized in Figure 3.12, Table 3.3. The best fit for Fe$^{2+}$-αKG:PHD2 at pH 6.5 EXAFS data consists of six N/O donor ligands of which two are imidazoles from multiple-scattering analysis. Using the chelate model, C atoms are found at 2.75 Å and 2.85 Å.

![EXAFS analysis](image)

**Figure 3.12** EXAFS analysis. Left: Unfiltered, $k^3$-weighted EXAFS spectra Fe(PHD)-αKG at pH 6.5 (top) and at pH 8.5 (bottom) and fits (black lines). Right: Fourier-transformed EXAFS data and fits.

The best fit for Fe$^{2+}$-αKG:PHD2 at pH 8.5 EXAFS data also consists of six N/O donor ligands of which two are imidazoles from multiple-scattering analysis (Table 3.3). But the average bond lengths are shorter than those at pH 6.5. Using the chelate model, two C atoms are found at 2.62 and 2.72 Å. Considering the Fe-N/O bond lengths at pH 6.5 and 8.5 it can be predicted that the N/O donor at 1.96 Å could be due to the hydroxide formation (Fe-OH will be shorter than Fe-OH$_2$). This is in agreement with the decrease in
Fe $K$-edge energy due to the increase of pH from 6.5 to 8.5 (OH will make Fe center more electron rich than OH$_2$).

**Solvent Isotope Effects on PHD2**

In lieu of the discovery of a titratable $pK_a$, visible differences in the spectra at pH 6.5 and pH 8.5 and in no small part based upon the radial bond length changes in the acid-base forms of PHD2 we turned to SIE experiments. SIE may potentially help to determine if further mechanistic information could be obtained with emphasis on the step involving the six coordinated water molecule release from iron, see figure 3.13.

![Chemical Reaction Diagram](image)

Figure 3.13 Individual steps believed to be involved in the catalysis of PHD2. Based upon pH and SIE studies focus is on $k_5$ where the release of water is thought to occur opening an open coordination site for O$_2$ activation with the iron(II) state maintained.

PHD2 was assayed under the identical conditions as that for determining pH effects on its catalytic activity by the collection of full Michaelis-Menten type kinetic curves (Figure 3.14).
Figure 3.14 Kinetic fits for PHD2 at varied pD. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2 mM ascorbic acid and ODIDD concentrations from 0-50 µM. Product formation was calculated using the equation $[\text{ODDD}^\text{OH}] = \chi_{\text{ODDD-OH}} \times [\text{ODDD}]_0$ for all initial rates which comprise the kinetic curves.

The exception is the replacement of H$_2$O for D$_2$O in all buffers and stocks of required materials. The solvent isotope effects on $k_{\text{cat}}$, $K_M$, and $k_{\text{cat}}/K_M$ were measured to test the relative rate constants for water binding and release to other steps in the chemical mechanism. The resulting data indicated an inverse solvent isotope effect where $k_{\text{catH}} / k_{\text{catD}}$ is 0.91 ± 0.03 and the observed $pK_a$ is 7.89 ± 0.03 which results in a $pK_a$ shift of 0.67 ± 0.04 from that at H$_2$O.(Figure 3.15). Based on fractionation factors for similar metal-
water complexes this $\Delta pK_a$ shift of 0.67±0.04 is agreement for other metalloenzymes such as the zinc-bound water in alcohol dehydrogenase ($\Delta pK_a$ 1.04), xanthine oxidase ($\Delta pK_a$ ~1), and carbonic anhydrase I and II ($\Delta pK_a$ 0.37-0.54)\textsuperscript{101, 121-124}. The kinetic parameters obtained are listed in table 3.4.

Figure 3.15 Solvent isotope effects, ($D_2O$, triangles, $H_2O$, squares) $pK_a$ fits for PHD2. Data is comprised of full Michaelis-Menten curves for each data point. Fully protonated form, 2.99 ± 0.08, $pK_a$ 7.22 ± 0.03, deprotonated 0.31 ± 0.02. Fully deuterated form 3.3 ± 0.06, $pK_a$ 7.89 ± 0.03, dedeuterated form 0.34 ± 0.04. The observed solvent isotope effect on $k_{cat}$ is 0.91 ± 0.03.
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<td>9.05</td>
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<td>0.21 ± 0.22</td>
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<td>0.30 ± 0.10</td>
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Table 3.4 Kinetic parameters for PHD2 under different pD values. All data was obtained by linear regression of the initial rates collected under varied ODDD concentration (1-50 µM), and then fitted to the Michaelis-Menten equation to obtain $k_{\text{cat}}$ and $K_M$.

A similar event occurs with regards to the accuracy of the $K_M$ as it did in H$_2$O buffers in which current detection methods hinder the ability to definitively assign a viable value to this parameter. In H$_2$O $K_M$ values range from 0.18-3.13 µM, in D$_2$O $K_M$ ranges from 0.21-2.33 µM. The exceptionally low range of $K_M$ is only observed in the pL range above
eight. In short this makes it difficult to know with certainty the specificity constant $k_{\text{cat}}/K_M$. A plot of $K_M$ showing the observed scattering is show in Figure 3.16.

![Figure 3.16 $K_M$ under various pD’s.](image)

**Figure 3.16** $K_M$ under various pD’s. All assays include 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 2 mM ascorbic acid and ODDD concentrations from 0-50 µM. Product formation was calculated using the equation $[\text{ODDD}^{\text{OH}}] = \chi_{(\text{ODDD-OH})} \times [\text{ODDD}]_0$. The $K_M$ was derived from fitting the data to the Michaelis-Menten equation.

Overall the $K_M$ values with the exception of a few outliers are somewhat similar and an averaging of the $K_M$ generates a value of $1.73 \pm 0.57$ which is over all much lower than that calculated for the H$_2$O $K_M$ value of $1.67 \pm 0.32$ µM. If we plot $k_{\text{cat}}/K_M$ with the unadjusted values we are unable to obtain any gainful information. If we assume the $K_M$ does not actually shift about which is not unreasonable considering the nature of the data we obtain we are then able to provide a conservative idea of the impact D$_2$O imparts upon $k_{\text{cat}}/K_M$ (Figure 3.17).
As previously mentioned inverse solvent isotope effects which arise in enzyme-catalyzed reactions have been attributed to the acid dissociation constant of a thiol, a diffusion-sensitive conformational change arising from solvent viscosity effects, or the dissociation of metal-chelated water\textsuperscript{102-105}. PHD\textsubscript{2}\textsuperscript{177-426} contains seven cysteine residues all of which are distally located to the active site pocket. Based on this fact the inverse solvent isotope effect witnessed is excluded as originating from the acid dissociation of thiol groups. Thus, the other two plausible reasons giving rise to the observed inverse SIE are the dissociation of a metal-H\textsubscript{2}O bond or solvent viscosity effects. To confirm or rule out viscosity as the underlying cause we initiated experiments in which we increased the viscosity of the H\textsubscript{2}O buffers.

**Viscosity Assays**

It has been concluded in previous work that mere external viscosity changes to a reaction mixture are enough to elicit rate changes in an enzyme-substrate mechanism\textsuperscript{104, 105}. The impact generated by viscosity in some systems has been suggested to influence the rate-

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Figure 3.17  \(k_{\text{cat}}/K_{M}\) in D\textsubscript{2}O, A) \(k_{\text{cat}}/K_{M}\) using \(K_{M}\) generated from Michaelis-Menten fits. B) \(k_{\text{cat}}/K_{M}\) using an averaged \(K_{M}\) (1.73 ± 0.57) which assumes little if any fluctuation in the value as the actual \(K_{M}\) is below the detection limits of data collection.
limiting step which is in effect the rate of necessary transition states required to proceed past the energy barrier in order to achieve product accumulation. The viscosity of the medium also has the ability to impart an inverse solvent isotope effect\textsuperscript{125}. To eliminate the potential that the observed inverse SIE is simply the result of viscosity, assays which introduced a visocogen were conducted. The addition of 10% sucrose to a non-deuterated 50 mM HEPES pH 7.0 buffer was introduced to mimic the viscosity imposed by D\textsubscript{2}O. The relative viscosity ($\eta/\eta_0$) of D\textsubscript{2}O at 37°C is 1.31 mPa\textsuperscript{s}. The relative viscosity of a 10% sucrose solution at 20°C is 1.333 at 37°C it is approximately 1.3 mPa\textsuperscript{s} and therefore approximates the relative viscosity of D\textsubscript{2}O. The results support that the viscosity is not a factor in the catalytic differences observed between H\textsubscript{2}O and D\textsubscript{2}O (Figure 3.18). The control assays in standard MPH H\textsubscript{2}O buffer generate initial rate values of $2.07 \pm 0.05 \text{ min}^{-1}$, and in the same buffer with 10% sucrose the rate is $2.04 \pm 0.02 \text{ min}^{-1}$.

![Figure 3.18 The solvent viscosity impact on PHD2. Assay includes 0.3 µM PHD2, 15 µM ferrous ammonium sulfate, 200 µM αKG, 1 mM ascorbate and 10 µM ODDD. Activity is measured as previously disclosed. A maximal velocity of $2.07 \pm 0.05 \text{ min}^{-1}$ was obtained for the control assay, and in the same buffer with 10% sucrose the observed rate is $2.04 \pm 0.02 \text{ min}^{-1}$.]
Having now eliminated two of the three factors which may give rise to the rarely witnessed inverse SIE, acid-dissociation of a thiol group and viscosity we conclude that the resultant effect is due to the dissociation of the metal-aquo bond which shifts from Fe(II)-OH\textsubscript{2} to Fe(II)-OH.

**Conclusions**

Herein we report an observable dependence on pH for the catalytic mechanism of PHD2 based upon a titratable pK\textsubscript{a} of 7.22 ± 0.03. The kinetic parameter of k\textsubscript{cat} is determined to be 2.99 ± 0.08 min\textsuperscript{-1} in the fully protonated form and 0.31 ± 0.02 min\textsuperscript{-1} in the deprotonated state. As previously mentioned accurate assessment of k\textsubscript{cat}/K\textsubscript{M} was unobtainable due to the detection limits of the analysis method for substrate accumulation under 1 µM in the reaction vessel. Equivalent experiments in D\textsubscript{2}O support a pK\textsubscript{a} shift of 0.67 ± 0.04 and a k\textsubscript{cat} of 3.30 ± 0.06 min\textsuperscript{-1} in the fully protonated form and 0.34 ± 0.04 min\textsuperscript{-1} in the deprotonated form, with an observed SIE for k\textsubscript{catH}/k\textsubscript{catD} of 0.91 ± 0.03. A summary of this data is provided in (table 3.5).

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<td>H\textsubscript{2}O</td>
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<tr>
<td>D\textsubscript{2}O</td>
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Observed SIE k\textsubscript{H}/k\textsubscript{D} 0.91 ± 0.03

Table 3.5 Observed kinetic parameters of PHD2 in water and deturium, pK\textsubscript{a} and observed SIE.

When the kinetic parameters k\textsubscript{H}/k\textsubscript{D}, V\textsubscript{H}/V\textsubscript{D} or (V/K)\textsubscript{H}/(V/K)\textsubscript{D} are larger than unity (larger in H\textsubscript{2}O) a normal solvent isotope effect is observed. When the parameters are less than

79
unity (larger in D$_2$O), it is called an inverse solvent isotope effect. The inverse SIE is a rarely witnessed event and can be attributed to three underlying causes. The first cause is due to the acid dissociation of a thiol ligand followed second and thirdly by, a diffusion-sensitive conformational change arising from solvent viscosity effects, or the dissociation of a metal-chelated water$^{102-105}$ While PHD2 contains seven cysteine residues, they are all distally located to the active site pocket and therefore acid dissociation of a thiol group is ruled out as the cause of the inverse SIE. Viscosity experiments exclude the result as arising from solvent viscosity effects. Therefore, this leaves us with only one option, the dissociation of a metal-chelated water molecule. In support of this method we report the kinetic parameters listed in table 3.5 followed up by X-ray absorption spectroscopy. The resulting XANES and EXAFS data analysis support shortened radial bond lengths in the pH 8.5 sample relative to the pH 6.5 sample indicative of greater electron density at the metal site. For comparison, SyrB2, a non-heme, αKG-dependent halogenase is utilized. The underlying chemistry of this related enzyme employs a similar consensus mechanism as PHD2 except the end result is a halogenation reaction and not a hydroxylation. The measured bond length of the iron-water bond in SyrB2 is 2.10 Å with a bond dissociation energy of 6.0 Kcal/mol, and the bond length for the hydroxyl form is 1.88 Å with a dissociation energy of 52 Kcal/mol$^{110,111}$. It is not unreasonable based on the similarity of the iron-water bond lengths in both enzymes that the shift to iron-OH form in PHD2 would result in a similar shortened bond length observed at 1.96 Å and greater dissociation energy, thereby making less readily available the empty coordination site necessary for oxygen activation to occur. The variation in OH-iron bond lengths of SyrB2 and PHD2 at 1.88 Å and 1.96 Å respectively may in part result from a difference
at their facial triad in which the Glu/Asp residue coordinates to SyrB2 in a bidentate manner versus a mono-dentate fashion in PHD2. To further support our model we present data which indicates that PHD2 activity begins to sharply drop off in a pH range deemed biologically relevant (pH 7.2) while the stability of the enzyme is maintained. This drop in activity without loss in stability is observable in the range of the calculated pK_a for PHD2 at 7.22 ± 0.03 in H_2O as would be expected. This data coupled with the fact that the only observable intermediate in the catalytic cycle that has been observed for PHD2 is a Fe(II) form. At this point we need to revert back to our model for each step in the cycle (Figure 3.19)\textsuperscript{126}.

There are two points in the mechanism in which the accumulation of an Fe(II) intermediate is likely. The first is at k_5 which is where the water molecule departs, opening a site for O_2 activation. The second is k_10 and or k_11 where release of product and succinate is likely to occur. It seems more plausible that the release of succinate and product are more favorable as the rate determining step since the water release is going to have a more rapid dynamic equilibrium and therefore contribute less hindrance to the
forward commitment of the reaction. Despite this assumption the evidence supports that the dissociation of the metal-OH$_2$ ligand is at least in part a contributing factor towards halting the forward commitment of the overall catalytic reaction of PHD2.

When looking at comparable enzymes such as TauD no SIE is observed suggesting that the water-off form of the enzyme is favored as compared to the water-on form of PHD2 that generates a small inverse SIE and that product release in TauD is the slow step of the reaction$^{113}$. It is highly probably that product release is the rate-limiting step for PHD2 as well with a small contribution from the metal-aquo ligand. In Xanthine hydroxylase, also Fe(II), αKG dependent, SIE experiments support little effect on $K_M$, as is the case with PHD2 and a 40% reduction on $V_{max}$ when H$_2$O was substituted with D$_2$O suggesting an exchangeable proton in the rate-determining step of the overall reaction$^{127}$. However the decrease in $V_{max}$ is more likely the result of general acid/base side chain than the metallocenter which can give rise to an inverse SIE as appears to be the case with PHD2 where the reaction is faster in D$_2$O. PHD2 unlike many of its related counterparts reacts very slowly with O$_2$ which may help explain the observed SIE. In TauD the introduction of substrate increased the coupled turnover 1000-fold from that of the uncoupled reaction in the absence of substrate, in PHD2 only a 30-fold increase is observed and the uncoupled activation of O$_2$ is on the order of 0.013 s$^{-1}$.$^{126}$ This is likely indicative of PHD2’s role as an oxygen sensor and the virtually non-existent uncoupled turnover (Appendix figure A1) provide further support for the water-on form of PHD2 which may limit the activation of oxygen by blocking the coordination site on the iron. Overall the dilatory reaction of PHD2 distinguishes it from any closely or distantly related enzymes. The observed inverse SIE witnessed for PHD2 has not been reported for any closely
related enzymes like other prolyl/proline hydroxylases nor any Fe(II), αKG dependent hydroxylases that is linked to the metallocenter further distinguishing it in its class. There are reports of inverse SIE’s for metalloenzymes but the result has been linked to viscosity effects alone\textsuperscript{104, 125}. The viscosity experiments conducted in this body of work have eliminated it as the underlying cause of the inverse SIE.
APPENDIX: Supplementary Data

**Abbreviations**

4NCat, 4-nitrocatechol

αKG, alpha-ketoglutarate;

BuCat, 4-tert-butylcatechol

CNP, 3-cyano-6-methyl-2(H) pyridinone

CS2, clavaminate synthase-2

CTAD, C-terminal transactivation domain of HIF-1α;

DHP, 2,3-dihydroxypyridine

ESI-MS, electrospray ionization mass spectrometry;

FIH-1, the factor inhibiting HIF

HIF, Hypoxia Inducible Factor

Hop-COOH, 5-hydroxy-4-oxo-4H-pyran-2-carboxylic acid

Hop-Me, 3-hydroxy-2-methyl-4-pyrone

Hop-OH, 5-hydroxy-2-hydroxymethyl-4-pyrone

HOPO, 3-hydroxy-1,2-dimethyl-4(1H)-pyridinone

HPCD, homoprotocatechuate 2,3-dioxygenase

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

MeCat, 4-methylcatechol

MLCT, metal-to-ligand charge transfer

NOP, 2-hydroxypyridine 1-oxide

ODDDD, oxygen dependent degradation domain

p300, transcriptional coactivator
PHD2, Prolyl Hydroxylase Domain 2

SIE, solvent isotope effect

TauD, taurine dioxygenase
pGEX-4T-1-PHD2 DNA Sequence and Plasmid Insertion Details

pGEX-4T-1 sequence

Full length: 4969 bp; PHD2 insert: 744 bp

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The green codon signifies the start codon for the GST-pGEX sequence, the primers highlighted in yellow indicate the forward and reverse annealing sites for the pGEX sequencing primers. The red sequences are BamH1 and EcoR1 restriction enzyme cut sites respectively.
**Sequence Alignment of Full Length PHD2 versus PHD2 Catalytic Domain used for all Experimental Procedures**

100.0% identity in 250 residues

| PHD2 Cut | 1 | GGLRPNGQTTPALKLaley1VPCMNKHGICVVDFFLGKETGQIGDEVRALHDTGKFT |
| PHD2 Full | 177 | GGLRPNGQTTPALKLaley1VPCMNKHGICVVDFFLGKETGQIGDEVRALHDTGKFT |
| PHD2 Cut | 61 | DQQLVSQKSSDKDIRGDKITWEGKEPGCETIGLMMSSGMLIRHCNGKLYGKYNGRT |
| PHD2 Full | 237 | DQQLVSQKSSDKDIRGDKITWEGKEPGCETIGLMMSSGMLIRHCNGKLYGKYNGRT |
| PHD2 Cut | 121 | KAMVACYPGNSTGYVRHVDNPGDRCVTCIYYLNKDWDKVSFGILRIFPEGKAFQFADI |
| PHD2 Full | 297 | KAMVACYPGNSTGYVRHVDNPGDRCVTCIYYLNKDWDKVSFGILRIFPEGKAFQFADI |
| PHD2 Cut | 181 | EPKFDRLLFFWSDRNPHEVQPAYATRYAITWYFDADERAKVKLYTGEKGRYRNLNK |
| PHD2 Full | 357 | EPKFDRLLFFWSDRNPHEVQPAYATRYAITWYFDADERAKVKLYTGEKGRYRNLNK |
| PHD2 Cut | 241 | PSDSVKGDVF |
| PHD2 Full | 417 | PSDSVKGDVF |

The full length PHD2 sequence is as follows:

| MANDSGGGPGG | PSPERDRGY | CELCGKMEI | LRCRSCSSF | YCCENHGRGD | WKKHKLVCGG |
| SEGALHGVBG | PHQISQAPP | AAVPPRAGA | REPRKAARR | DNASTGDAK | KVAKPPADP |
| AAAASPCRAA | AGGSGAAVA | EAEPGKEKAAA | ARSSLFQEEKA | NLYPSNTPG | DALSPPGLR |
| PNGQTPLPA | LKLALLEYV | CMNKHGICV | DDFLGKETGG | GIGDEVRALH | DTGFTDGG |
| VSGQSSSDKD | IRGDKIIWIE | GKEPGCETIG | LLMSSGMLI | RHNCNGKLYG | KINIKTKAMV |
| ACYPNGBTGY | VRHVDNPGD | GRCVTCYIY | NKDWDаксНС | GILRIFPEGK | AQQFAIEPKF |
| DRLLFFWSDR | RNPHEVQPAY | ATRYAITWY | FDADERAK | VKLYTGEKGV | RVLNYPSDS |
| VGKDV | | | | | |

87
**PHD2 Executes a Tightly Coupled Reaction in the Presence of Prime Substrate**

As PHD2 is deemed one of the primary oxygen sensors it would seem likely that it the overall reaction mechanism is tightly coupled. By tightly coupled we imply that turnover results in equivalent amounts of the products succinate and hydroxyl proline. If we witness production of succinate greater than that for hydroxyl proline this suggests that the enzyme activates oxygen at the iron center generating a highly reactive intermediate that is capable of hydroxylating any viable target within a defined radius. If this were the case, it would make PHD2 a poor oxygen regulator as the result of this uncoupled reaction would lead to cellular damage from oxidative products.

To test the coupling of PHD2 to hydroxyl proline assays were performed in which the succinate production was measured and compared to the accumulation of hydroxylated ODDD. A succinate detection kit was employed (Succinic Acid, UV-method, Boehringer Mannheim / R-Biopharm) following the manufactures protocol. Detection of ODDD-OH follows the previous protocols mentioned in this thesis.

The resulting coupling ratio was determined to be $0.93 \pm 0.1$, indicating that reaction is tightly coupled as would be thought for an enzyme that functions as an oxygen sensor. This assay also provided a secondary support method validating our mass spectrometry method of determining initial rate values (Figure A1).
Figure A1  Succinate production as compared to ODDD-OH production to determine coupling ratio. PHD2 1.0 µM, ferrous ammonium sulfate 20 µM, αKG 500 µM, 2 mM ascorbate, 80 µM ODDD.


30. Metzen, E.; Berchner-Pfannschmidt, U.; Stengel, P.; Marxsen, J. H.; Stolze, I.;
    Fandrey, J., Intracellular localisation of human HIF-1 alpha hydroxylases: implications
31. Huang, J.; Zhao, Q.; Mooney, S. M.; Lee, F. S., Sequence determinants in
    hypoxia-inducible factor-1alpha hydroxylation by the prolyl hydroxylases PHD1,
32. Berra, E.; Benizri, E.; Ginouves, A.; Volmat, V.; Roux, D.; Pouyssegur, J., HIF
    prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-
34. Muller, I.; Kahnert, A.; Pape, T.; Sheldrick, G. M.; Meyer-Klaucke, W.; Dierks,
    T.; Kertesz, M.; Uson, I., Crystal structure of the alkylsulfatase AtsK: insights into the
    catalytic mechanism of the Fe(II) alpha-ketoglutarate-dependent dioxygenase
    Welford, R. W.; Elkins, J. M.; Oldham, N. J.; Bhattacharya, S.; Gleadle, J. M.; Ratcliffe,
    P. J.; Pugh, C. W.; Schofield, C. J., Hypoxia-inducible factor (HIF) asparagine
    hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin
    Schofield, C. J., Hypoxia-inducible factor asparaginyl hydroxylase (FIH-1) catalyses
    Bouros, D.; Bougioukas, G.; Harris, A. L.; Gatter, K. C., Expression of prolyl-
    hydroxylases PHD-1, 2 and 3 and of the asparagine hydroxylase FIH in non-small cell
38. Saban, E. Controlled Oxygen Activation in Human Oxygen Sensor FIH.
    University of Massachusetts-Amherst, Amherst, 2011.
39. Costas, M.; Mehn, M. P.; Jensen, M. P.; Que, L., Jr., Dioxygen activation at
    mononuclear nonheme iron active sites: enzymes, models, and intermediates. Chem Rev
    2004, 104, (2), 939-86.
40. Hausinger, R. P., Fell/alpha-ketoglutarate-dependent hydroxylases and related
41. Solomon, E. I.; Brunold, T. C.; Davis, M. I.; Kemsley, J. N.; Lee, S. K.; Lehnert,
    N.; Neese, F.; Skulan, A. J.; Yang, Y. S.; Zhou, J., Geometric and electronic
    structure/function correlations in non-heme iron enzymes. Chem Rev 2000, 100, (1), 235-
    350.
42. Vaillancourt, F. H.; Yeh, E.; Vosburg, D. A.; O'Connor, S. E.; Walsh, C. T.,
    Cryptic chlorination by a non-haem iron enzyme during cyclopropyl amino acid
43. Hutton, H. R.; Boyd, G. S., The metabolism of cholest-4-en-3-one-7-alpha-ol by


82. Durrant, J. D.; de Oliveira, C. A. F.; Andrew McCammon, J., Pyrone-Based Inhibitors of Metalloproteinases Types 2 and 3 May Work as Conformation-Selective Inhibitors. Chemical Biology & Drug Design, no-no.
90. Chen, Y.-H. The Active Site Chemistry of Factor Inhibiting HIF-1, Coordination, Bonding, and Reaction. University of Massachusetts, Amherst, 2009.


