SPECTROSCOPIC AND THEORETICAL STUDIES OF MONONUCLEAR NON-HEME IRON ENZYMES: INSIGHT INTO THE ROLE OF THE FACIAL TRIAD AND SUBSTRATE EFFECTS ON REACTIVITY

A DISSERTATION
SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
AND THE COMMITTEE ON GRADUATE STUDIES
OF STANFORD UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

Adrienne R. Diebold
March 2011
I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Edward Solomon, Primary Adviser

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Steven Boxer

I certify that I have read this dissertation and that, in my opinion, it is fully adequate in scope and quality as a dissertation for the degree of Doctor of Philosophy.

Lynette Cegelski

Approved for the Stanford University Committee on Graduate Studies.

Patricia J. Gumport, Vice Provost Graduate Education

This signature page was generated electronically upon submission of this dissertation in electronic format. An original signed hard copy of the signature page is on file in University Archives.
ABSTRACT

Mononuclear non-heme iron enzymes are an important class with a wide range of medical, pharmaceutical and environmental applications. Within this class, the oxygen activating enzymes use Fe\textsuperscript{II} to activate O\textsubscript{2} for reaction with the substrate. The focus of this thesis is on two major themes of the oxygen activating enzymes - the role of the (2His/1 carboxylate) facial triad and the initial O\textsubscript{2} reaction steps of \(\alpha\)-keto acid-dependent dioxygenases - using a combination of spectroscopic techniques and DFT calculations. For ferrous systems, abs/CD/MCD/VTVH MCD studies define the geometric and electronic structure of the ferrous site. In combination with DFT calculations, a structure/function picture of the ferrous sites is developed. To extend these studies to the initial steps of O\textsubscript{2} binding, studies with NO as an O\textsubscript{2} analogue (\{FeNO\}\textsuperscript{7}/\{FeO\textsubscript{2}\}\textsuperscript{8}) utilize EPR/abs/CD/MCD/VTVH MCD spectroscopy with DFT calculations to elucidate important effects of the substrate on the \{FeNO\}\textsuperscript{7} bond. These effects are used in the computational extension to the experimentally inaccessible O\textsubscript{2} bound complexes giving insight into the initial steps of O\textsubscript{2} binding and activation. Taken together, these studies shed light on the rational for facial triad ligation at the Fe\textsuperscript{II} site in the oxygen activating enzymes and how the Fe\textsuperscript{II} ligand set tunes the specific reactivity of these enzymes.

A common theme in the oxygen activating enzymes is the facial triad (2 His/1 carboxylate) ligation of Fe\textsuperscript{II} by the protein. Recently, a number of enzymes have been discovered which do not use this facial triad, including a subset with a 3His triad. The ligand fields of both the resting facial and 3His triads are very similar; however, the 3His triad shows a decreased pKa for bound water. Both Dke1, a 3His triad enzyme, and 4-hydroxyphenylpyruvate dioxygenase (HPPD), a facial triad enzyme, bind the native substrate of the other allowing a comparison between the substrate-bound forms of the 3His and facial triads. Using acac, the native substrate of Dke1, the major difference between the acac-bound Dke1 and HPPD complexes lies in the energy of the MLCT transition, reflecting the difference in charge at the site. For 4-hydroxyphenylpyruvate (HPP), the native substrate of HPPD, the reduced charge in resting Dke1 causes HPP to enolize forming the dianion. Dke1-HPP(enolate) reacts
with O₂ to form the C2-C3 cleavage product while HPPD-HPP(keto) decarboxylates with standard α-keto acid reactivity. Studies of the 3His triad in comparison to the facial triad evaluate both the functionality of the 3His triad and the rational for a conserved 2His/1 carboxylate facial triad motif in the α-keto acid-dependent enzymes.

The α-keto acid-dependent dioxygenases utilize an α-keto acid cofactor in the initial steps of the reaction with O₂. These early, O₂-dependent steps are not accessible experimentally and thus are not well understood. The α-keto acid dependent dioxygenase HPPD was used to study the effects of the α-keto acid on reactivity. HPPD utilizes the fused α-keto acid-phenol, HPP as its substrate. The \{FeNO\}⁷/\{FeO-2\}⁸ methodology was used to evaluate the initial steps of the O₂ reaction. Comparison of the NO complexes of resting HPPD and HPPD-HPP shows that α-keto acid is a strong donor which promotes electron density transfer to NO. This effect is seen in the O₂ complex through a new S=1 bridged-binding mode for O₂ which avoids the less energetically favorable one-electron reduction of O₂. This intermediate generates a new pathway for the initial steps of the O₂ reaction in α-keto acid dependent dioxygenases.

Most of the mononuclear non-heme iron enzymes catalyze oxygenase chemistry, inserting O₂ into the substrates and cofactors on which they act. Isopenicillin N Synthase is unusual in that it catalyzes oxidase chemistry in the 4e⁻ reduction of O₂ to H₂O with oxidation of the substrate ACV. However, ACOV, a substrate analogue which lacks an amide nitrogen, catalyzes oxygenase chemistry. The Fe\textsuperscript{III}-hydroperoxide intermediate generated after O₂ activated H-atom abstraction from the substrate (the common first step) can undergo one of three different pathways for O-O cleavage: homolytic cleavage, heterolytic cleavage or nucleophilic attack. The O-O potential energy surfaces for these three pathways show that the hydroperoxide hydrogen bonds to the amide nitrogen of ACV polarizing the σ* orbital of the peroxide toward the proximal oxygen, facilitating heterolytic cleavage. In the substrate analogue ACOV, this hydrogen bond is no longer present, leading to nucleophilic attack on the substrate intermediate C-S bond and the experimentally observed
oxygenase reactivity. Thus specific interactions with each substrate govern the resulting oxidase or oxygenase reactivity.
PREFACE

This dissertation focuses on the role of the triad coordinating the Fe^{II} center and how the substrate directs reactivity in mononuclear non-heme iron enzymes. Chapter 1 presents background information on 1) the role of metals in biological systems with specific focus on biological iron, 2) the specific enzymes studied in this thesis, and 3) the spectroscopic and computational methodologies used to study these enzymes.

Chapter 2 presents a study of the effects of the second sphere protein residues in the diketone cleaving dioxygenase, Dke1. It was a collaborative effort with Dr. Grit Straganz at the University of Graz in Austria and her student Sigrid Egger. Dr. Straganz and Sigrid Egger were responsible for the protein expression, Fe^{II} binding studies and the kinetic analysis. I performed the pH-dependent CD and MCD spectroscopic experiments. Dr. Straganz provided the protein for the CD/MCD spectroscopic experiments. This work was published *Biochemistry* (2010, 49, 996-1004).

Chapter 3 examines the 3His triad in Dke1 and the difference between the 3His triad and the facial (2His/1 carboxylate) triad. This work is an extension of the collaboration with Dr. Grit Straganz. She provided Dke1 for the spectroscopic studies. I performed the spectroscopic studies and did the electronic structure calculations. Michael Neidig provided preliminary spectroscopic data. Professor Graham Moran and his student Panqing He provided HPPD for spectroscopic studies. This work has been published in *Biochemistry* (2010, 49, 6945-6952).

Chapter 4 finishes the study of Dke1, evaluating the specific role of charge at the Fe^{II} center and how it directs reactivity of α-keto acids. Dr. Grit Straganz supplied Dke1 for spectroscopic study. I performed the spectroscopic studies and electronic structure calculations.

Chapter 5 studies the initial steps of α-keto acid-dependent dioxygenase reactivity. HPPD for spectroscopic study was provided by Professor Graham Moran and his students Panqing He and Michael Kavana. Michael Neiding did preliminary MCD experiments on the HPPD-HPP-NO complex and Christina Brown-Marshall did
preliminary EPR on the HPPD-HPP-NO complex, performed preliminary electronic structure calculations, and provided some preliminary analysis. I completed the spectroscopic and electronic structure calculations and performed the full analysis.

Chapter 6 finishes the reaction coordinate of IPNS and considers the effect of substrate analogues on the direction of reactivity to oxidase or oxygenase. Christina Brown-Marshall performed electronic structure calculations to examine the O-O bond cleavage and did the primary analysis. I performed electronic structure calculations to characterize the dipole interaction and evaluate a 4th reaction pathway, and I completed the analysis. This work has been published in *Biochemistry* (2010, 49, 1176-1182).
ACKNOWLEDGEMENTS

Reflecting back on all of the people who have helped me throughout my graduate career, I have a lot of people to thank. First and foremost, I have to thank Ed, my advisor, for giving me the opportunity to pursue this thesis and from whom I have learned a great deal about bioinorganic spectroscopy and academic science. I would also like to thank my current and former thesis committee members – Professors Steven Boxer, Lynette Cegelski, Matt Kanan, Cliff Wang and Dan Stack – for their time and effort in assisting me with this work.

As a spectroscopist, I have had an insatiable need for protein samples to study. My work is deeply indebted to Dr. Grit Straganz at the University of Graz, Professor Graham Moran at the University of Wisconsin-Milwaukee, Professor John Lipscomb at the University of Minnesota, Professor Michael Maroney at the University of Massachusetts-Amherst and all of their students for keeping me supplied with protein in the high concentration and volume that my studies required.

When I arrived in the Solomon Lab, I knew nothing about MCD or computational chemistry. I am very thankful to Monita Pau, Michael Neidig, and Chrisssy Brown-Marshall for teaching me about MCD, DFT and the data analysis particular to non-heme iron systems. I would also like to thank co-monomuclear non-heme iron members Sam, Shaun, Ken, Lei and Mathieu who have been excellent sounding boards. The broad scope of spectroscopy that we employ requires a lot of instrument up keep. Thanks to all of the crew members who kept the instruments maintained, helped me to run samples, and kept the computer cluster running. Thanks is due to Adam Tenderholt for taking the time to write software that has made my data analysis much more streamlined and to Adam and Matt Kieber-Emmons for always being willing to field a barrage of questions and ideas about calculations and general science. Thank you also to all of the Solomon Lab members past and present for your support, camaraderie and frequent coffee runs. The atmosphere in this lab has always been collaborative and welcoming.

Personally, I would like to thank all of my friends at Stanford, particularly Adam and Sam, for being there for me and making life a little more interesting. Ariel
has been with me since we met freshman year of college and has been a constant source of encouragement, workout buddy and great best friend. Thank you my parents and brothers who have supported me in everything I have done (even though they don’t understand why I would want to do it in the first place). And finally, to Ben for always listening to me and never letting me accept the status quo.
1 Introduction: Nonheme iron enzymes and methodology

1.1 Mononuclear non-heme iron enzymes ................................................................. 2
  1.1.1 Metal sites in biology ................................................................................. 2
  1.1.2 Mononuclear non-heme iron enzymes .................................................... 3
  1.1.3 Enzymes studied ...................................................................................... 6
1.2 Spectroscopic and Computational Methods ...................................................... 8
  1.2.1 Spectroscopic methods ............................................................................ 8
  1.2.2 Ferrous methodology .......................................................................... 12
  1.2.3 {FeNO}^7/{FeO_2}^8 methodology ......................................................... 16
  1.2.4 Computational methods ........................................................................ 20
1.3 Scope of the thesis .......................................................................................... 21
1.4 References .................................................................................................... 22

2 Kinetic and CD/MCD Spectroscopic Studies of the Atypical, 3-His Ligated, Non-hem Fe^{II} Center in Diketone Dioxygenase: The Role of Hydrophilic Outer Shell Residues in Catalysis

2.1 Introduction .................................................................................................... 29
2.2 Materials and Methods .................................................................................. 31
  2.2.1 Materials ................................................................................................ 31
  2.2.2 Methods .................................................................................................. 32
    2.2.2.1 Site-directed mutagenesis and enzyme preparation ...................... 32
    2.2.2.2 Fe^{II} content and protein concentration ..................................... 33
    2.2.2.3 Steady state kinetics ....................................................................... 34
    2.2.2.4 Substrate binding kinetics .............................................................. 35
    2.2.2.5 CD and MCD spectroscopy ........................................................... 35
2.3 Results ............................................................................................................ 36
  2.3.1 Kinetic parameters and pH profile of WT Dke1 .................................. 36
  2.3.2 Kinetic parameters and pH profiles of second sphere variants of Dke1 ........................................................................................................... 39
## Table of Contents

4.3.1.3 UV-vis absorption and MCD of Dke1-HPP ................................... 94  
4.3.1.4 Resonance Raman ........................................................................ 95  
4.3.2 Computational ......................................................................................... 96  
4.3.2.1 Geometry optimized structures....................................................... 97  
4.3.2.2 Electronic structure of enolized HPP ............................................. 99  
4.4 Analysis .......................................................................................................... 100  
4.4.1 Spectral assignments ....................................................................... 100  
4.4.2 Reaction coordinate ......................................................................... 106  
4.5 Discussion....................................................................................................... 109  
4.6 Acknowledgements ........................................................................................ 111  
4.7 References ...................................................................................................... 111  
4.8 Supporting Information .................................................................................. 115  

5 Understanding the Reactivity of α-keto Acid Dependent Dioxygenases: Application of the \{FeNO\}⁷/{FeO₂}⁸ Methodology to the Initial Steps of O₂ Reactivity

5.1 Introduction .................................................................................................... 118  
5.2 Materials and Methods ................................................................................... 121  
5.3 Results ............................................................................................................ 123  
5.3.1 Spectroscopic......................................................................................... 123  
5.3.2 Computational - \{FeNO\}⁷ ..................................................................... 128  
5.4 Analysis .......................................................................................................... 132  
5.4.1 Correlation of calculations to experiment ............................................. 132  
5.4.2 Comparison to B3LYP/LACVP ............................................................ 136  
5.4.3 Extension to \{FeO₂\}⁸ ............................................................................. 138  
5.4.4 Completion of α-keto acid reaction coordinate..................................... 143  
5.5 Discussion....................................................................................................... 146  
5.6 Acknowledgements ........................................................................................ 147  
5.7 References ...................................................................................................... 148  
5.8 Supporting Information .................................................................................. 153

6 Reaction Coordinate of Isopenicillin N Synthase: Oxidase versus Oxygenase Activity

6.1 Introduction .................................................................................................... 162  
6.2 Methods .......................................................................................................... 164  
6.3 Results ............................................................................................................ 165  
6.4 Discussion ...................................................................................................... 172  
6.5 Acknowledgements ....................................................................................... 175  
6.6 References ................................................................................................. 175  
6.7 Supporting Information .................................................................................. 178

xiii
LIST OF TABLES

CHAPTER 1
Table 1.1 List of relevant binuclear non-heme iron enzymes, their reactivities, and active site structures ................................................................. 4

CHAPTER 2
Table 2.1 Primers and template-DNA used in this study to generate Dke1 variants ................................................................................................... 33
Table 2.2 Steady state kinetic properties of WT Dke1 and variants.............. 39
Table 2.3 Kinetic first-order rate constants of substrate ligation for WT Dke1 and variants from stopped flow kinetic studies ......................... 43

CHAPTER 4
Table 4.1 C_0/D_0 ratios for HPPD-HPP and Dke1-HPP .......................................... 103

CHAPTER 5
Table 5.1 Key geometric and electronic structure parameters for E-NO and E-%KA-NO.......................................................................................... 129
Table 5.2 TD-DFT transition energies and directions of transition moment dipoles........................................................................................................... 133
Table 5.3 ΔSCF Fe^{III} d-d ligand field transition energies................................................................................................................................. 135
Table 5.4 NO \textsuperscript{-} to 2π* character in Fe d_{yz} and d_{xz} ................................................................. 136
Table 5.5 Key geometric and electronic structure parameters for E-%KA-O\textsubscript{2} complexes .............................................................. 139
Table 5.6 Steps to S=1 peroxo-bridge ................................................................ 142
Table S5.1 Key geometric and electronic structure parameters for HPPD-HPP-NO 1 and 2........................................................................ 153
Table S5.2 ΔSCF Fe^{III} d-d ligand field transition energies for HPPD-HPP-NO 1 ................................................................................................. 153
Table S5.3 Key geometric and electronic structure parameters for E-NO and E-%KA-NO with B3LYP/LACVP and BP86+10%HF/Gen ................ 153

CHAPTER 6
Table S6.1 Key computational results .................................................................. 179
Table S6.2 Spin densities from elongation of the O-O bond with the dipole present ........................................................................................................ 179
Table S6.3 Cartesian coordinates of optimized IPNS-ACV-Fe\textsuperscript{II}-Peroxide (Model 1) .................................................................................. 179
Table S6.4 Cartesian coordinates of optimized IPNS-ACOV-Fe\textsuperscript{II}-Peroxide (Models 2&3) ................................................................. 181
Table S6.5 Cartesian coordinates from the transition state of IPNS-ACV-Peroxide O-O bond cleavage (Model 1).......................... 182
Table S6.6  Cartesian coordinates of optimized IPNS-ACV-β Lactam FeIV-oxo (Model 1)........................................................................................................................................183
Table S6.7  Cartesian coordinates of optimized IPNS-Thiocarboxylate FeII (Model 2)................................................................................................................................................184
LIST OF ILLUSTRATIONS

CHAPTER 1
Figure 1.1 Biological environments for Fe ................................................................. 2
Figure 1.2 General mechanistic strategy of α-keto acid-dependent
dioxygenases ........................................................................................................ 5
Scheme 1.1 Structures of the facial triad and 3His triad ............................................. 6
Scheme 1.2 Reaction catalyzed by Dke1 .................................................................... 6
Scheme 1.3 Reaction catalyzed by HPPD .................................................................. 7
Scheme 1.4 Reaction catalyzed by IPNS .................................................................... 8
Figure 1.3 FeII d-orbital splitting and representative ligand field MCD spectra ...... 13
Figure 1.4 Splitting of the 5T2 ground state ................................................................ 14
Figure 1.5 Saturation magnetization isotherms generated for an S=2, non-
Kramers system .................................................................................................... 15
Figure 1.6 5K, 7T MCD spectrum of Fe-EDTA-NO with Gaussian resolution
of the transitions .................................................................................................. 18
Figure 1.7 Electronic structure of {FeNO}7 models ................................................... 19

CHAPTER 2
Figure 2.1 Metallocenter and H-bonding network in Dke1 ........................................ 30
Figure 2.2 kcat app/KM app and kcat app profiles for wild type Dke1 and
Glu98ÆGln variant ................................................................................................. 38
Figure 2.3 Stopped flow traces of the Dke1-metallocetner with 1,1-difluor-2,4-pentanedione (DPD) ................................................................. 42
Figure 2.4 CD and LT MCD spectra for WT Dke1 .................................................. 45
Figure 2.5 CD and LT MCD spectra for E98Q Dke1 .............................................. 47
Figure S2.1 Correlation of the diketonate’s εHOMO and the rates of Dke1-FeII-
diketonate complex decay for WT Dke1 and variants ...................................... 56
Figure S2.2 kcat-pH and kcat/KM-pH correlations for WT Dke1 and variants ............ 57
Figure S2.3 Stopped flow binding curves of PD to WT Dke1 and variants .......... 59
Figure S2.4 CD spectra of WT Dke1 and E98Q at varying pH ................................ 59
Figure S2.5 Overlay of the MCD spectra of WT Dke1 and E98Q at pD 9.5 ........... 60
Figure S2.6 Autooxidation of E98Q .......................................................................... 60
Figure S2.7 Determination of the rate constant for oxidation of free FeII in Tris
buffer, 20 mM at pH 7.5 ..................................................................................... 61

CHAPTER 3
Scheme 3.1 Breakdown of acetylacetone via Dke1 ................................................... 64
Figure 3.1 CD/MCD/VTVH CMD of Resting FeII-Dke1 ........................................... 68
Scheme 3.2 Experimentally derived Ligand Field diagrams for resting Dke1
and CS2 and acac-bound Dke1 and HPPD ............................................................ 69
Figure 3.2 CD/MCD/VTVH MCD of acac-bound complexes .................................. 71
Figure 3.3 UV-vis absorption/CD/MCD spectra of acac-bound complexes .......... 73
Figure 3.4 Molecular orbital energy level diagrams for 5C 3His-acac and
facial triad-acac ................................................................................................. 74
Figure 3.5  Molecular orbital contours for the donor and acceptor orbitals for MLCT ..................................................................................................... 74
Figure S3.1  VTVH MCD isotherms for Dke1-acac at 11,100 cm$^{-1}$ and HPPD-acac at 12,300 cm$^{-1}$ ................................................................. 83
Figure S3.2  CD/MCD of resting Fe$^{II}$-HPPD ................................................................. 84
Figure S3.3  HOMO and LUMO of free acac ligand .............................................. 84
Figure S3.4  TD-DFT predicted spectra for the acac-bound complexes .......... 85
Figure S3.5  Structures used to calculate water affinity ........................................ 86

CHAPTER 4
Scheme 4.1  Native reaction catalyzed by Dke1 ......................................................... 88
Scheme 4.2  Dioxygen dependent cleavage of PP catalyzed by Dke1 or the facial triad .................................................................................................. 89
Figure 4.1  VTVH-MCD of Dke1-HPP .......................................................................... 93
Figure 4.2  UV-visible absorption of MCD spectra .................................................. 94
Figure 4.3  Resonance Raman shift for Dke1-HPP and the O$_2$ exposed control ...... 96
Scheme 4.3  Tautomerization of HPP .................................................................. 96
Figure 4.4  Geometry optimized structures of Dke1-HPP(keto) and Dke1-HPP(enolate) ......................................................................................... 97
Figure 4.5  Molecular orbital energy level diagrams for Dke1-HPP(keto) and Dke1-HPP(enolate) ........................................................................... 98
Figure 4.6  Molecular orbital contours for selected $\beta$ orbitals of Dke1-HPP(keto). ............................................................................................... 98
Figure 4.7  Molecular orbital contours for selected $\beta$ orbitals of Dke1-HPP(enolate) ............................................................................................... 99
Figure 4.8  Comparison of bonding orbitals available for HPP(keto) and HPP(enolate) in complex with Dke1 .................................................... 100
Figure 4.9  TD-DFT predicted absorption spectra ................................................. 101
Figure 4.10  Comparison of MLCT and LMCT in the keto and enolate forms of HPP ......................................................................................... 102
Figure 4.11  Intensity mechanism for the MLCT transition observed in $\alpha$-keto acid bound facial triads ................................................................. 104
Figure 4.12  Application of the mechanism for intensity from the MLCT transition observed in $\alpha$-keto acid bound facial triads to the Dke1-HPP(enolate) LMCT ................................................................. 105
Figure 4.13  Three potential mechanistic pathways for the O$_2$ reaction with Dke1-HPP(enolate) ........................................................................... 107
Figure 4.14  Reaction coordinate for the O$_2$-dependent cleavage of HPP(enolate) ......................................................................................... 108
Scheme 4.4  Metal-mediated O-O cleavage in Dke1-HPP(enolate) ......................... 109
Figure 4.15  Schematic of initial C-C cleavage of HPP(keto) and HPP(enolate).... 111
Figure S4.1  LMCT transitions for pseudo-A MCD intensity in Dke1-HPP
LMCT ........................................................................................................... 115
Figure S4.2 Molecular orbital energy level diagrams for the Dke1-HPP(enolate) reaction coordinate ................................................................. 116
Figure S4.3 Geometry optimized structure of ether-Fe\textsuperscript{III}-oxo ................................................................. 116

CHAPTER 5
Scheme 5.1 Proposed mechanism for a-keto acid dependent dioxygenases............. 118
Scheme 5.2 Reaction catalyzed by HPPD .................................................................. 119
Figure 5.1 Molecular orbital energy level diagram for \{FeNO\}\textsuperscript{7} model complexes .................................................................................................................. 120
Figure 5.2 EPR of \{FeNO\}\textsuperscript{7} enzyme complexes .............................................. 124
Figure 5.3 Temperature dependence of \{FeNO\}\textsuperscript{7} S=3/2 EPR signal .............. 124
Figure 5.4 Room temperature UV-vis absorption spectra of HPPD-NO and HPPD-HPP-NO .................................................................................. 125
Figure 5.5 VTVH MCD of \{FeNO\}\textsuperscript{7} .................................................................. 126
Figure 5.6 Computational models for S=3/2 \{FeNO\}\textsuperscript{7} ........................................ 129
Figure 5.7 Molecular orbital diagrams for S=3/2 E-NO and E-\alpha\textsuperscript{-}KA-NO ............. 130
Figure 5.8 Molecular orbital contours for geometry optimized structures of S=3/2 E-NO .................................................................................................. 130
Figure 5.9 Molecular orbital contours for geometry optimized structure of S=3/2 E-\alpha\textsuperscript{-}KA-NO ...................................................................................... 131
Figure 5.10 Molecular orbital contours of \alpha\textsuperscript{-}KA available for bonding to Fe ........................................ 132
Figure 5.11 TD-DFT predicted absorption spectra ......................................................................................................................................................................... 133
Figure 5.12 Transitions in E-NO and E-\alpha\textsuperscript{-}KA-NO ...................................................................................... 134
Figure 5.13 Comparison of geometry optimized structures of E-NO and E-\alpha\textsuperscript{-}KA-NO for the BP86+10%/Gen and B3LYP/LACVP functional and basis set combinations .................................................................................. 137
Figure 5.14 Computational models for \{FeO\textsubscript{2}\}\textsuperscript{8} ...................................................................................... 139
Figure 5.15 Molecular orbital diagrams for three spin states of E-\alpha\textsuperscript{-}KA-O\textsubscript{2} ............ 140
Figure 5.16 Molecular orbital contours for geometry optimized structure of S=1 E-\alpha\textsuperscript{-}KA-O\textsubscript{2} ...................................................................................... 141
Figure 5.17 Formation of S=1 Fe\textsuperscript{IV}-O\textsubscript{2}\textsuperscript{2-} bridge ...................................................................................... 141
Scheme 5.3 Nucleophilic interaction between the occupied O\textsubscript{2} \pi\textsuperscript{*} (ip) orbital and the unoccupied \alpha\textsuperscript{-}KA \pi orbital ................................................................................................. 143
Figure 5.18 2-D spin crossover surface ........................................................................... 144
Figure 5.19 Electron flow for initial steps of E-\alpha\textsuperscript{-}KA-O\textsubscript{2} reaction .................................................................................................................. 145
Figure 5.20 Free energy diagram for initial steps of E-\alpha\textsuperscript{-}KA-O\textsubscript{2} reaction ...................................................................................... 145
Figure S5.1 Fe-EDTA-NO room temperature UV-vis absorption spectrum .............................................................................................................................. 153
Figure S5.2 Fe-EDTA-NO and HPPD-NO 5K, 7T MCD spectra with Gaussian resolution .............................................................................................................................. 154
Figure S5.3 VTVH MCD isotherms for ligand field transitions ........................................ 154
Figure S5.4 Simulated VTVH MCD for HPPD-HPP-NO 5650 cm\textsuperscript{-1} band .......... 154
Figure S5.5 Computational models for HPPD-HPP-NO ..................................................................................................................................................................... 155
Figure S5.6 Molecular orbital diagrams for three computational models of S=3/2 \alpha\textsuperscript{-}keto acid bound \{FeNO\}\textsuperscript{7} ................................................................................................. 155
Figure S5.7 TD-DFT predicted absorption spectra for computational models of S=3/2 α-keto acid................................................................................................................... 156
Figure S5.8 Comparison of the d_{z^2} and d_{x^2-y^2} orbitals between E-NO and E-αKA-NO.................................................................................................................................................. 156
Figure S5.9 Molecular orbital diagrams for E-NO and E-αKA-NO using B3LYP/LACVP.................................................................................................................................................................. 157
Figure S5.10 Comparison of TD-DFT predicted absorption spectra for BP86+10%HF/Gen and B3LYP/LACVP for both E-NO and E-αKA-NO.............................................................................................................. 157
Figure S5.11 Molecular orbital contours for S=2 E-αKA-O_2........................................ 158
Figure S5.12 HPPD-HPP-O_2 1 S=1 bridged structure................................................. 158
Figure S5.13 Molecular orbital diagram for S=1 bridged HPPD-HPP-O_2 1.................. 159
Figure S5.14 Choice of O-O or C-C coordinate for spin crossover............................. 159
Figure S5.15 Spin crossover .......................................................................................... 160
Figure S5.16 Molecular orbital diagram for S=1 and S=2 E-αKA-O_2 at the crossing point................................................................................................................................. 160

CHAPTER 6
Scheme 6.1 The four electron oxidative double ring closure of ACV to form Isopenicillin N .................................................................................................................................................. 162
Scheme 6.2 Three reaction pathways for Fe^{II}-Hydroperoxide.................................... 163
Figure 6.1 Models used to calculate three reaction pathways of Fe^{II}-Hydroperoxide.................................................................................................................................................. 166
Figure 6.2 Molecular orbital diagrams of IPNS-ACV-Fe^{II}-Hydroperoxide................. 166
Figure 6.3 Geometric structures of IPNS-Fe^{II}-Hydroperoxide complexes with O-O bond elongation................................................................................................................................. 167
Figure 6.4 Donor and acceptor orbitals for homolytic cleavage, heterolytic cleavage and nucleophilic attack................................................................................................................................. 168
Figure 6.5 Energetics of O-O bond cleavage .................................................................. 169
Figure 6.6 Alpha hydroperoxide σ* orbitals................................................................. 170
Scheme 6.3 Rotation of the peroxide for nucleophilic attack......................................... 171
Figure 6.7 Donor and acceptor orbitals and product for beta-lactam ring closure......................................................................................................................................................................... 172
Scheme 6.4 Attack by the distal and proximal oxygen of the hydroperoxide.............. 174
Figure S6.1 Alleviation of backbonding with O-O bond elongation ............................ 178
Chapter 1

Introduction: Mononuclear non-heme iron enzymes and methodology
1.1 Mononuclear non-heme iron enzymes

1.1.1 Metal sites in biology

Metal ions play an important role in many biological processes. In the simplest case, Na\(^+\) and K\(^+\) balance osmotic pressure in cells and generate gradients and electrochemical potential differences. The Na\(^+\)/K\(^+\) ATPase pump creates membrane potentials that are used to generate action potentials for nerve impulses. Metals play structural roles such as K\(^+\) associated with the polyG tale of telomeres, Mg\(^{2+}\) associated with the phosphate backbones of DNA and RNA and with NTP’s and Zn\(^{2+}\) bound to the DNA transcription factor zinc finger proteins. Muscle contraction and the triggering of many signaling cascades rely on Ca\(^{2+}\). Metal ions also play a role in catalysis both with and without redox processes. Zn\(^{2+}\) (carbonic anhydrase) and Mn\(^{2+}\) (acid phosphatases) are used in Lewis acid catalysts. Manganese is also found both in superoxide dismutase which scavenges reactive oxygen species in the cell and in the 4Mn cluster of photosystem II, a crucial piece of photosynthesis. An iron-molybdenum cluster is required for nitrogen fixation and vitamin B\(_{12}\) contains cobalt (1). For redox catalysis processes in biological systems, iron and copper are the most abundant metal ions used and are employed in a wide range of chemical processes. Just a few examples of important pathways that rely on copper containing proteins include the production of melanin by tyrosinase, neurotransmitter production by DβM, and oxidative respiration using cytochrome c oxidase (2-4).

![Diagram](image1.png)

Figure 1.1 Biological environments for Fe

Biological systems use iron in several different environments (Figure 1.1). Protoporphyrin IX coordinates an iron center to make heme for both redox and non-redox processes. In addition to O\(_2\) transport in hemoglobin and myoglobin, iron is
coordinated as heme in cytochrome c, of the previously mentioned cytochrome c oxidase, and in P450, an enzyme with widespread use and types of oxidase reactions and whose uncontrolled production is associated with cancer (4, 5). Iron is also found as a redox cofactor in iron-sulfur clusters including 2Fe2S, 3Fe4S, 4Fe4S and Rieske clusters. These redox cofactors are a crucial component of ferredoxins and cytochrome b complexes (6). The last common environment for biological iron is non-heme iron. These can be either mononuclear or binuclear sites which are ligated to the protein through amino acid residues. Binuclear non-heme iron enzymes are involved in a number of important reactions including the conversion of ribose to deoxyribose (7). Mononuclear non-heme iron enzymes also are involved in a number of important enzymatic reactions and will be discussed further below.

1.1.2 Mononuclear non-heme iron enzymes

Mononuclear nonheme iron enzymes are an important class with diverse and important biological function. They are involved in the biosynthesis of antibiotics including penicillins and cephalosporins (8, 9), associated with genetic diseases (10-12), and involved in the treatment of some cancers (13). These enzymes all use an iron center to catalyze the formally spin forbidden reaction of O₂ with organic substrates. As such, the class can be divided further into two subclasses, oxygen activating and substrate activating, depending on the route used to overcome the spin forbiddenness of the reaction. Table 1.1 shows a representative subset of these enzymes with the types of reactions they catalyze. The oxygen activating enzymes, which include the pterin-dependent, α-keto acid-dependent, Rieske dioxygenases and extradiol dioxygenases, use a ferrous center to activate dioxygen for reaction. Alternatively, the substrate activating enzymes, including the lipoxygenases and intradiol dioxygenases, use a ferric center to activate the substrate for attack by dioxygen (7).
Table 1.1 Major classes of Mononuclear non-heme iron enzymes

<table>
<thead>
<tr>
<th>A. Oxygen Activating (Fe^{II})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Extradiol Dioxygenases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
<tr>
<td><strong>Pterin-Dependent Hydroxylases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
<tr>
<td><strong>α-KG-Dependent Dioxygenases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
<tr>
<td><strong>Rieske Dioxygenases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
<tr>
<td><strong>Bleomycin</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Substrate Activating (Fe^{III})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Lipoxygenases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
<tr>
<td><strong>Intradiol Dioxygenases</strong></td>
</tr>
<tr>
<td>![Chemical Reaction Diagram]</td>
</tr>
</tbody>
</table>
One of the major components of the oxygen-activating subclass are the \( \alpha \)-keto acid-dependent dioxygenases which use an \( \alpha \)-keto acid cofactor to supply the necessary extra electrons for reaction with dioxygen. This class of enzymes has been extensively studied leading to the mechanistic strategy shown in Figure 1.2 (7).

![Figure 1.2 General mechanistic strategy of \( \alpha \)-keto acid-dependent dioxygenases](image)

(Adapted from Ref 7.)

The resting, Fe\(^{II}\)-bound site has 6 ligands – three protein-derived amino acids constituting the facial triad (2His/1 carboxylate) and 3 water-derived ligands – arranged in a distorted octahedral geometry. The binding of \( \alpha \)-keto acid displaces two of the water ligands, but retains a coordinatively saturated Fe\(^{II}\) site. Substrate binding at the active site leads to displacement of the final water ligand and an open coordination site available for O\(_2\) binding (7). The first trappable intermediate in the \( \alpha \)-keto acid-dependent dioxygenases is the Fe\(^{IV}=O\) generated by decarboxylation of the \( \alpha \)-keto acid and subsequent O-O bond cleavage. This intermediate has now been characterized in several enzymes including TauD (14), prolyl-4-hydroxylase (15), tyrosine hydroxylase (16), and the halogenase enzyme CytC3 (17). This Fe\(^{IV}=O\) is the oxidant which acts on the substrate. That the O\(_2\) binding and initial reaction steps are too fast to be observed spectroscopically or trapped kinetically has caused this section of the enzyme mechanism to be termed the ‘gray area’ of non-heme iron chemistry.
(18). This ‘gray area’ now presents the most interesting steps for study in the reaction pathway.

Within the oxygen-activating subclass, a common Fe$^{II}$ binding motif is often utilized. Two protein-derived histidine residues and a monodentate carboxylate (Asp or Glu) ligate Fe$^{II}$ on one face of a distorted octahedron (Scheme 1, left). This ‘facial triad’ ligation (as it will be called through this thesis) has been proposed to be of importance to enzyme function (19).

Recently a few Fe$^{II}$-dependent enzymes with modifications to the facial triad have been identified. The $\alpha$-ketoglutarate-dependent halogenase enzymes (SyrB2 and CytC3) lack the carboxylate residue to ligate the Fe$^{II}$ (20, 21). The halogen anion of the reaction is found coordinated to Fe$^{II}$ in place of this carboxylate group. Cysteine dioxygenase (CDO) and the diketone-cleaving dioxygenase Dke1 have a 3 histidine triad instead of a facial triad (Scheme 1, right) (22, 23). The effect of the change in coordination from the facial triad to the 3His triad is one major theme in this thesis.

1.1.3. Enzymes Studied

$\textit{Dke1}$ - The diketone-cleaving dioxygenase, Dke1, from \textit{Acinetobacter johnsonii} cleaves acetylacetone (acac) with incorporation of molecular oxygen to form methyl glyoxal and acetate (Scheme 2) (23).
The crystal structure of resting Dke1 has been solved (PDB id: 3bal). Dke1 belongs to the superfamily of cupin fold proteins which includes a number of metalloproteins such as quercetin dioxygenase and acireductone dioxygenase (24). In the crystal structure, a Zn$^{2+}$ ion is ligated by 3 histidine residues showing a metal binding site which is replaced by the catalytically active metal ion, Fe$^{II}$, during turnover. Comparison of the amino acid sequence in Dke1 with that of the facial triad enzymes CS2, IPNS and TauD shows that in place of the aspartate or glutamate conserved among facial triad enzymes, Dke1 possesses a histidine residue (23). The active site pocket of Dke1 is very large and hydrophobic enabling a wide variety of substituted β-diketones to access the active site. Studies have shown that Dke1 will turnover a number of β-diketones and their reaction rate has been correlated to the energy of the HOMO of the β-diketone (25, 26). Through study of its 3His triad, Dke1 presents a system with which to compare and contrast the role of the highly conserved facial triad. Furthermore, insight into the reactivity catalyzed by Dke1 sheds light onto the mechanism of enzymatic cleavage of aliphatic C-C bonds.

**HPPD** - Hydroxyphenylpyruvate dioxygenase (HPPD) is an α-keto acid dependent enzyme that catalyzes the reaction of 4-hydroxyphenyl pyruvate (HPP) with dioxygen (27), and defects in HPPD are associated with the genetic disease tyrosinemia (10). HPPD is unusual among the α-keto acid dependent dioxygenases in that the α-keto acid moiety is fused to the substrate. Reaction with dioxygen (Scheme 3) releases CO$_2$ and yields homogentisate a crucial intermediate in the tyrosine catabolism pathway (27).

![Scheme 1.3 Reaction catalyzed by HPPD](image)
A crystal structure of the resting, Fe$^{II}$-bound form of HPPD has been solved (PDB id: 1cjx). In it, the Fe$^{II}$ is ligated by the standard facial triad of 2 histidine residues and an aspartate residue (28). A number of studies have been carried out to investigate the factors governing the later stages of reactivity in HPPD, but little has been done with the initial α-keto acid dependent steps.

**IPNS - Isopenicillin N Synthase (IPNS)** catalyzes the formation of isopenicillin N, a precursor to β-lactam antibiotics such as the penicillins and cephalosporins. The substrate, δ-(L-α-aminoadipoyl)-L-cysteine-D-valine (ACV), undergoes 4e$^-$ oxidation to form both the β-lactam and thiazole rings of isopenicillin N (scheme 4) (29).

[Scheme 1.4 Reaction catalyzed by IPNS]

IPNS is unusual among the oxygen-activating MNHFe enzymes in that it does oxidase chemistry with the 4 e$^-$ reduction of dioxygen to water and not the much more commonly observed oxygenase chemistry where dioxygen is incorporated into the substrate. The thiolate ligation to the Fe$^{II}$ center has been shown to stabilize a ferric-superoxide upon dioxygen binding, facilitating the first H-atom abstraction which is the abstraction of the β-methylene H atom of the thiolate of ACV to generate an Fe$^{III}$-hydroperoxo species (30). Completion of this reaction is evaluated in this thesis.

1.2. Spectroscopic and Computational Methods

1.2.1 Spectroscopic Methods

A wide variety of spectroscopic methods can be employed to study the active sites of metalloproteins. EPR and ENDOR, X-ray spectroscopies (XAS and EXAFS), and Mössbauer probe the ground state properties of the active site. For an understanding of the excited states, UV-vis absorption, resonance Raman, nuclear
resonance vibrational spectroscopy (NRVS), circular dichroism (CD) and magnetic
circular dichroism (MCD) are most commonly used. Absorption, CD and MCD are
the techniques most widely applied in this thesis and so a brief description of their
origin is given. This is followed by their application and the spectroscopic approach to
studying ferrous and \{FeNO\}_7 systems used in this thesis.

Electronic absorption spectroscopy probes transitions between the ground
state, \( \Psi_g \), and excited state, \( \Psi_e \). Theoretically, this transition results from the
interaction of a photon with an electron via the transition moment operator (\( \hat{M} \)). The
experimentally observed absorption band is characterized by the energy dependence (\( \nu \)
in cm\(^{-1} \)) of the molar extinction coefficient (\( \varepsilon \) in M\(^{-1}\) cm\(^{-1} \)). The theoretical description
is related to the experimentally observed transition through the oscillator strength, \( f \),
given in equation 1a-b.

\[
\begin{align*}
(a) \quad f_{\text{exp}} &= 4.32 \times 10^{-9} \int \varepsilon(\nu) d\nu \\
(b) \quad f_{\text{theo}} &= 4.702 \times 10^{-9} \nu \left[ \Psi_e^* \hat{M} \Psi_g d\tau \right]^2
\end{align*}
\]

From these equations, it is apparent that transition intensity requires a non-zero
transition moment integral. When the wavelength of light is much greater then the
radius of the electron orbital, the terms of the transition moment operator are given by
the multipole expansion (equation 2), with each term reduced by \( \sim 10^{-3} \) compared to
the previous term.

\[
\hat{M} = \hat{M}_{\text{electric dipole}} + \hat{M}_{\text{magnetic dipole}} + \hat{M}_{\text{electric quadrupole}} + \ldots
\]

For the NIR-visible-UV region of interest in these studies, \( \hat{M}_{\text{electric dipole}} \) dominates. As
\( \hat{M}_{\text{electric dipole}} \) does not act on spin, \( \Delta S=0 \) for spin-allowed transitions. Thus for
electronic absorption transitions in the NIR-visible-UV, the transition must have non-
zero electric dipole transition moment intensity and \( \Delta S=0 \).

CD and MCD spectroscopies measure the differential absorption of left and
right circularly polarized light by a sample.

\[
\Delta \varepsilon = \varepsilon_L - \varepsilon_R = \Delta A / bC
\]

These methods have the advantage over traditional linearly polarized absorption
spectroscopy of different selection rules and phase sensitive detection, allowing the
study of low intensity, otherwise obscured transitions and the potential for better
resolution of overlapping bands. Optical activity is required for intensity in CD spectroscopy; however, in a magnetic field, optical activity is induced by the Faraday effect with the net result that all molecules will exhibit MCD activity (31). These two techniques are explained further below.

In CD spectroscopy, the transition $|A⟩ \rightarrow |J⟩$ must be simultaneously electric and magnetic dipole allowed in the same direction. Intensity in CD is described by equation 4, where $R$ is the rotation strength, $m$ is the electric dipole operator and $μ$ is the magnetic dipole operator.

$$R \propto \text{Im}(\langle A | \bar{m} | J⟩ \cdot ⟨J | \bar{μ} | A⟩)$$

(4)

Only the imaginary part of this equation is considered as the magnetic dipole operator is pure imaginary. Inspection of molecular symmetry character tables indicates that $R$ will be non-zero for the point groups C$_n$, D$_n$, O, T, and I. In the low symmetry environment of a protein, the active site will have C$_1$ symmetry and thus CD intensity (31).

MCD intensity, under non-saturating conditions, is given in equation 5:

$$\frac{ΔA}{E} = (3.27 \times 10^{38}) \frac{α^2 Cl}{n} βH \left[ A_1 \left( \frac{∂f(E)}{∂E} \right) + B_0 + C_0 \frac{kT}{E} f(E) \right]$$

(5)

where $ΔA$ is the difference between absorption of left- and right-circularly polarized light, $E$ is $hν$, $α$ is the electric permeability, $C$ is the concentration, $l$ is the pathlength, $n$ is the index of refraction, $β$ is the Bohr magneton, $H$ is the applied magnetic field, $f(E)$ is the bandshape in absorption (which is commonly a Gaussian) and $∂f(E)/∂E$ is the first derivative of the bandshape. It is initially apparent that the intensity is a sum of three terms. The first term, $A_1$, will have a derivative bandshape, while the second and third terms, $B_0$ and $C_0$, will have an absorption bandshape that has no temperature dependence for the B-term and an inverse temperature dependence for the C-term. The equations describing $A_1$, $B_0$, and $C_0$ are given in Equations 6a-c for molecules with $z$ oriented along the magnetic field. From these equations, A and C-terms require degenerate ground or excited states and two non-zero perpendicular transition moments. B-term intensity in MCD results from field-induced mixing between states.
From these MCD equations, the selection rules for MCD are $\Delta m_l = +1$ for absorption of left circularly polarized light and $\Delta m_l = -1$ for absorption of right circularly polarized light. A comparison of the relative intensities of the three terms is also insightful. The ratio of A:B:C terms in MCD goes as $(1/\Gamma):(1/\Delta E):(1/kT)$ where $\Gamma$ is the full width at half-height and $\Delta E$ is the energy difference between mixing states. At room temperature, a typical ratio of the three terms is 10:1:50; at 4.2K, however, this ratio is 10:1:3300 giving rise to a dominant C-term at low temperatures. As noted earlier, C-term intensity (and A-term as well) requires orbital degeneracy. In the low symmetry sites commonly found in metalloproteins, orbital degeneracy has been removed and therefore MCD transitions are electric dipole allowed, but the moment is in only one direction (instead of 2 perpendicular directions). Spin orbit coupling between excited states gives the necessary mixing to give rise to MCD C-term intensity for systems with $S \neq 0$ ground states. This happens by one of two pathways. In the first, spin-orbit coupling between two states close in energy gives rise to two MCD transitions of equal and opposite sign, termed a pseudo-A transition. True A-term intensity and pseudo-A behavior can be distinguished by the temperature dependence (or lack thereof) of the bands. The second intensity mechanism results from spin-orbit coupling of the ground state with low lying excited states (deviation from the sum rule) (31, 32). The application of CD and MCD theory to ferrous and ferric systems gives a wealth of information about the geometric and electronic structure at the metal
center and the surrounding coordination environment and is applied in the next sections.

1.2.2 Ferrous Methodology

An understanding of the coordination environment and geometric and electronic structure of the metalloprotein active site is important for understanding reactivity. Most ferrous proteins do not have charge transfer (CT) transitions in the visible or near UV region and higher energy CT transitions are obscured by intense background absorption by the protein. Furthermore, the Fe$^{II}$ d-d transitions, which occur in the NIR, are parity forbidden and have very low $\varepsilon$ values. Additionally, Fe$^{II}$ has an S=2, non-Kramers (even electron) ground state which is inaccessible by EPR, due to large ZFS, making ground state information difficult to obtain. An approach has been developed to use CD and MCD spectroscopy to obtain information about both the ground and excited states of Fe$^{II}$ in the metalloprotein active site and to define the coordination environment of Fe$^{II}$ (7, 33, 34).

A high-spin, d$^6$ Fe$^{II}$ site has a $^5$D ground state. In the presence of an $O_h$ ligand field, the 5-fold degeneracy of the d-orbitals splits into $^5T_{2g}$ (ground state) and $^5E_g$ states (Figure 1.3A, left). These states are further split in the low symmetry of the protein environment and with different coordination environments (Figure 1.3A). For a distorted octahedral (6-coordinate (6C)) site with biologically relevant N and O ligands, the splitting between the $^5E$ and $^5T_2$ states ($10D_q$) is approximately 10,000 cm$^{-1}$. The $^5E$ state is also split, by ~2,000 cm$^{-1}$ (Figure 1.3A, far left). Removal of an axial ligand, giving a square pyramidal site, brings the $d_{z^2}$ orbital down in energy relative to the $d_{x^2-y^2}$ (Figure 1.3A, middle left). For a 5C trigonal bipyramidal geometry, now the $d_{x^2-y^2}$ orbital is lowered in energy (Figure 1.3, middle right). Finally, for a 4C site (Figure 1.3A, far right), $10D_q$ is much smaller ($10D_{qTd} \sim 4/9$ $10D_{qOh}$) and the $^5E$ state is now the ground state.
A study of a set of structurally defined model complexes has validated correlation between the predicted $^5T_2$ and $^5E$ splittings from ligand field theory and the transitions observed in the NIR MCD spectra (Figure 1.3B). For a distorted 6C site (Figure 1.3B top panel), two transitions are observed, centered at ~10,000 cm$^{-1}$ and split by ~2000 cm$^{-1}$. A square pyramidal 5C site (Figure 1.3B, second panel) exhibits one transition > 10,000 cm$^{-1}$ and a second transition at low energy (~5,000 cm$^{-1}$). In a trigonal bipyramidal site, a single transition is observed at < 10,000 cm$^{-1}$ with its low energy pair below the energy range of the experimental setup (Figure 1.3B, third panel). Finally, the smaller 10Dq of a 4C, distorted tetrahedral site (10Dq$_{Td} = -(4/9)10Dq_{Oh}$) gives rise to two transitions low in energy (Figure 1.3B, bottom panel). Thus, evaluation of the NIR CD and MCD spectra of ferrous active sites defines both the
coordination environment at the Fe\textsuperscript{II} center and the excited state (\(\text{^5E}\)) splitting of the ligand field (7, 32-34).

The triply-degenerate Fe\textsuperscript{II} \(\text{^5T}_2\) ground state is also split in the low symmetry environment of the protein by axial (\(\Delta\)) and rhombic (\(V\)) ligand field splitting. These states are further split by spin-orbit coupling within the states. This spin orbit coupling gives rise to axial and rhombic zero field splitting (ZFS) and a total of 15 sublevels (5 spin-degenerate levels x 3 orbitally-degenerate levels) as seen in Figure 1.4 (for a system with \(-D\)).

![Figure 1.4 Splitting of the \(\text{^5T}_2\) ground state](Adapted from Ref 34.)

For a negative ZFS system, \(M_s = \pm 2\) is lowest in energy. Rhombic distortion splits and mixes the \(M_s = +2\) and \(M_s = -2\) wavefunctions to produce \(|X\rangle = (|+2\rangle + |-2\rangle)/\sqrt{2}\) and \(|Y\rangle = (|+2\rangle - |-2\rangle)/\sqrt{2}\) split by an amount \(\delta\). For the ferrous, non-Kramers ion, \(\delta\) is typically greater than the microwave energy used in EPR which is why ferrous systems are EPR silent.

In order to define the splitting of the ground state, analysis of variable temperature, variable field MCD (VTVH MCD) has been developed. As noted previously, the dominant intensity in low temperature MCD spectra results from C-term behavior which increases in intensity with increasing field and decreasing
temperature until the saturation of the signal. By plotting the intensity of the MCD signal at a single energy as a function of field and temperature (using the unitless parameter $\beta H/2kT$), saturation magnetization isotherms are generated (Figure 1.5).

![Figure 1.5 Saturation magnetization isotherms generated for an S=2, non-Kramers Fe$^{\text{II}}$ system.](Adapted from Ref 34.)

In non-Kramers systems, a nesting (spread) of the isotherms is observed, and the degree of nesting is proportional to the magnitude of $\delta$. As mentioned previously, the $M_s = \pm 2$ levels are mixed in the absence of a magnetic field. Application of a magnetic field further splits this doublet by an amount $g_\parallel \beta H$ (Figure 1.4) and, in the limit of large magnetic fields, the wavefunctions become pure $|+2\rangle$ and $|-2\rangle$ states.

Equation 7 is used to relate $\delta$ and $g_\parallel \beta H$ to the MCD intensity. Numerical fitting of the intensity of the VTVH MCD isotherms using equation 5 gives values for $\delta$ and $g_\parallel$.

$$\Delta \varepsilon = \sum_{i, \text{doublets}} \left\{ A_{\text{sat lim}} \int_0^{\pi/2} \left( g_{\parallel} \cos^2 \theta - \sqrt{2} \left( \frac{M_z}{M_{xy}} \right)_i g_{\perp} \sin^2 \theta \right) \frac{\beta H}{\Gamma_i} \alpha_i \sin \theta d\theta \right\} + \sum_{i, \text{doublets}} B_i H \gamma_i + \sum_{m, \text{singlets}} B_m H \eta_m \quad (7)$$

where

$$\Gamma_i = \sqrt{\delta_i^2 + \left( g_{\parallel} \beta H \cos \theta \right)^2 + \left( g_{\perp} \beta H \sin \theta \right)^2}$$

$$\alpha_i = \frac{\left( e^{-\left(E_i - \Gamma_i/2\right)/kT} - e^{-\left(E_i + \Gamma_i/2\right)/kT} \right)}{\sum_{j, \text{doublets}} \left( e^{-\left(E_j - \Gamma_j/2\right)/kT} - e^{-\left(E_j + \Gamma_j/2\right)/kT} \right) + \sum_{n, \text{singlets}} \left( e^{-E_n/kT} \right)}$$
\[
\gamma_i = \frac{1}{\tau} \left( e^{-(E_i - \delta_i/2)/kT} + e^{-(E_i + \delta_i/2)/kT} \right); \quad \eta_m = \frac{1}{\tau} \left( e^{-E_m/kT} \right)
\]

\[
\tau = \sum_{j, \text{doublets}} \left( e^{-(E_j - \delta_j/2)/kT} + e^{-(E_j + \delta_j/2)/kT} \right) + \sum_{n, \text{singlets}} \left( e^{-E_n/kT} \right)
\]

While negative ZFS systems are more common among ferrous active sites, tetragonal compression can lead to positive ZFS sites. In these sites, \( M_s = 0 \) is lowest in energy. \( M_s = \pm 1 \) is the next energy level, and the \( | -1 \rangle \) and \( | +1 \rangle \) states will split in a similar fashion to the description of the \( M_s = \pm 2 \) sublevels. The 0 and one member of the \( \pm 1 \) sublevel makes an effective \( \pm 2 \) doublet when the magnetic field is perpendicular to \( z \), but with a splitting, “\( \delta \)” \( \gg \delta_{\text{negative ZFS}} \). For a positive ZFS system, \( g_\parallel = 8 \). The value of \( \delta \) and the energy of the lowest energy “singlet” (the remaining member of the \( \pm 1 \) level) is used to obtain the ZFS parameters \( D \) and \( E \).

Both \( \delta \) and \( g_\parallel \) and \( D \) and \( E \) are used to obtain the axial and rhombic ligand field splitting parameters (\( \Delta \) and \( V \), respectively), thus defining the splitting of the \( ^5T_2 \) ground state. Following this approach, excited state MCD spectroscopy is used to define both the ground and excited state splittings of ferrous sites (7, 32-34).

1.2.3 \( \{\text{FeNO}\}^7/\{\text{FeO}_2\}^8 \) Methodology

For the oxygen activating enzymes, the initial steps involving \( \text{O}_2 \) have been termed the ‘gray area’ of non-heme iron dioxygenase reactivity (18). To date, only two Fe-O-O intermediates have been observed (one in an extradiol dioxygenase and one in a Rieske dioxygenase) (35, 36), and they have yet to be characterized. In the \( \alpha \)-keto acid dependent dioxygenases, the first intermediate trapped after addition of \( \text{O}_2 \) is an \( \text{Fe}^{IV}=\text{O} \) which forms after decarboxylation of the \( \alpha \)-keto acid (14). To study these elusive steps of the reaction, computational approaches have been employed (18, 37, 38). One difficulty with a pure computational approach, however, is a lack of physical data with which to validate the computational model. If a stable analogous model could be studied both spectroscopically and computationally, it would provide a model system which reflects the catalytic system. Computational studies going forward with this experimentally calibrated model would then be better equipped to account for the unique effects of a particular system or substrate.
For dioxygenase enzymes, a logical analogue to O$_2$ is nitric oxide (NO). NO has one less electron than dioxygen making it less reactive. Therefore, it forms very stable, reversible complexes with non-heme ferrous centers which are both chromophoric and half-integer spin (39, 40). In the notation of Enemark and Feltham, these complexes are termed {FeNO}$^7$ for the 7 total valence e$^-$s (6 d e$^-$s from Fe$^{II}$ and 1 e$^-$ from the NO radical) (41). A number of model complex and protein {FeNO}$^7$ sites have been characterized with a variety of spectroscopic techniques. EPR and Mössbauer studies have shown that the vast majority of non-heme {FeNO}$^7$ sites have S=3/2 ground states (42-46). This S=3/2 state is best described as a high spin Fe$^{III}$ antiferromagnetically coupled to NO$^-$ (vide infra). The strong Fe-N$^{NO}$ bond (from crystallography and EXAFS) gives a tetragonally compressed distortion to the iron center and defines the electronic z-axis of the site (47). These {FeNO}$^7$ sites can be further characterized by a variety of spectroscopic techniques including magnetic Mössbauer, x-ray absorption methods, EPR, UV-vis absorption, resonance Raman, CD, MCD, and VTVH MCD.

To better understand this unusual S=3/2 {FeNO}$^7$ site and correlate its electronic structure to spectroscopic data, extensive studies were undertaken on the NO complexes of Fe-EDTA and Fe-[Me$_3$TACN]-(N$_3$)$_2$ (47). From Fe K-edge XAS and EXAFS data, (in comparison with well described reference compounds) the oxidation state of the Fe center is best described as Fe$^{3+}$. The observed S=3/2 EPR spectrum is described by the spin Hamiltonian given in equation 8, where D is the axial ZFS parameter, E is the rhombic ZFS parameter, and $g_0 = 2.0$.

$$\hat{H}_{\text{spin}} = D[S_x^2 - 5/4 + E / D(S_y^2 - S_z^2)] + g_0 \beta \hat{S} \cdot \hat{H}$$  \hspace{1cm} (8)

At the axial limit, the S=3/2 spin state splits into two doublets, $M_s = \pm 1/2$ and $M_s = \pm 3/2$, with $M_s = \pm 1/2$ lowest in energy and the doublets split by an amount 2D. The EPR intensity arises from transitions from the $M_s = \pm 1/2$ doublet with $g_\parallel = 2.0$ and $g_\perp = 4.0$. Deviation from the axial limit cause $g_\perp$ to split leading to rhombic ZFS and a measurable E/D value (47).

In the UV-vis absorption/CD/MCD 5 bands which arise from the Fe-NO interaction are observed. (The Fe-EDTA-NO 5K, 7T MCD spectrum with Gaussian
resolution of the bands is shown for reference in Figure 1.6.) These bands have been assigned as the Fe$^{III}$ d-d, ligand field bands (bands 1 and 2) and NO$^{-}$ to Fe$^{III}$ d CT transitions (bands 3-5) (47).

![Figure 1.6 5K, 7T MCD spectrum of Fe-EDTA-NO with Gaussian resolution of the transitions](image)

Figure 1.6 5K, 7T MCD spectrum of Fe-EDTA-NO with Gaussian resolution of the transitions

VTVH MCD can also be used to get the polarization of the transition (and hence information about the origin of the transition). For randomly oriented samples, the intensity of the VTVH MCD isotherms is modeled by equation 9 which, using the $g_0$, D and E/D values obtained from EPR, is fit to obtain the effective transition moments, $M_{ij}^{\text{eff}}$. These effective transition moments are related to % x, y, or z polarization by equation 8 (shown for % x polarization) and the cyclic permutations of the indices for % y and % z polarization to obtain the polarization of the transition being probed by VTVH MCD (48).

$$\frac{\Delta \varepsilon}{E} = \frac{\gamma}{4\pi S} \int_0^{2\pi} \int_0^{\pi} \sum_i N_i \left( I_z \langle S_z \rangle_i M_{xy}^{\text{eff}} + I_y \langle S_y \rangle_i M_{xz}^{\text{eff}} + I_x \langle S_x \rangle_i M_{yz}^{\text{eff}} \right) \sin \theta d\theta d\phi$$ (9)

$$\% x = 100 \times \left[ \frac{\left( M_{xy}^{\text{eff}} M_{yz}^{\text{eff}} \right)^2}{\left( M_{xy}^{\text{eff}} M_{xz}^{\text{eff}} \right)^2 + \left( M_{xy}^{\text{eff}} M_{yz}^{\text{eff}} \right)^2 + \left( M_{xz}^{\text{eff}} M_{yz}^{\text{eff}} \right)^2} \right]$$ (8)

VTVH MCD taken of band 5 of Fe-EDTA-NO is z-polarized. A z-polarized transition is consistent with a NO$^{-}$ and Fe$^{III}$ d CT transition as the short Fe-N$^{\text{NO}}$ bond defines the z-axis of the molecule.
Spin unrestricted DFT calculations on these \{FeNO\}^7 complexes yield a molecular orbital energy level diagram shown in Figure 1.7A with selected orbital contours shown in Figure 1.7B (for Fe-Me₃TACN-(N₃)₂-NO). The calculated electronic structure is best described as Fe^{III}-NO⁻ (from the 5 unoccupied \(\beta\) Fe d orbitals and 2 unoccupied \(\alpha\) NO orbitals) which is consistent with the experimental description given above (49).

![Figure 1.7 Electronic structure of \{FeNO\}^7 models. (A) Energy level diagram showing Fe d and NO\(^{-} \ 2p^*\) orbitals. (B) Unoccupied Fe and NO\(^{-}\) orbital contours. (Adapted from Ref 49.)](image)

The ultimate goal of these NO studies is to generate a well grounded computational model for extension to the initial steps of O\(_2\) reactivity for non-heme iron enzymes. Using these experimentally calibrated \{FeNO\}^7 computational models NO is replaced by O\(_2\) in the active site generating an \{FeO\(_2\)\}^8 complex. The reaction of this complex with the substrate is then modeled computationally. The reaction pathway of O\(_2\) with IPNS has been modeled using this \{FeNO\}^7/\{FeO\(_2\)\}^8...
methodology. The IPNS \( \{\text{FeNO}\}^7 \) studies showed new spectroscopic features which reflected strong donation by the thiolate of the substrate. The effect of the thiolate on the \( \{\text{FeO}_2\}^8 \) complex resulted in a strong stabilization of the (usually highly endergonic) \( \text{Fe}^{\text{III}}\text{O}_2^- \) and an FMO aligned for H-atom abstraction (the first step in the mechanism). This methodology is applied in Chapter 5 to understand the initial steps of the \( \text{O}_2 \) reaction of \( \alpha \)-keto acid dependent dioxygenases.

1.2.4 Computational Methods

A theoretical study of metalloprotein active sites compliments the spectroscopic approaches described above. The computational models are used to aid in the assignment of specific transitions in the experimental spectra. They also provide insight into the geometric and electronic structure of the active site leading to a better understanding of the effects of protein-derived ligands and exogenous substrates. Proposed mechanisms, whether corroborated by experimental intermediates or wholly computationally derived, are evaluated energetically with insight into the electron flow and possible intermediates. Important insight is gained as to the feasibility of a mechanistic pathway and what factors govern reactivity.

The field of computational chemistry has advanced rapidly in recent years with improvements in both the variety of approaches available and the size and level of description that is economically accessible to explore. In these studies, quantum chemical calculations at the density functional theory level (DFT) are employed to gain insight into the geometric and electronic structures of enzyme active sites and their mechanisms of reactivity. Previous work on the experimentally calibrated \( \{\text{FeNO}\}^7 \) computational models examined a variety of functional and basis set combinations to determine which was most appropriate for use in these systems (49). From those results and comparisons made in this work (see chapter 5), the best description of the \( \{\text{FeNO}\}^7 \) system was obtained with the BP86 functional with 10% Hartree-Fock exchange included and a triple-\( \zeta \) plus polarization basis set to describe \( \text{Fe-N-O} \) and the active part of the substrate (e.g. the \( \alpha \)-keto acid moiety) and a double-\( \zeta \) plus polarization basis set for all other atoms. This functional and basis set combination also agrees well with the experimental data for the ferrous systems
studied and has been used consistently throughout this thesis. In building the active site models, the histidine and aspartate or glutamate protein-derived residues were truncated at the β-carbon of the amino acid and fixed with respect to each other to simulate the constraints of the protein backbone. As a real protein system does not exist in the gas phase, solvation effects were considered where appropriate. Full descriptions of the computational models and procedures are detailed in the specific chapters.

1.3 Scope of the thesis

This thesis presents a series of studies which explore the effects of the endogenous and exogenous ligands to the Fe\textsuperscript{II} center. Specifically, the focus is on the effects of the 3His triad vs the facial triad and the resulting implications on reactivity as well as the role of the substrate in directing the reactivity of these non-heme Fe\textsuperscript{II} sites. Chapter 2 examines the effects of second sphere residues in Dke1. Chapter 3 extends the study of Dke1 to substrate binding and compares the unusual 3His triad in Dke1 with the more commonly observed facial triad. In Chapter 4, the exploration of the 3His triad in Dke1 is extended to the α-keto acid, HPP. The reaction catalyzed by Dke1 with HPP results in an atypical cleavage pattern of the α-keto acid which occurs because of the difference in charge between the 3His and facial triads. Chapter 5 evaluates the reaction coordinate of α-keto acids using the \{FeNO\}\textsuperscript{7}/\{FeO\textsubscript{2}\}\textsuperscript{8} methodology to understand the effects of binding α-keto acid which lead to a new O\textsubscript{2} binding mode. Finally, in Chapter 6, the reaction coordinate of IPNS (whose initial steps were validated previously with the \{FeNO\}\textsuperscript{7}/\{FeO\textsubscript{2}\}\textsuperscript{8} methodology) is completed and substrate analogues are used to gain insight into the unusual oxidase activity of IPNS. Taken together, these studies shed light on the rational for facial triad ligation at the Fe\textsuperscript{II} site and how the Fe\textsuperscript{II} ligand set and substrate or cofactor tunes the specific reactivity of these enzymes.
1.4 References


Chapter 2

Kinetic and CD/MCD Spectroscopic Studies of the Atypical, 3-His Ligated, Non-heme Fe$^{II}$ Center in Diketone Dioxygenase: the Role of Hydrophilic Outer Shell Residues in Catalysis

2.1 Introduction

Mononuclear non-heme Fe$^{II}$ centers are important for the catalysis of a variety of highly specific O$_2$-dependent reactions (1-3). A common rationale is often employed in these enzymes: a facial triad of two histidines and one glutamate/aspartate together with two or three water molecules ligate the resting Fe$^{II}$ cofactor. In the presence of all reaction components, a catalytically competent 5-coordinate (5C) metal center is formed, which allows O$_2$ binding and reduction, coupled with substrate oxidation (3). The facial triad is considered to play various roles including (i) creating a high affinity Fe$^{II}$ binding site, (ii) providing a geometric scaffold that allows O$_2$ coordination to the Fe$^{II}$ center upon cosubstrate and/or substrate binding to the enzyme, and (iii) maintaining the appropriate redox-potential of the metal center.

The facial triad is embedded in the protein environment, which typically affords selective binding and appropriate positioning of the substrate and, if required, cosubstrate. The active site pocket may also provide proteinogenic residues that promote reaction steps via acid-base catalysis or H-bonding interactions. In the reaction mechanism of extradiol catechol ring-cleaving dioxygenases, results from detailed pre-steady state and steady state kinetic, mutational and computational studies have demonstrated roles for specific hydrophilic active site residues in substrate binding, O$_2$ reduction and C-C bond cleavage (4-7). Additionally, the H-bonding network in the active site will contribute to the de/stabilization of outer and inner shell water which can participate in catalysis. Metal-bound waters, furthermore, directly determine the metal center coordination number and, consequently, cofactor stability and the catalytic competence of the metal center. While a 6C metal center is believed to protect Fe$^{II}$ from uncoupled oxidation by O$_2$ when only cofactor is present, formation of a 5C center upon ligation of all (co)substrates is required for efficient catalysis. The facial triad itself has been shown to contribute directly to the H-bonding network of non-heme iron enzymes. In the α-ketoglutarate dependent dioxygenases, the carboxylate ligand of the facial triad plays a role in stabilizing water coordination through a H-bonding interaction between the non-coordinating oxygen of the carboxylate and the coordinated water (8).
In recent years, a small number of O$_2$-dependent non-heme iron enzymes have been found that exhibit distinct metal binding motifs (9). The rationale for these deviations and the resulting consequences are under active investigation. In the case of the α-ketoglutarate- and O$_2$-dependent halogenases SyrB2 (10) and CytC3 (11), the natural substitution of the metal ligating carboxylate of the facial triad with alanine leaves space for a halide ion, which is the substrate in the halogenation reaction. This mechanistically driven deviation from the facial triad motif comes at the cost of losing a high affinity Fe$^{II}$ binding site in the resting enzyme.

In the C-C bond cleaving dioxygenase Dke1, which displays a high affinity 3 His Fe$^{II}$ binding site (Figure 2.1, PDB entry: 3bal (12)), a clear rationale for this deviation in metal binding motif, which it shares only with cysteine dioxygenase, is still missing.¹ We have studied the role of the first coordination sphere of Dke1, which constitutes the primary enzyme of the acetylacetone degradation pathway in *Acinetobacter johnsonii* (13, 14), in metal binding and catalysis.

![Figure 2.1 Metallocenter and H-bonding network in Dke1, the primary enzyme of the acetylacetone degradation pathway in *A. johnsonii* (PDB entry: 3bal, (12)). First sphere histidines and hydrophilic active site residues are labeled accordingly. Note the structure is for the Zn substituted form and no H$_2$O was resolved. Carbons, oxygens, nitrogens and sulfurs are depicted in gray, red, blue and yellow, respectively. H-bonds colored blue.](image)

¹ Sequence alignments with cysteine dioxygenase suggest a 3-His metal binding motif also for cysteamine- and 3-mercaptopropioate dioxygenase. Direct evidence from protein structures however is not available.
Spectroscopic investigations, employing a combination of near-infrared circular dichroism (CD), magnetic circular dichroism (MCD), and variable-temperature, variable-field (VTBH) MCD spectroscopies have revealed that Dke1 follows the general mechanistic strategy of O₂ activation by the facial triad ligated Fe²⁺ center, showing a 6C resting site and partial conversion to a 5C species upon substrate ligation (15). Mutation of the first coordination sphere to a facial triad leads to the destruction of the high-affinity metal binding site (16) preventing study of a restructured facial triad.

Here we report a detailed investigation of the role of the outer shell residues in the enzyme’s structural stability and reactivity. Previous steady state and single-turnover enzyme kinetics together with quantitative structure-activity relationship (QSAR) studies have allowed dissection of particular steps of the chemical reaction and have implied a chemical mechanism (17,18). The O₂ reduction is the rate limiting step of the enzymatic reaction under physiological conditions, and O₂ reduction and C-O bond formation are concerted. In this study, these methods are combined with mutational and CD and MCD spectroscopic methods to gain detailed insight into the interplay of second-shell residues and the structure and function of the 3-His metal center. Results reveal marked effects of particular outer shell residues on cofactor stability, active site geometry, substrate binding and O₂ reduction rates. The roles of the respective amino acid residues in specific reaction steps are discussed.

2.2 Materials and Methods

2.2.1 Materials

2,4-Pentanedione (PD), 1,1,1-trifluoro-PD (TFPD), 4,4-difluoro-1-phenyl-1,3-butanedione (DFPB), 4,4,4-trifluoro-1-phenyl-1,3-butanedione (TFPB), phenyl-1,3-butanedione (PB) and other chemicals were obtained from Sigma Aldrich (St. Louis, MO, U.S.A.) except 1,1-difluoro-2,4-pentanedione (DFPD) which was from Matrix Scientific (Columbia, SC). All substrates were purchased at highest available quality (>97% pure) if not otherwise stated. Pfu DNA polymerase was from Promega (Madison WI). The other enzymes for molecular biology were purchased from MBI
Fermentas (St. Leon-Rot, Germany). The expression vector pKYB1 was from New England Biolabs (Beverly, MA).

2.2.2 Methods

2.2.2.1. Site-directed mutagenesis and enzyme preparation

Site-directed mutations were introduced via a standard two stage PCR protocol (19). Two separate primer extension reactions were performed using the respective forward and reverse oligonucleotide primer (Table 2.1) and plasmid vector pKYB1-

*dkel Strep* (20) as a template. The latter contains the full-length *dkel* gene fused to an oligonucleotide encoding a 10-amino acid C-terminal affinity tag (21). Note that we have demonstrated, previously, that heterologous expression and the affinity tag do not alter the biochemical and catalytic properties of Dke1 (20), which we originally isolated from *A. johnsonii* (13, 14). For the introduction of multiple mutations the respective mutation-bearing analogous vector was used. The PCR protocol used 150 ng of template in a total volume of 25 μL. Four cycles of separate amplification with 3 units of *Pfu* DNA polymerase (Promega) were followed by 18 cycles of amplification of the combined reaction mixtures using an annealing temperature of 62 °C. The final extension phase was 15 min at 72 °C. After digestion of the template DNA with *DpnI*, the amplified plasmid vectors were transformed into electro-competent *E. coli* BL21(DE3) cells (Stratagene, La Jolla, CA) according to standard procedures. Plasmid DNA from positive clones was isolated via a miniprep kit (Wizard Plus SV Minipreps, Promega) and subjected to dideoxy sequencing of the entire *dkel* gene, to verify that the clone bore the desired mutation and that no errors had been introduced into the sequence during amplification via PCR.
Table 2.1 Primers and template DNA used in this study to generate Dke1 variants. Mutations introduced are underlined in the primer sequence.

| Target-variant | Template: a | Primer (5’→3’)
|----------------|-------------|---------------------------|
| T107A WT       |             | fw: 5'-CACATTGGCTTTCCTTTCTC-3’
|                |             | rev: 5'-CGCAAGAGAAAGGCTTTTTACATGCAATG-3’|
| E98Q WT       |             | fw: 5’-CCAAGCTACGGTTTCAGGTGCATTGC-3’
|                |             | rev: 5’-GCAATGCACCTGAAAGACTGAAAACCCTGAGCTTTG-3’|
| E98A WT       |             | fw: 5’-CCAAGCTACGGTTTCAGGTGCATTGC-3’
|                |             | rev: 5’-CAATGCACCTGAAAGACTGAAAACCCTGAGCTTTG-3’|
| R80A WT       | E98A        | fw: 5’-GAAAAATGGAAGTGGCTGTCGATG-3’
|                |             | rev: 5’-CTTGCTCCGCCACCCACTTCCTTC-3’|
| E98A_R80A     | E98A        | fw: 5’-CACATGTGCCGCCCCGGGGATTTTTCCTGACTAAGGG-3’
|                |             | rev: 5’-CCCTTAGTCAGGAAAACTCCCCGCGGCCAGCATG-3’|
| Y70F WT       |             | fw: 5’-CATGCTGGCCCGGGGAATTTTTCCTGACTAAGGG-3’
|                |             | rev: 5’-CCCTTAGTCAGGAAAACTCCCCGCGGCCAGCATG-3’|
| E98A_R80A_Y70F WT | E98A_R80A | fw: 5’-CATGCTGGCCCGGGGAATTTTTCCTGACTAAGGG-3’
|                |             | rev: 5’-CCCTTAGTCAGGAAAACTCCCCGCGGCCAGCATG-3’|
| Y70A WT       | E98A        | fw: 5’-CATGCTGGCCCGGGGAATTTTTCCTGACTAAGGG-3’
|                |             | rev: 5’-CCCTTAGTCAGGAAAACTCCCCGCGGCCAGCATG-3’|

a The template was the pKYB1- dke1 Strep vector, bearing the respective, outlined mutation.
b Wild type plasmid.

Production of recombinant wild-type Dke1 and variants of Dke1 was done according to the procedures described previously (20). Purification was carried out according to reported protocols, using affinity chromatography with a 1 mL Strep-Tactin column (IBA, Goettingen, Germany) and subjected to buffer exchange by three passages of desalting via NAP-25 columns (GE Healthcare, Chalfont St. Giles, Great Britain). using 20 mM Tris-HCl buffer (pH 7.5). Protein solutions were concentrated (≥10 mg/mL, ≥95 % purity) with Vivaspin centrifugation concentrator tubes (Vivascience, Hannover, Germany) and stored at -20 °C.

2.2.2.2. FeII content and protein concentration

The content of protein-bound FeII in purified enzyme preparations was determined spectrophotometrically, whereby the formation of a colored solution complex between FeII and ferene S (ε592 nm = 35.5 mM-1 cm-1) (22, 23) was monitored in an overnight measurement. All assays were performed at 25 °C in 20 mM Tris-HCl buffer (pH 7.5), containing ferene S in excess (20 mM) and 2 mM ascorbic acid as a reductant, to keep FeII in its reduced form over time. Blanks in the absence of protein were measured and subtracted. Protein concentrations were determined by measuring
the absorbance at 280 nm of the appropriately diluted purified enzyme preparation in Tris buffer (20 mM, pH 7.5) and applying a theoretically calculated absorption coefficient from the respective sequence based on the method of Edelhoch (24) and the parameters of Pace (25) using the program ProtParam (http://www.expasy.ch). Note that this method, which uses native protein, gave the same results as the standard procedure, which uses denaturing conditions (6M Guanidine-HCl buffer, 20 mM KH₂PO₄/K₂HPO₄, pH 6.5) within an experimental error of 5%. The method was furthermore validated using the BCA Method (Pierce, Rockford, IL). Apparent first-order iron detachment rates were determined using Microcal Origin.

2.2.2.3 Steady state kinetics

The standard assay of Dke1 activity was carried out at 25° C using 0.2 mM PD dissolved in air-saturated 20mM Tris-HCl buffer (pH 7.5). The rate of substrate depletion was measured spectrophotometrically at 280 nm and 25° C with a DU 800 UV–Vis Spectrophotometer (Beckmann Coulter, Inc., Fullerton, CA).

Steady-state kinetic studies with PD and TFPD at different pH values were performed in 100 mM MES (pH 5.0 -6.5) and Tris (pH 6.5 -10.5) buffer, whereby the absorbance trace at the wavelength of maximum substrate absorbance was monitored (18). The pH dependence of extinction coefficients had been determined previously (18) and reaction rates were calculated accordingly. Specific activities and apparent \( k_{cat}^{app} \) values were generally related to the concentration of Fe⁡II containing enzyme active sites, which were calculated from the iron content in the gel filtrated protein preparation as described (vide supra).

\( K_{M}^{app} \) and \( k_{cat}^{app} \) values were obtained in two ways. (i) A series of classical initial rate measurements at varied substrate concentrations and a non-linear fit of the Henri–Michaelis–Menten equation for a single-substrate enzymatic reaction to the data was performed. (ii) To validate initial rate data, the integrated form of the Michaelis-Menten equation to expanded reaction time courses was applied, whereby 50 μM of substrate was used and rates were corrected for the decrease in O₂.

---

² Given that Dke1 activity is insensitive to a increase of ionic strength, as tested at salt concentrations up to 1 M NaCl, the change of ionic strength at various pH values was neglected.
It was shown that both approaches of kinetic analysis yielded consistent estimates for the apparent catalytic center activity ($k_{cat}^{app}$) and Michaelis–Menten constant ($K_M^{app}$) at 260 µM O$_2$, whereby the integrated approach allows for a better assessment of rates at low substrate concentrations.

2.2.2.4. Substrate binding kinetics.

Formation of the enzyme-Fe$^{II}$–substrate complex was assessed with a stopped-flow UV-Vis-spectrophotometer (SF61DX2, Hi-Tech) housed in argon-flushed glove box (Belle Technology), monitoring the prototypical low energy transition of the substrate ligated metal center at the wavelength of its maximum absorbance, $\lambda_{max}$ (18). Stock solutions of substrate (300 µM) and enzyme (300 µM Fe$^{II}$, 600 µM enzyme subunits) were used, which had been made anaerobic by repeated flushing with nitrogen. Note that the substrate concentration was fully saturating at the steady state ($K_M^{app} < 10$ µM). The reaction was conducted at 25 °C in an air-saturated 20mM Tris-HCl buffer (pH 7.5).

Binding kinetics showed biphasic behavior and were best fit via the following formula describing double-exponential decay:

$$A = a_0 - a_1 e^{-(k_1 t)} - a_1' e^{-(k_1' t)}$$

where $A$ is the absorbance, $a_0$ is the total amplitude, $a_1$ and $a_1'$ are the amplitudes of the respective binding-steps that proceed with first-order rate constants of $k_1$ and $k_1'$ respectively. All measurements were carried out in triplicate and resulting rate constants were averaged.

2.2.2.5. CD and MCD spectroscopy.

Apo-enzyme was prepared by dialyzing Dke1 against 20 mM EDTA in 20 mM Tris-HCl buffer (pH 7.5) at 4 °C for 2 days using a Slide-A-Lyzer cassette (Pierce, Rockford, IL), with repeated buffer exchange and another 2 days against the Tris buffer without EDTA. The resulting apo-enzyme was then buffer exchanged into 100 mM Tris-HCl in D$_2$O at the appropriate pD, using a 4 mL Amicon Ultra-4 filter with a 10 kDa cutoff membrane (Millipore), and the protein was then brought to a monomer concentration of ~1-2 mM. Reagents were made anaerobic by purging with argon gas on a Schlenk line. Dke1 was made anaerobic by alternating cycles of evacuation and argon purge on the Schlenk line at 0 °C. All samples were prepared under an inert
atmosphere inside a N$_2$-purged ‘wet box’ to maintain an O$_2$ free environment. Ferrous ammonia sulfate was added in microliter quantities to a final concentration of 90% of the monomer concentration. The samples were then transferred into an anaerobic cuvette and incubated for ~1 h in the glove box, prior to measurement. Saturating amounts of deuterated sucrose were added anaerobically to protein preparations as a glassing agent for MCD measurements to a final concentration of ~1mM active sites. CD measurements were taken without and with sucrose to ensure that the Fe$^{II}$ site was unaffected by the glassing agent. (Note that sucrose, rather than glycerol, was used as a glassing agent as the latter affected the NIR CD spectrum of the Fe$^{II}$ active site.)

Near-IR (600-2000 nm) CD and MCD data were recorded on a Jasco J-200D spectropolarimeter with a liquid N$_2$-cooled InSb detector and equipped with an Oxford Instruments SM4000-7 tesla (T) superconducting magnet. CD measurements were taken at 5 °C in an anaerobic cuvette. CD spectra were corrected for buffer and protein baselines by subtraction, and MCD spectra were corrected for the natural CD and zero-field baseline effects by averaging the positive and negative field data at a given temperature.

2.3 Results

2.3.1 Kinetic parameters and pH profile of WT Dke1

The steady state kinetic parameters of WT Dke1 at pH 7.5 towards a range of dicarbonyl substrates have been investigated previously (17, 18, 25). In order to gain information about the ionizable groups involved in catalysis, $k_{cat}^{app}$ and $K_M^{app}$ values were determined using PD for WT Dke1 between pH 5 and 10. Figure 2.2A shows these values as a function of pH.

The log($k_{cat}^{app}/K_M^{app}$) versus pH plot, showed a clear bell shape with inflections in the acidic and basic pH regions with slopes of +1 and -1. Data were fit to equation 2:

\[
Y = \log\left[\frac{C}{1+K_1/[H^+]+[H^+]/K_2}\right]
\]

3 It should be noted that Dke1 could not be saturated with O$_2$ up to cO$_2$ of 1.2 mM, preventing the determination of kinetic constants under saturating conditions.
where $Y$ is $\log(k_{\text{cat}}^{\text{app}})$ or $\log(k_{\text{cat}}^{\text{app}} / K_M^{\text{app}})$, $C$ is the pH-independent value of $Y$, and $K_1$ and $K_2$ are the macroscopic dissociation constants for the ionizable groups being titrated. $[H^+]$ is the proton concentration. From this fit, $pK_a$ values of $6.3 \pm 0.4$ and $8.4 \pm 0.4$ were determined. Given that the $k_{\text{cat}}^{\text{app}}$ mirrors the O$_2$ reduction rate, which is the first step of the chemical reaction (18), this indicates that some precatalytic step is influenced by either enzyme or substrate ionization and is therefore pH-dependent. It should be noted that the substrate, PD, has a $pK_a$ of 8.9 and is considered to bind as a monoanion to the metal center (18,26). As a result, a change in its protonation state may well affect substrate affinity and substrate binding rates.

The $k_{\text{cat}}^{\text{app}}$ values are constant over the pH range investigated, with only a slight decrease of the $k_{\text{cat}}^{\text{app}}$ observed above pH 10 and below pH 5.5, where protein instability began to interfere with activity measurements. Because of this protein instability, it was not possible to determine whether the slight curvature in the extremes of the plots reflects a protonation equilibrium. Data were fit by equation 2 (vide supra) and the resulting curve eliminated the possibility of a $pK_a$ in the range of pH 4.2 – 10.0 (Figure 2.2A).
To test if the protonation state of the substrate is affecting the enzyme kinetics, $k_{cat}/K_M^{app}$-pH profiles for Dke1 were determined using TPD, the trifluorinated substrate analogue of PD, which has a $pK_a$ of 6.6 (18, 26), more than 2 log units lower than that of PD. The resulting $k_{cat}^{app}/K_M^{app}$-pH profile showed an inflection in the basic region with a $pK_a$ value of 8.1 ± 0.2, (Table 2.2 and Figure S2.2B of the Supporting Information), the same as that for PD within experimental error. Therefore, the basic $pK_a$ found in the $k_{cat}^{app}/K_M^{app}$-pH profile was clearly not due to substrate deprotonation. At pH < 6.0, no reliable data could be obtained due to enzyme inactivation and slow reaction rates and a low extinction coefficient with TPD.
Table 2.2 Steady state kinetic properties of Dke1 WT and variants.a

<table>
<thead>
<tr>
<th>Variant</th>
<th>kcat&lt;sub&gt;app&lt;/sub&gt; [s&lt;sup&gt;-1&lt;/sup&gt;]</th>
<th>Km&lt;sub&gt;app&lt;/sub&gt; [mM s&lt;sup&gt;-1&lt;/sup&gt;]</th>
<th>Fe&lt;sup&gt;II&lt;/sup&gt; [%]</th>
<th>pK&lt;sub&gt;a2&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>6.6</td>
<td>9.1±1.5 (21)</td>
<td>70</td>
<td>8.4±0.4 (8.1±0.2)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>E98A</td>
<td>0.074</td>
<td>4.1±2</td>
<td>60</td>
<td>- / &gt; 10</td>
</tr>
<tr>
<td>E98Q</td>
<td>0.050</td>
<td>2.9±2</td>
<td>71</td>
<td>- / &gt; 10</td>
</tr>
<tr>
<td>R80A</td>
<td>0.126</td>
<td>37±15</td>
<td>50</td>
<td>9.0 ± 0.5</td>
</tr>
<tr>
<td>Y70F</td>
<td>1.27</td>
<td>8.5±3</td>
<td>64</td>
<td>9.1 ± 0.5</td>
</tr>
<tr>
<td>Y70A</td>
<td>0.13</td>
<td>35±15</td>
<td>51</td>
<td>n. d.</td>
</tr>
<tr>
<td>T107A</td>
<td>0.1</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>5</td>
<td>n. d.</td>
</tr>
<tr>
<td>E98A_R80A</td>
<td>0.098</td>
<td>35±15</td>
<td>47</td>
<td>n. d.</td>
</tr>
<tr>
<td>E98A_R80A_Y70F</td>
<td>0.033</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>38</td>
<td>n. d.</td>
</tr>
<tr>
<td>E98A_R80A_Y70A</td>
<td>0.007</td>
<td>n.d.&lt;sup&gt;c&lt;/sup&gt;</td>
<td>68</td>
<td>n. d.</td>
</tr>
</tbody>
</table>

a Kinetic constants were determined in air-saturated buffer, at pH 7.5 and 25 °C. The typical iron content of an enzyme preparation (Fe<sup>II</sup>) is given and pK<sub>a2</sub> values obtained for the “basic” inflection in the kcat<sub>app</sub>/Km<sub>app</sub>-pH correlation of WT and each variant are given. The kcat<sub>app</sub> values shown are relative standard deviation of < 20 %. The experimental error of all Fe<sup>II</sup> contents was < 10 % and corresponded to their total iron determined by ICP-MS (error < 1%).

b The pK<sub>a</sub> for the substrate TPD is given in brackets.

c No variation of catalytic rates at substrate concentrations of 400µM and 600 µM were obtained; therefore, the resulting velocities were considered to mirror enzyme saturation and, therefore, kcat<sub>app</sub>.

2.3.2 Kinetic parameters and pH profiles of second sphere variants of Dke1

2.3.2.1 Kinetic parameters

To gain insight into the role of H-bonding residues in dicarbonyl cleavage, hydrophilic amino acid residues with a potential role in catalysis were identified and subjected to mutagenesis. The structure of Dke1 reveals a mononuclear metallocenter, ligated by three histidines, His62, His64 and His 104, in an active site pocket predominantly lined with hydrophobic residues and accessible via a hydrophobic tunnel (PDB entry: 3bal). Only four hydrophilic amino acid residues are present in the substrate binding pocket. A hydrophilic gate formed by Arg80, Glu98 and Tyr70 connects the hydrophobic cavity with the exterior (Figure 2.1). The glutamate residue, Glu98, is a second-shell residue, positioned adjacent to the metal-ligating His104, with its proximal carboxylate O in H-bonding distance of the ε<sup>2</sup>-NH group of the histidine (2.82 Å). Furthermore, Glu98 forms a salt bridge with Arg80. This structure H-bonds to a water molecule, which is further H-bonded to Tyr70, forming the
hydrophilic gate. The fourth hydrophilic residue found in the active site is Thr107, which is situated in the outer coordination shell of Fe$^{II}$. All four residues were subjected to mutagenesis, and the resulting protein variants were characterized to elucidate their role in catalysis.

Variants were constructed, expressed and purified where Tyr70, Arg80, Glu98 and Thr107 were individually and in combination substituted with alanine. Additionally, the ‘structurally conservative’ mutants Tyr70→Phe and Glu98→Gln were produced. Iron contents were determined for all variants. Notably, for Thr107→Ala, the iron content was <5% in all preparations, and reconstitution of this enzyme variant with Fe$^{II}$ could not be achieved, indicating a disruption of the metal binding site. All other variants showed iron contents of >50% after purification, indicative of an intact metal binding site and allowing further characterization.

The steady state kinetic properties of all variants and typical Fe$^{II}$ content are given in Table 2.2 and summarized below. The substitution of Glu98 with alanine showed a remarkable, ~100-fold reduction in $k_{cat}^{app}$ (0.074 s$^{-1}$). The analogous substitution with glutamine resulted in a similarly diminished reaction rate ($k_{cat}^{app} = 0.050$ s$^{-1}$). A comparable decrease of $k_{cat}^{app}$ by 2 orders of magnitude was also found for the Arg80→Ala variant ($k_{cat} = 0.126$ s$^{-1}$). The substitution of Tyr70 with alanine resulted in a 60 fold decrease in $k_{cat}^{app}$ while the structurally conservative mutation to phenylalanine lead to a minor, 3-fold decrease of the catalytic rate. No major changes were found for the $K_M^{app}$ values of the variants investigated. Thus, mutations perturbing the “hydrophilic gate”, formed by Glu98, Arg80 and Tyr70, led to a reduction of $k_{cat}^{app}$ by 2 orders of magnitude. Dke1 variants bearing double and triple mutations were constructed in order to characterize the interplay of the particular mutations. Simultaneous substitution of Glu98 and Arg80 with alanine resulted in $k_{cat}^{app}$ values comparable to the respective single mutations. The additional exchange of Tyr70, thus completely abolishing the hydrophilic gate, had an additional diminishing effect on $k_{cat}^{app}$.

In a previous study on WT Dke1, it was determined that the reduction of O$_2$, which coincided with the enzyme-substrate complex decay rate ($k_{decay} = k_2 [O_2]$), constituted the rate limiting step of catalysis ($k_{cat}^{app}$). This rate was characterized by a
strong dependence of $k_{\text{cat}}^{\text{app}}$ on the potential of the substrate to donate electrons which is mirrored by the energy level of its highest occupied molecular orbital ($\varepsilon_{\text{HOMO}}$) (18).

Analogous correlations of $k_{\text{decay}}$ with the $\varepsilon_{\text{HOMO}}$ of the substrate, obtained for the variants described above, show that the nature of the rate-limiting step has not changed upon mutation (see Figure S2.1 of the Supporting Information).

2.3.2.2 pH profiles

In order to investigate the apparent role of ionizable groups in the precatalytic steps of reactivity in Dke1, pH profiles of the steady-state kinetic parameters for the variants Glu→98Ala, Arg80→Ala and Tyr70→Phe were determined. Importantly, the $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$–pH profiles of the Glu→98Ala and Glu→98Gln variants of Dke1 showed an apparent elimination of both the acidic and basic pK$_a$ effects (Figure 2.2B and Figure S2.2A of the Supporting Information). Alternatively, for the variants Arg80→Ala and Tyr70→Phe, the basic pK$_a$ values were conserved, demonstrating that these residues are not associated with the basic $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$–pH curve inflection (Figures S2.2C and S2.2D of the Supporting Information). As in WT Dke1, the $k_{\text{cat}}^{\text{app}}$ rates were not pH-dependent in all cases, over the entire pH range investigated. Results from $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$–pH profiles are summarized in Table 2.2. The $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$–pH profiles and $k_{\text{cat}}^{\text{app}}$–pH profiles for all variants are given in the Supporting Information.

2.3.3 Stopped flow kinetic analysis of substrate binding

In order to further define the pH-dependence of the precatalytic steps and, in particular, the apparent elimination of the pH dependence of the $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$–pH profiles for the Glu98 variants, the substrate net-binding rates of WT Dke1 and variants were determined by stopped flow kinetic measurements. Ligation of the substrate to the metal center was monitored by measuring the formation of an absorption band which evolves upon ligation of a diketonate to the Fe$^{\text{II}}$ cofactor and has been suggested previously to result from a MLCT transition (18).

---

4 Protein instability precludes measurements of the $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$ above pH 10. The curvature in the $k_{\text{cat}}^{\text{app}}/K_{M}^{\text{app}}$ pH profile at high pH cannot be fit with equation 2, ruling out a pK$_a$ < 10.
Substrate binding was assessed using PD and DPD as ligands. The latter substrate binds more slowly than PD and allows for the initial, fast binding phase to be monitored (18). Variation of ligand concentration (0.15–5 mM) did not lead to a change in the binding rate under the conditions used (inset, Figure 2.3A), which implies that ligation of the diketonate to the metal cofactor was assessed under saturating conditions, and substrate ligation is not the first kinetic step of substrate binding. This is in line with a scenario where the rates observed mirror the net rate of exchange of water and substrate ligands at the Fe$^{II}$ center and not prebinding steps.

Figure 2.3 Stopped flow traces of the Dke1-metallocenter with 1,1-difluor-2,4-pentanedione (DPD). Traces were averaged from triplicate measurements. Panel A shows substrate binding rates for WT Dke1 (violet) and the outer sphere variants Tyr70Phe (blue), Arg80Ala (teal), Glu98Ala (dark grey), Glu98Gln (black). The inset shows substrate ligation to the Fe$^{II}$ center at varying DPD concentrations. Panel B shows the pH dependence of substrate binding to WT Dke1 and the E98Q variant: traces for WT Dke1 at pH 7.5 (violet) and at pH 9.5 (red), traces for the Glu98ÆGln variant at pH 7.5 (black) and at pH 9.5 (blue). Analogous rates for binding of 2,4-pentanedione are presented in the Supporting Information.
Rates of binding of DPD to the variants Glu98→Gln, Glu98→Ala, Tyr70→Phe and Arg80→Ala were measured at pH 7.5 (Figure 2.3A and Table 2.3). Generally, curves were best fit with a double exponential decay, indicating a biphasic binding event. In the E98Q and E98A variants, the binding velocity is decreased by 2 ($k_1$) and 3 ($k_1'$) orders of magnitude, respectively. The Tyr70→Ala and Arg80→Ala substitutions only caused a moderate, 10-fold, reduction in binding rates. Binding curves with PD as a ligand showed the same general trend as found for DPD (see Figure S2.4A of the Supporting Information), where, with the exception of the Glu98 variants, the initial binding phase was too fast to be assessed. Results are summarized in Table 2.3.

### Table 2.3 Kinetic first-order rate constants of substrate ligation for WT Dke1 and variants from stopped flow kinetic studies.a

<table>
<thead>
<tr>
<th>Dke1 variant</th>
<th>Substrate</th>
<th>pH</th>
<th>$k_j$ s$^{-1}$</th>
<th>$k_j'$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>PD</td>
<td>7.5</td>
<td>&gt;2000</td>
<td>180</td>
</tr>
<tr>
<td>WT</td>
<td>DPD</td>
<td>7.5</td>
<td>55</td>
<td>10</td>
</tr>
<tr>
<td>E98Q</td>
<td>PD</td>
<td>7.5</td>
<td>1.3</td>
<td>0.15</td>
</tr>
<tr>
<td>E98Q</td>
<td>DPD</td>
<td>7.5</td>
<td>0.28</td>
<td>0.02</td>
</tr>
<tr>
<td>E98A</td>
<td>DPD</td>
<td>7.5</td>
<td>0.23</td>
<td>0.008</td>
</tr>
<tr>
<td>R80A</td>
<td>PD</td>
<td>7.5</td>
<td>56</td>
<td>-</td>
</tr>
<tr>
<td>R80A</td>
<td>DPD</td>
<td>7.5</td>
<td>13.5</td>
<td>0.75</td>
</tr>
<tr>
<td>Y70F</td>
<td>PD</td>
<td>7.5</td>
<td>7.15</td>
<td>-</td>
</tr>
<tr>
<td>Y70F</td>
<td>DPD</td>
<td>7.5</td>
<td>1.9</td>
<td>0.30</td>
</tr>
<tr>
<td>WT</td>
<td>PD</td>
<td>9.5</td>
<td>330</td>
<td>25</td>
</tr>
<tr>
<td>WT</td>
<td>DPD</td>
<td>9.5</td>
<td>1.6</td>
<td>0.13</td>
</tr>
<tr>
<td>E98Q</td>
<td>PD</td>
<td>9.5</td>
<td>1.7</td>
<td>0.12</td>
</tr>
<tr>
<td>E98Q</td>
<td>DPD</td>
<td>9.5</td>
<td>0.3</td>
<td>-</td>
</tr>
</tbody>
</table>

a Data were obtained at 25°C and at air saturation. The experimental error of all rate constants is >20%.

To further investigate the elimination of the basic pK$_a$ effect on the $k_{cat}^\text{app}/K_M^\text{app}$-pH profile upon Glu98 substitution, binding kinetics for WT Dke1 and the Glu98→Gln variant were determined at varying pH values. Measurements were performed at pH 7.5 and 9.5, which are approximately one log unit below and one unit above the pK$_a$ of 8.4, respectively, determined from the $k_{cat}^\text{app}/K_M^\text{app}$-pH profile of WT
Dke1 (*vide supra*). The resulting curves obtained for DPD ligation are shown in Figure 2.3B and the associated rate constants are given in Table 2.3. The low- and high-pH forms of the Glu98→Gln variant clearly show different binding characteristics, with the low pH form showing a biphasic binding behavior with a pronounced slower phase compared to the apparently monophasic high-pH form.

In WT Dke1, the analogous shift resulted in a ~30 ($k_1$) and ~80-fold ($k_{1'}$) decrease in binding rates. PD binding curves, again, showed the same trend as found for DPD (see Figure S2.4B of the Supporting Information), however, fast substrate ligation rates and a probable change of substrate protonation state in the pH range investigated influence the substrate binding rates and complicate the data analysis. The sensitivity and robustness of substrate binding rates of WT Dke1 and the Glu98 variants, respectively, to a pH shift from 7.5 to 9.5 mirror their $k_{cat}^{app}/K_M^{app}$-pH profile characteristics.

Taken together, the results from stopped flow kinetics show that Glu98 promotes substrate ligation when the pH is less than the $pK_a$ 8.4. Given that a $pK_a$ of 8.4 is extraordinarily high to be associated with glutamic acid ionization and when the fact that there is no other ionizable group in the active site is considered, one explanation for this effect is a possible role of metal bound water in the basic $pK_a$ process. (Note that Fe$^{II}$-bound water usually shows a $pK_a$ of ~9, depending on the environment.) A combination of near-infrared (NIR) CD and MCD was employed to directly probe the coordination at the Fe$^{II}$ site as a function of pH in WT Dke1 and its E98Q variant.

### 2.3.4 NIR-CD and MCD spectroscopy of the metal center in WT Dke1 and its Glu98Gln variant

CD and MCD are spectroscopic techniques that provide information about the coordination environment and ligand field at a metal cofactor site (27,28). In this way, these techniques can report changes at the active site complementary to the kinetic results. WT Dke1 and the E98Q variant were studied with CD and MCD spectroscopy to explore the pH dependence observed in the kinetic measurements. (pD values are used here as the data must be obtained in D$_2$O.) A detailed description of the effects of
the 3His ligand set on the Fe$^{II}$ center in Dke1 relative to the two-His, one-carboxylate facial triad is forthcoming (15) and will not be covered here.

**WT Dke1.** CD spectra of WT Dke1 were taken between pD 7.0 and 9.5. The CD spectrum at pD 7.0 shows two transitions, one at 8,000 (positive feature) and one at 10,500 cm$^{-1}$ (negative feature). With an increase in pD, the spectrum shifts to one dominant positive peak at $\sim$ 11,000 cm$^{-1}$ (Figure 2.4A and Figure S2.4A of the Supporting Information). A plot of $\Delta\varepsilon$ versus pD for the transition at 11,300 cm$^{-1}$ fits to a titration curve with a pK$_a$ of 8.2 ± 0.2 (Figure 2.4A, inset).

![Figure 2.4 CD and LT MCD data for WT Dke1.](image)

**Figure 2.4 CD and LT MCD data for WT Dke1.** (A) CD for pD 7.0 (purple) and 9.5 (green). The inset gives intensity at 11,300 cm$^{-1}$ as a function of pD. (B) LT MCD at 5K and 7T for pD 7.0 (purple, $\Delta\varepsilon \times 1.5$) and 9.5 (green).

To investigate whether the signals observed in CD were due to a change in the ligand field at the iron center or to a conformational change in the protein, low temperature MCD spectra were taken of the pD 7.0 and 9.5 samples. At pD 7.0, two
positive transitions are observed centered at ~10,100 cm\(^{-1}\) and split by ~1900 cm\(^{-1}\), which is characteristic of a 6 coordinate site. (From extensive studies on structurally defined Fe\(^{II}\) model complexes, 6 coordinate Fe\(^{II}\) exhibits 2 transitions in the ~10,000 cm\(^{-1}\) region, split by ~2,000 cm\(^{-1}\), 5 coordinate shows 1 transition at ~10,000 cm\(^{-1}\) and one at ~5,000 cm\(^{-1}\) and 4 coordinate only shows transitions in the 5,000 – 7,000 cm\(^{-1}\) region.) (27,28) When the pD is increased to 9.5, a very similar two transition pattern is observed; however, the transitions are shifted up in energy by ~300 cm\(^{-1}\) compared to those at pD 7.0 (Figure 2.4B).

**Dke1 Glu98→Gln variant.** To further explore the apparent effect of Glu98 on the p\(K_a\) of substrate binding, CD and MCD experiments were conducted in parallel to those described above for the wild type enzyme. The CD spectra for the E98Q variant show one main peak at ~11,000 cm\(^{-1}\) that shifts up in energy (to ~11,300 cm\(^{-1}\)) and up in intensity with increasing pD (Figure 2.5A and Figure S2.4B of the Supporting Information). The inset of Figure 2.5A shows a plot of this intensity versus pD for E98Q Dke1. These data can be fit to a pD equilibrium with a p\(K_a\) of 8.2 ± 0.2. The low temperature MCD spectrum of the E98Q variant at pD 7.0 shows a significant spectral change compared that of the to wild type. A low energy feature at ~5,000 cm\(^{-1}\) is now observed, characteristic of the presence of a 5C Fe\(^{II}\) species. The more intense asymmetric peak centered at ~9,700 cm\(^{-1}\) suggests two overlapping contributions and since a 5C component can only contribute two transitions in the NIR spectral region, the third peak indicates the additional presence of a 6C Fe\(^{II}\) component. When the pD is increased to 9.5 in the E98Q variant, the spectrum changes to two transitions centered at ~10,400 cm\(^{-1}\). The spectrum indicates that only a 6C Fe\(^{II}\) species is now present. Additionally, these transitions occur at the same energies as those observed for the WT enzyme at pD 9.5 (Figure S2.5 of the Supporting Information).
Figure 2.5 CD and LT MCD data for E98Q Dke1. (A) CD for pD 7.0 (red, $\Delta \varepsilon \times 4$) and 9.5 (teal). The inset gives intensity at 11,300 cm$^{-1}$ as a function of pD. (B) LT MCD at 5K and 7T for pD 7.0 (red) and 9.5 (teal).

2.3.5 Autooxidation studies of the E98Q variant

To determine whether the change of metal center geometry to a partial five-coordinate species had an impact on the autooxidation rate of the Glu98Gln variant, the O$_2$ consumption of the resting Glu98Gln variant was monitored at 25 °C. Notably, iron detachment rates (~ 0.0003 s$^{-1}$), estimated by the fereneS method (23), are 2-3 orders of magnitude faster than apparent autooxidation rates determined for WT Dke1 at air saturation (18). Therefore free iron will be present in equilibrium throughout the enzymic auto-oxidation ($K_{d, Fe}= 5$ µM for WT Dke1 (16)), and consequently, the specific rate of autooxidation ($k_{autox}^{app}$) will be complex, representing the oxidation of both bound and unbound iron. For the E98Q variant a biphasic trace for O$_2$ consumption was observed, with a fast decay of ~ 15 µM O$_2$ followed by a slow rate of O$_2$ consumption (Figure S2.6 of the Supporting Information). The first, fast, phase
of O$_2$ consumption shows the typical oxidation rate of unbound Fe$^{II}$ ($k_{Fe} = 0.016$ mM$^{-1}$ s$^{-1}$, Figure S2.7 of the Supporting Information) which is generally expected to be present in Fe$^{II}$-containing enzyme preparations in equilibrium. The second phase reflects the auto-oxidation of the E98Q variant and has a specific rate of autooxidation ($k_{autoox}^{app}$) of $1.2 \times 10^{-6}$ s$^{-1}$ related to Fe$^{II}$. This value compares well to the slightly higher rate of autooxidation in WT Dke1 ($k_{autoox}^{app} = 7.5 \times 10^{-6}$ s$^{-1}$) (18), showing that the apparent autooxidation rate in the Glu98Gln variant is not elevated compared to WT Dke1.

2.4 Discussion

The hydrophilic residues in the substrate binding pocket of Dke1 are crucial for enzyme function in terms of (i) cofactor stability, (ii) stabilization of the 6C geometry at the metal center and (iii) promotion of the transition state for O$_2$ reduction.

*Effect of second-shell residues on cofactor stability in Dke1.* Thr107, stabilizes Fe$^{II}$ binding and mutation leads to loss of cofactor affinity and function. In the crystal structure of Dke1 (Figure 2.1), Thr107 is in H-bonding distance of putative metal-bound waters (note that first shell water molecules are missing in the crystal structure), suggesting a stabilization of the water ligands. Destabilization of the metal center by 1 order of magnitude following mutation of a second-shell threonine has been reported previously for the 3-His ligated metallocenter of Zn$^{II}$-dependent carbonic anhydrase. In this case, the crystal structure of the variant showed subtle geometric changes in the water shell (29,30). In Dke1, for which a K$_d$ of 5 μM for Fe$^{II}$ has been determined previously (16), a decrease in affinity by 1 order of magnitude will lead to elimination of the high-affinity metal binding site.

Alternatively, Glu98, which is also located in the second-shell of the metal center and interacts with the ε²-nitrogen of His 104 by a 2.86 Å H-bond, shows no stabilizing effect on the metal center. The Glu98 variants of Dke1 actually lead to a 4-fold decrease in Fe$^{II}$ detachment rates from 0.002 s$^{-1}$ to 0.0005 s$^{-1}$. Second sphere carboxylate residues H-bonded to the non-coordinated nitrogen of a metal-ligated histidine have been reported to increase metal-cofactor stability when the carboxylate
acts as a strong H-acceptor (31,32). In Dke1, the H-bonding interaction of Glu98 with Arg80 (Figure 2.1) lowers the pKₐ of Glu98 and likely prevents a similar role for Glu98 here.

**Stabilization of the 6C Fe⁺² center.** A detailed kinetic analysis of WT Dke1 revealed new insight into the precatalytic steps of diketonate cleavage. Taking into account the proposed mechanism of Dke1 (18), where the diketonate coordinates to the Fe⁺² cofactor and the substrate ligated metal center reduces O₂ via an encounter complex, precatalytic steps mirror the event of substrate binding. The $k_{cat}^{app}/K_M^{app}$ pH-profile shows a bell shape with inflections in the acidic (pKₐ 6.3) and basic (pKₐ 8.4) ranges, corresponding to a pH dependence of binding rates, as $k_{cat}^{app}$ values are insensitive to pH in the range investigated (Figure 2.2). Protein instability prevented a detailed characterization of the low pH effect. The high pKₐ was characterized further by stopped flow measurements and CD and MCD spectroscopy.

Transient substrate binding kinetics of WT enzyme generally showed a > 30 fold decrease of the rate of substrate ligation to Fe⁺² at pH 9.5 compared to that at pH 7.5, which parallels the pH effect seen in the $k_{cat}^{app}/K_M^{app}$ profile. Note that substrate binding rates determined were independent of DPD concentration in the range of 0.15 mM to 5 mM showing that substrate ligation to the metal center is not the first kinetic step of substrate binding. It is therefore reasonable to view the velocities determined as net rates of the ligand binding to the metal center and independent of access to the site. A reduction in the rate of binding at high pH suggests a stronger ligand set at the Fe⁺² center. This is consistent with deprotonation of one of the water ligands at the Fe⁺² center. The CD pH titration, which directly probes the Fe⁺² center, revealed a pKₐ of 8.2 for WT Dke1. The shift of the ligand field in the MCD data to higher energy (Figure 2.4B) indicates a change at the Fe⁺² center to a stronger ligand. These data support a coordinated water-hydroxide equilibrium in WT Dke1. Hydroxide will be displaced more slowly than water by the substrate ligand, leading to decreased binding rates.⁵ This is mirrored by an increased $K_M^{app}$ value in the enzyme kinetics of Dke1.

---

⁵ Note that the MCD spectra show a complete conversion to the PD-ligated metal center at pD 7.0 (14) In the kinetics, extinction coefficients of the enzyme-substrate complex are not significantly changed at high pH or with DPD as a ligand. This indicates that despite a shift in net binding rates, at equilibrium, the level of substrate ligation to the metal center is > 90%.
and, consequently, the inflection of the $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$-pH profile. A water-hydroxide equilibrium at this pH range is not commonly found for mononuclear non-heme Fe$^{\text{II}}$ sites and represents a significant difference between the 3His ligation to Fe$^{\text{II}}$ in Dke1 and the facial triad Fe$^{\text{II}}$ ligation seen for many other ferrous enzymes. This depressed pK$_a$ of bound water likely reflects the reduced level of donation from the third histidine ligand compared to the carboxylate found in the standard facial triad enzymes.

Investigation of the Glu98$\rightarrow$Gln variant by CD spectroscopy showed a pH-dependent signal change with a pK$_a$ at pH $\sim$8.2, analogous to the wild-type enzyme. In the MCD data of E98Q Dke1 at pH 7.0, a mixture of 5C and 6C species is present. The presence of this 5C Fe$^{\text{II}}$ species indicates that there is a reduced affinity for H$_2$O in the absence of the carboxylate. Previous work has suggested that a carboxylate hydrogen bond can stabilize water binding to an Fe$^{\text{II}}$ site. The MCD data for the E98Q variant at high pH show a completely 6C site. Hydroxide binds more tightly than water to the Fe$^{\text{II}}$ center, and increasing the pH results in deprotonation of the water ligand giving an hydroxide that stays bound due to its increased affinity for Fe$^{\text{II}}$. Combined, these data strongly support the model in which the pK$_a$ reflects deprotonation of a metal-bound water ligand. Therefore, Glu98 plays a role, directly or indirectly, in the stability of the bound water.

In the $k_{\text{cat}}^{\text{app}}/K_M^{\text{app}}$-pH profile of E98Q, both of the pK$_a$ effects have been eliminated. The acidic pK$_a$ may mirror slowed binding due to the protonation of Glu98; however, protein instability prevented further exploration of this effect. Pre-steady state kinetic substrate binding studies of the Glu98$\rightarrow$Gln and Glu98$\rightarrow$Ala variants showed a marked, $>100$ fold, decrease in net binding rates. As opposed to the wild-type enzyme, substrate ligation rates at pH 7.5 and pH 9.5 were in the same time regime (Figure 2.3B) with $k_1$ values of $0.30 \pm 0.02$ s$^{-1}$ and $0.28 \pm 0.03$ s$^{-1}$, respectively, consistent with the pH profile results that substrate binding velocities show no marked pH dependence between pH 7.5 and 9.5. The MCD data on E98Q show that the water-hydroxide equilibrium observed in WT is still present in this variant. Thus, the elimination of the pH effect on the E98Q variant is not due to the direct elimination of
a water-hydroxide equilibrium at the Fe$^{II}$ active site but a more indirect effect of a protein structural change due to the mutation in the vicinity of the site.

Effect of the hydrophilic gate. Glu98, Arg80 and Tyr70 form a hydrophilic gate that allows small hydrophilic molecules like water and the metal ion cofactor, to access the hydrophobic active site. Mutation of each residue to the smaller alanine destroys this gate. O$_2$ reduction rates in these variants are diminished by 2 orders of magnitude, confirming their crucial role in catalysis. The $k_{cat}^{app}$ rates are insensitive to pH in the range investigated, showing that acid-base catalysis is not involved in the rate limiting step of O$_2$ reduction. One role of the gate may be in providing an H-bond that stabilizes the developing negative charge on O$_2$ in the transition state of the rate determining step of O$_2$ reduction, a scenario invoked for the early steps of the mechanism in Mn$^{II}$ dependent catechol dioxygenase (33). Loss of this H-bond can cause a 100 to 1000-fold reduction in the rate (10-20 kJ/mol). In the crystal structure of Dke1, Glu98, Arg80 and Tyr70 are oriented such that they could position one water molecule to provide this H-bond. Alternatively, destruction of the gate will allow more water molecules to enter the active site and this may interfere with O$_2$ reduction. Further studies will be required to characterize the impact of active site hydrophilicity on catalysis.

2.5 Acknowledgements

GDS gratefully acknowledges funding of this work by the FWF (Austrian Science Fund, project P18828) and support of the joint work by Max Kade Foundation. EIS acknowledges NIH GM 40392 for support. The authors thank Eva-Maria Imp for technical assistance. ICP-MS analyses were performed at the Institute of Analytical Chemistry at TU Graz.
2.6 References


2.7 Supporting Information

Figure S2.1 Correlation of the diketone’s $\varepsilon_{\text{HOMO}}$ and the rates of Dke1-Fe$^{II}$-diketone complex decay for the wild-type Dke1 and the variants Tyr70→Phe, Arg80→Ala, Glu98→Gln for the substrates 2,4-pentanedione (PD), 1,1-difluor-2,4-pentanedione (DPD), 1,1,1-trifluor-2,4-pentanedione (TPD), 1-phenyl-4,4-difluor-1,3-butanedione (DFPB), 1-phenyl-4,4,4-trifluor-1,3-butanedione (TFPB). Note that for the mutants Tyr70→Ala, double mutant Arg80→Ala Glu98→Gln and triple mutants Arg80→Ala Glu98→Gln Tyr→Ala/Tyr→Phe a > 100 fold reduction of O2 consumption rates for the substrate DPD, compared to PD, was confirmed.
(a) $k_{cat}/K_m$ – pH correlations for E98A with PD:

\[
\begin{align*}
\log k_{cat}/(k_{cat}/K_m) &= f(H^+ [M]) \\
H^+ [M] &= 10^{-5} - 10^{-11}
\end{align*}
\]

(b) $k_{cat}/K_m$ – pH correlations for Dkel wild type with TPD:

\[
\begin{align*}
\log k_{cat}/(k_{cat}/K_m) &= f(H^+ [M]) \\
H^+ [M] &= 10^{-5} - 10^{-11}
\end{align*}
\]

$pK_a = 8.1$
Figure S2.2 $k_{\text{cat}}/K_m$–pH correlations for Dke1 and variants. (A) The E98A mutant and 2,4-pentanedione (PD) as a substrate; (B) WT Dke1 with 1,1,1-trifluoro-2,4-pentanedione (TPD) as a substrate; (C) The Y70F mutant and PD; (D) The R80A mutant and PD.
**Figure S2.3** Stopped flow binding curves of PD (A) to WT Dke1 (violet), Arg80→Ala (green), Tyr70→Ala (blue) and Glu98→Gln at 25 °C, pH 7.5 and (B) to WT Dke1 at pH 7.5 (violet) and pH 9.5 (pink) and the Glu98→Gln variant at pH 7.5 (black) and pH 9.5 (gray).

**Figure S2.4** CD spectra of WT Dke1 (A) and E98Q (B) at varying pH.
Figure S2.5 Overlay of the MCD spectra of WT (green) and E98Q (teal, $\Delta \varepsilon \times 0.6$) Dke1 at pD 9.5.

Figure S2.6 Autooxidation of the mutant E98Q. A preparation of E98Q (400 μL of 4.3 mM Dke1 subunits, 60% Fe$^{II}$ occupation, 4 °C) was added to 1.6 mL of 20 mM Tris buffer (pH 7.5), into a stirred, sealed vial which was situated in a thermostat at 25 °C. Residual air was displaced by the enzyme preparation. Stirring was switched off and O$_2$ consumption rates were recorded via a Microtox-O$_2$-sensor. The initial phase of the resulting, biphasic curve gave a second-order rate constant of $k_{Fe} = 0.016 \pm 0.002$ mM$^{-1}$ s$^{-1}$ (inset), when fit to equation S1 (see Figure S2.7). The subsequent, slow phase was fit using a linear correlation, which gave a $k_{autox}^{app} = 1.2 \times 10^{-6}$ s$^{-1}$, when related to the concentration of Fe$^{II}$ ligated active sites.
Figure S2.7 Determination of the rate constant for oxidation of free Fe$^{II}$ in 20 mM Tris buffer (pH 7.5). In literature, autooxidation in aqueous media has been described as a process that is first-order in respect to both O$_2$ and Fe$^{II}$ concentration, whereby the rate constant strongly depends on the respective buffer and pH (E. J. Roeckens, R. E. Van Grieken, *Marine Chemistry* (1983) 13, 195-202). Autooxidation rates for free iron were determined in the range 15 μM – 60 μM using the same conditions as for determination of autooxidation rates of WT Dke1 and the mutant E98Q. O$_2$ consumption was found to be equimolar with Fe$^{II}$ oxidation. Resulting traces were fit to equation S1: $\frac{d[Fe^{II}]}{dt} = \frac{d[O_2]}{dt} = -k_{Fe}[O_2][Fe^{II}]$ (Software: Berkeley Madonna). A $k_{Fe}$ value of 0.015 ± 0.002 mM$^{-1}$ s$^{-1}$ was obtained, which was constant throughout the range investigated.
Chapter 3

The 3His Triad in Dke1: Comparisons to the Classical Facial Triad

*Reproduced with permission from Biochemistry, 2010, 49, 6945-6952. Copyright © 2010 American Chemical Society.
3.1 Introduction

Mononuclear nonheme iron enzymes (MNHFe’s) are an important class that are involved with a wide range of medical, pharmaceutical, and environmental applications. They catalyze a diverse range of chemical reactions, most commonly hydroxylation, but also oxidative ring closure, desaturation, carbon-carbon bond and aromatic ring cleavage, hydrogen atom abstraction and halogenation.\(^{(1, 3)}\) The oxygen activating enzymes – that include the extradiol dioxygenases, pterin-dependent dioxygenases, Rieske dioxygenases and \(\alpha\)-keto acid dependent dioxygenases – use Fe\(^{II}\) to activate O\(_2\) for attack on the substrate.\(^{(1)}\) They usually share a common structural motif for Fe\(^{II}\) coordination: a protein-derived facial triad composed of two histidine residues and one carboxylate moiety (hereafter referred to as the facial triad).\(^{(4, 5)}\) Additionally, along with the substrate, most of these enzymes require a cofactor to deliver the reducing equivalents necessary for reactivity.

Recently, a few exceptions to this facial triad motif have been observed. In the halogenases, the carboxylate is absent and a halogen ion takes its place in the coordination sphere. Diketone-cleaving dioxygenase, Dke1,\(^{(6)}\) and cysteine dioxygenase, CDO,\(^{(7-9)}\) contain a 3 histidine triad, while carotenoid oxygenase contains a 4 histidine motif. These His-only active site enzymes act directly on their substrates, without the need for a cofactor. Recent work on the NO complex of cyst-bound CDO \(^{(10)}\) found an S=1/2 site while the facial triad enzyme ES-NO complexes generally have high spin, S=3/2 ground states.\(^{(11-13)}\) Thus there can be a ligand field (LF) difference between 3His and facial triad sites and, of course, there are differences between carboxylate and histidine as a ligand. The diketone-cleaving dioxygenase Dke1 from \emph{A. johnsonii} cleaves acetylacetone (acac) with incorporation of molecular oxygen to yield methyl glyoxal and acetate (Scheme 1).\(^{(6)}\) Dke1 will turnover a variety of diketone substrates and their reaction rates correlate with the energy of the HOMO. The substrate binds directly to the Fe\(^{II}\) site in Dke1 which accelerates the rate of reaction with O\(_2\) by \(\sim 10^6\) fold. These results indicate the importance of the presence of the Fe\(^{II}\)–\(\beta\)-diketone complex in O\(_2\) reactivity.\(^{(14, 15)}\)
Scheme 3.1 Breakdown of acetylacetone via Dke1

This study elucidates the geometric and electronic structure of the 3 His triad in Dke1 and the effects of the acac ligand in binding to the Fe$^{II}$ site. Additionally, the differences between the canonical facial triad motif and the 3 His triad are evaluated with comparisons to two well studied facial triad enzymes, clavaminate synthase (CS2)\(^{(16)}\) and hydroxyphenylpyruvate dioxygenase (HPPD)\(^{(17, 18)}\). In particular, HPPD was chosen for comparison as previous study has shown that it binds β-diketones.\(^{(19)}\)

A combination of spectroscopic techniques and density functional theory (DFT) calculations are used to quantitatively evaluate differences between the 3 His triad and the facial triad. Near IR (NIR) circular dichroism (CD), magnetic circular dichroism (MCD) and variable-temperature, variable field (VTVH) MCD probe the coordination environment and LF at the Fe$^{II}$ site as explained in the first section of the results. UV-vis absorption, CD and MCD probe the metal-to-ligand charge transfer (MLCT) transition (of Fe$^{II}$ to acac) and give insight into the Fe$^{II}$-acac bond. These studies provide an understanding of the geometric and electronic structure of the 3 His triad in Dke1 and the binding of acac to this Fe$^{II}$ site. They also give insight into the utility of the commonly observed facial triad motif in reactivity.

3.2 Materials and Methods

3.2.1 Sample Preparation

Dke1 and HPPD were purified according to previously published procedures.\(^{(6, 20, 21)}\) Apo-dke1 and apo-HPPD were exchanged into deuterated buffer (100 mM Tris-HCl or 100 mM HEPES, respectively) at a pD = 7.0 using an Ultrafree-4 filter with a 10kDa cutoff membrane (Millipore) to a concentration of 2-4 mM. All reagents were used as received without further purification. Buffer,
acetylacetone, ferrous ammonium sulfate, glycerol-d₃ and sucrose were made anaerobic by purging with Ar on a schlenk line. Dke1 and HPPD were made anaerobic by alternating cycles of vacuum and purging with Ar at 273K. All samples were prepared in an inert atmosphere, N₂ purged “wet box” to maintain an O₂ free environment. Ferrous ammonium sulfate was dissolved in buffer and added to Dke1 or HPPD in microliter quantities to a concentration of 90% of the enzyme (monomer) concentration to avoid free iron in the sample. Acetylacetone was added in microliter quantities to a concentration of 10-25 fold excess over the protein concentration. Glycerol-d₃ (~60% v/v) or sucrose (175% w/v) was added to the sample as a glassing agent for MCD measurements, giving a final sample concentration of 1-2 mM. Sucrose was partially deuterated by dissolving in D₂O (1:10 w/v) and incubated overnight allowing for proton/deuteron exchange, then lyophilized to remove excess water. CD measurements were taken without and with the presence of the glassing agent to determine if the glassing agent affects the iron site. For Dke1, a small change in the NIR CD signal was observed with glycerol but not with sucrose. However MCD measurements on Dke1 with both glycerol and sucrose were identical, indicating that this conformational change does not reflect a change at the iron site. Furthermore, increasing the concentration of acetylacetone in the sample prevented this change in CD without affecting the MCD data. Data presented for Dke1 are with glycerol as the glassing agent because the resulting data have a better signal to noise ratio than the partially deuterated sucrose. HPPD was not affected by glycerol and all HPPD MCD samples were made with glycerol-d₃.

3.2.2 Spectroscopic studies

NIR (600-2000 nm) CD and MCD spectra were taken on either a Jasco J-200D or Jasco J-730 spectropolarimeter with a liquid N₂ cooled InSb detector (Teledyne Judson Technologies) and an Oxford Instruments SM-4000-7T superconducting magnet. UV-vis (300-900 nm) CD and MCD spectra were taken on a Jasco J-810D spectropolarimeter equipped with an extended S20 photomultiplier tube and a SM-4000-7T superconducting magnet. UV-vis Abs spectra were taken on an Agilent 8453 diode array spectrometer. CD and Abs spectra were taken at 278K in an anaerobic
cuvette and were corrected for buffer and protein baseline effects by subtraction. Natural CD features and baseline effects were excluded from the MCD spectra by taking the average of the magnitudes of the positive and negative field data. For VTVH MCD, a calibrated Cernox resistor (Lakeshore Cryogenics, calibrated 1.5 – 300 K), inserted into the sample cell, was used for accurate temperature measurement. The VTVH MCD data obtained were normalized to the intensity maximum, and ground state parameters were obtained using previously published procedures.(22, 23)

3.2.3 Computational methods

The Dke1 active site was modeled using the crystal structure (PDB ID: 3bal) as a starting point. The δ-coordination of one of the histidine residues observed in the crystal structure was preserved. The HPPD active site was modeled using the HPPD-NTBC crystal structure as a starting point (PDB ID: 1t47). Histidine residues were truncated to methyl imidazole and aspartate was truncated to propionate for the models. Constraints imposed by the protein backbone were simulated in both cases by fixing the relative positions of the β-carbons of the backbone. The coordination at the active site was completed with either coordinated water or a monoanionic, bidentately coordinated acetylacetone ligand.

Density functional theory (DFT) calculations were performed using the Gaussian 03 program(24, 25) with the spin unrestricted functional BP86(26) with 10% Hartree-Fock Exchange and under tight convergence criteria. The triple-ζ basis set, 6-311G*, was used to describe the Fe and the conjugated O-C-C-C-O moiety of acetylacetone; the double-ζ basis set, 6-31G*, was used to describe all other atoms. The structures were optimized and found to be stable with no imaginary frequencies > 30 cm⁻¹ (which are associated with the constraints placed on the β-carbons). Effects of the protein environment were included by applying the polarized continuum model (PCM)(27) with a dielectric constant, ε = 4.0, to calculate solvated energies for the complexes. These solvated single point calculations were done with the 6-311+G(2d,p) basis set. The energies given include thermal and Zero-point corrections. Orbital compositions were determined with QMForge(28), and molecular orbitals were visualized with Molden version 4.1(29). Time-dependent DFT (TD-DFT)
calculations were performed to compare to the Fe$^{II}$-acac CT spectra and SWizard(30, 31) was used to parse the results.

3.3 Results

NIR CD and MCD spectroscopy probe the energy splitting of the five d orbitals by the ligand environment. In a symmetric six-coordinate (6C), site the d orbitals split into a ground t$_{2g}$ set and excited e$_{g}$ set separated by $\sim 10,000$ cm$^{-1}$ for non-heme ligands. The e$_{g}$ set is further split in energy by the low symmetry protein environment. 6C Fe$^{II}$ sites exhibit two transitions in the 10,000 cm$^{-1}$ region split by $\sim 2,000$ cm$^{-1}$. For a five-coordinate site (5C), the splitting is larger leading to transitions in the $\sim 10,000$ cm$^{-1}$ and 5,000 cm$^{-1}$ region. For four-coordinate (4C) sites, the ligand field (LF) is weak leading to transitions in the 5,000 – 7,000 cm$^{-1}$ region. For the high spin d$^{6}$ center in $O_h$ symmetry, the extra electron in the t$_{2g}$ orbitals gives a $^{5}$T$_{2g}$ ground state. This is also energy split due to the low symmetry of the protein environment leading to a non-Kramers doublet ground state defined by its zero field splitting (ZFS) $\delta$ and $g_{||}$ values. These can be obtained from VTVH MCD data and related to the splitting of the t$_{2g}$ set of d orbitals ($\Delta$, the axial splitting, and V, the rhombic splitting) as described in Ref 23.

3.3.1 Resting Dke1

The 278 K CD spectrum of apoDke1 is featureless. Upon addition of 0.9 equivalents of Fe$^{II}$, two transitions are observed in the CD spectrum, a positive feature at 8,000 cm$^{-1}$ and a negative feature at 10,500 cm$^{-1}$. When cooled to 5K, these features sharpen and shift up in energy to $\sim 9,000$ cm$^{-1}$ and $\sim 11,000$ cm$^{-1}$ (Figure 3.1A). The 7T, 5K MCD spectrum shows two transitions (Figure 3.1B, green), centered at 10,100 cm$^{-1}$, split by 1600 cm$^{-1}$. These can be resolved into transitions at 9,100 cm$^{-1}$ and 10,900 cm$^{-1}$ consistent with the low temperature CD and indicative of a distorted 6C iron center. To characterize the ground state of this 6C site, VTVH MCD data were taken at 8850 cm$^{-1}$ (arrow in Figure 3.1B and plotted in Figure 3.1C). The data fit to a negative zero field splitting with $\delta = 4.6 \pm 0.2$ cm$^{-1}$ and $g_{||} = 9.1 \pm 0.3$ which leads to $\Delta = -300 \pm 100$ cm$^{-1}$ and $|V| = 140 \pm 40$ cm$^{-1}$. 
A comparison of the MCD spectrum of Dke1 to the MCD spectrum of a ‘typical’ facial triad enzyme, clavaminate synthase (CS2)(16), shows no significant spectral difference (Figure 3.1B, red). The ground state of Fe-loaded Dke1 also does not show significant differences from the ground state of CS2 ($\delta = 4.5 \pm 0.15$ cm$^{-1}$ and $g_\parallel = 9.2 \pm 0.1$ giving $\Delta = -400 \pm 100$ cm$^{-1}$ and $|V| = 190 \pm 50$ cm$^{-1}$). These similarities of the resting sites are shown quantitatively in the experimentally-derived ligand field energy level diagram given in Scheme 2 (left).
Scheme 3.2 Experimentally derived Ligand Field Diagrams for resting Dke1 and CS2 (left) and acac-bound Dke1 and HPPD (right). For resting Dke1 and CS2, the $^5E_g$ and $^5T_{2g}$ states and splittings are marked in blue. For acac-bound Dke1 and HPPD, the $^5E_g$ and $^5T_{2g}$ states and splittings for the 6C site are marked in green. The $^5E_g$ splitting for the 5C sites is indicated in orange. (The $^5T_{2g}$ splittings for 5C acac-bound Dke1 and HPPD are not shown because the $^5T_{2g}$ splittings for Dke1-acac (5C) could not be obtained as described in the text.)

3.3.2 Acac-bound Dke1 and HPPD

3.3.2.1 NIR studies of acac-bound Dke1 and HPPD

Addition of acetylacetone (acac) to Fe$^{II}$-Dke1 leads to a large change in the NIR CD and MCD spectra, most notably, the presence of a LF transition at low energy. The 278K NIR CD spectrum of acac-bound Dke1 (Figure 3.2A) shows transitions at $< 6,000 \text{ cm}^{-1}$ and $13,000 \text{ cm}^{-1}$ with the $13,000 \text{ cm}^{-1}$ peak exhibiting a tail to lower energy. In the corresponding MCD spectrum (Figure 3.2B), three transitions are clearly observed: a low energy transition at $< 6,000 \text{ cm}^{-1}$ and two transitions at $9,300 \text{ cm}^{-1}$ and $11,000 \text{ cm}^{-1}$ with additional asymmetry at the high energy side of the $11,000 \text{ cm}^{-1}$ peak. From this asymmetry and the presence of a strong peak in the CD spectrum on the higher energy side of this band, it can be inferred that there are three transitions in this $8,000 – 13,000 \text{ cm}^{-1}$ energy region. As a single Fe$^{II}$ center can have
no more than two transitions in the NIR region, the presence of a total of four transitions indicates that more than one species is present. From the low energy band and three transitions in the higher energy region, a 5C and a 6C site are present. The 6C component has two transitions in the 10,000 cm\(^{-1}\) region, and the 5C component has one transition in the \(~10,000\) cm\(^{-1}\) region and the one transition at \(< 6,000\) cm\(^{-1}\). VTVH MCD data taken at 9025 cm\(^{-1}\) (arrow in Figure 3.2B and plotted in Figure 3.2C) fit to a negative zero field split system with ground state splitting parameters of \(\delta = 2.6 \pm 0.1\) cm\(^{-1}\) and \(g_{\parallel} = 9.0 \pm 0.1\) giving \(\Delta = -850 \pm 100\) cm\(^{-1}\) and \(|V| = 400 \pm 58\) cm\(^{-1}\), consistent with a 6C site but with a weak axial water ligand.\(^1\) VTVH MCD data taken at 11,100 cm\(^{-1}\) fit to the same parameters as obtained for the 9025 cm\(^{-1}\) transition (Supporting Figure S3.1) indicating that these are the two LF transitions of the 6C component. The difference in the ground state splitting parameters between resting Dke1 and the 6C component of Dke1-acac indicates that this 6C component results from having acac-bound and does not reflect unligated resting enzyme. Furthermore, assignment of the two middle transitions to a 6C site allows assignment of the transitions at \(< 6,000\) cm\(^{-1}\) and \(~ 13,000\) cm\(^{-1}\) to a 5C square pyramidal site. Ground state splitting parameters could not be obtained from VTVH MCD data for the 5C species due to noise in the low energy region and overlap with the 6C site for the higher energy transition.

\(^1\) The observed splitting of the \(^5\)E excited state indicates that the site is 6C. The ground state, however, shows a fairly large axial splitting indicative of an elongation of the axial Fe\(^{II}\)-H\(_2\)O bond.\(^1\)
To evaluate the effect of the 3His triad relative to the facial triad, spectroscopic studies on an acac-bound facial triad Fe\textsuperscript{II} enzyme were also performed. Hydroxyphenylpyruvate dioxygenase (HPPD) was chosen for these studies because the diketone herbicide NTBC, an inhibitor of HPPD, has been shown to bind to its Fe\textsuperscript{II} center.\(^{32, 33}\) The 278K CD spectrum of acac-bound HPPD (Figure 3.2D) is different from the spectrum of the resting enzyme (Supporting Figure S3.2)\(^{18}\) showing one predominant, asymmetric transition at \(~ 12,000 \text{ cm}^{-1}\). The 5K, 7T MCD spectrum
shows three transitions (Figure 3.2E): two positive features, at < 5,000 cm\(^{-1}\) and ~ 9,000 cm\(^{-1}\), and a negative feature at ~ 12,000 cm\(^{-1}\). Note that the positive intensity to higher energy is an intense CT transition in the MCD spectrum further described in the next section. Again, the presence of more than two transitions indicates that a mixture of two different Fe\(^{II}\) sites is present with the low energy positive-high energy negative bands characteristic of a 5C square pyramidal site.\(^2\) The middle transition is assigned to a 6C, distorted octahedral component where the second transition is likely masked by the large negative feature from the 5C site. VTVH MCD data for the 6C site taken at ~ 8,600 cm\(^{-1}\) (arrow in Figure 3.2E and plotted in Figure 3.2F) are best fit to a positive zero field split system (\(D = 14 \pm 0.5\) cm\(^{-1}\) and \(|E| = 2.7 \pm 0.1\) cm\(^{-1}\) giving \(\Delta = 300 \pm 100\) cm\(^{-1}\) and \(|V| = 192 \pm 64\) cm\(^{-1}\)). These splittings are different from those obtained for the 6C component of resting HPPD\(^{18}\) indicating that the 6C component of acac-bound HPPD is an acac-bound species and not unreacted resting. VTVH MCD saturation magnetization curves could not be obtained for the low energy band, however, data taken on the negative band at ~ 12,300 cm\(^{-1}\) (Supporting Figure S3.1) are best fit to a negative zero field split system characteristic of a 5C site (\(\delta = 2.3 \pm 0.2\) cm\(^{-1}\) and \(g_\parallel = 8.9 \pm 0.1\) giving \(\Delta = -1000 \pm 100\) cm\(^{-1}\) and \(|V| = 540 \pm 60\) cm\(^{-1}\)). The experimental ligand field energy splittings obtained from the above analysis for both Dke1-acac and HPPD-acac are shown in Scheme 2 (right side).

3.3.2.2 UV-vis spectra of acac-bound Dke1 and HPPD

The acac-Fe\(^{II}\) complex of Dke1 has a MLCT transition in the UV-vis region. The UV-vis absorption, CD and MCD spectra (Figure 3.3) of Dke1-acac and HPPD-acac were obtained to probe this MLCT transition for both classes of NH Fe enzymes with the same substrate. A single, broad transition assigned to this MLCT is observed at ~ 24,000 cm\(^{-1}\) in all three spectra of Dke1-acac. In HPPD-acac, this transition is shifted to lower energy by ~ 1,000 cm\(^{-1}\) and is less intense than the transition in Dke1-

\(^2\) Although most of the ferrous proteins that have been studied only show positively signed d-d transitions in MCD (due to ground state spin orbit coupling, see ref 22), in a few cases a negative higher energy transition is observed. The negative transition arises from spin orbit coupling between the components of the split E excited state due to a distortion of the LF which results in \(d_e/d_o\) mixing and occurs in distorted 5C, square pyramidal sites.\(^2\)
acac. A second transition is observed at higher energy (~ 28,000 cm⁻¹) for the acac complexes of both enzymes which is assigned as an intra-ligand transition (*vide infra*).

**Figure 3.3** UV-vis absorption/CD/MCD spectra of acac-bound complexes. (A) room temperature absorption spectra, (B) room temperature CD spectra, (C) 7T, 5K MCD spectra of Fe^{II}-Dke1-acac (maroon) and Fe^{II}-HPPD-acac (teal, x2).

### 3.3.3 Nature of the acac-Fe^{II} bond

The HOMO of the acac ligand is an out of plane π orbital with maximum density at the central carbon. The LUMO is an out of plane π*orbital with significant carbonyl character (Supporting Figure S3.3). These orbitals are positioned for good overlap with the Fe^{II} center similar to the bonding defined for the α-keto acid moiety in ref 33.
DFT studies of the 3His-acac (Dke1 model) and facial triad-acac (HPPD model) complexes were performed to aid in the assignment of the transitions and the description of bonding. Molecular orbital energy level diagrams (Figure 3.4, spin unrestricted, $\alpha$ orbitals on left, $\beta$ orbitals on left) of the 3His- and facial triad-acac complexes show that the acac HOMO and LUMO orbitals are close in energy to the Fe $d$ orbitals.

Figure 3.4 Molecular orbital energy level diagrams for 5C 3His-acac and facial triad-acac.

![Molecular orbital energy level diagrams for 5C 3His-acac and facial triad-acac.](image)

Figure 3.5 Molecular orbital contours for the donor and acceptor orbitals for MCLT.

![Molecular orbital contours for the donor and acceptor orbitals for MCLT.](image)
The contours in Figure 3.5 show that these are oriented to interact with the Fe$^{II}$ d orbitals. The amount of acceptor $\pi^*$ LUMO character in the donor $d_{xz}$ orbital reflects the amount of Fe$^{II}$ backbonding into the acac ligand, and this backbonding determines the strength of the MCLT transition. From the calculations, the amount of $\pi^*$ character in the donor $d_{xz}$ orbital is similar for both the 3His- and facial triad-acac bound structures (≈ 5-6%); however, the experimentally observed MLCT transition of acac-bound Dke1 is more intense than that of acac-bound HPPD (Figure 3.3). This intensity difference suggests that there is more Fe$^{II}$ backbonding with the 3His triad and that the calculations slightly underestimate the overlap in the 3His compared to the facial triad-acac complex.

Time-dependent DFT (TD-DFT) calculations were performed to correlate to the MLCT transition in the UV-vis absorption spectrum. As both the 5C and 6C sites will contribute to the MLCT transition, spectra were calculated for both coordination numbers in both the 3His- and facial triad-acac models. (All four predicted spectra and the Gaussian resolution of the major bands are in the Supporting Information, Figure S3.4.) The TD-DFT calculations predict the observed $d\pi \rightarrow acac \pi^*$ MLCT transition and an intraligand ($\pi$ to $\pi^*$, acac HOMO $\rightarrow$ LUMO) transition for the acac-bound models. Additionally, the TD-DFT calculations predict the energy shift observed experimentally with the MLCT transition for the 3His(Dke1)-acac complex higher in energy than this transition in the facial triad(HPPD)-acac complex by ~3,000 cm$^{-1}$.

### 3.4 Discussion

#### 3.4.1 The 3His Ligand Field

As imidazole is a stronger field ligand than carboxylate, a change in the Fe$^{II}$ coordinating ligand set from a carboxylate oxygen to histidine nitrogen could suggest that the ligand field would change as well. Additionally, the low spin, $S=1/2$, state that is observed in the cysteine-bound CDO-NO(10) complex potentially also suggests that the ligand field of the 3His may be higher than in the facial triad. However, this in fact is not observed for Dke1 as the experimentally derived ligand field splitting of the $d$ orbitals in Scheme 2 is very similar to that of the ‘typical’ 6C facial triad/H$_2$O
coordination observed experimentally in CS2. This reflects that only 1 out of 6 ligand positions is perturbed. Furthermore, DFT calculations with a facial triad-NO complex with cysteine bound in the orientation proposed previously for CDO (coordination through the thiolate and the amine) indicate that the facial triad-cysteine-NO complex would also be low spin. This supports the previous assertion that it is the cysteine ligand that drives the CDO-NO complex to a low spin state.\(^{(10)}\) Thus it is unlikely that the ligand field of the 3His triad would significantly affect the spin state energetics along the reaction coordinate relative to the facial triad.\(^3\)

\[\text{3.4.2 The Acac-Fe}^{II} \text{Bond}\]

Many of the resting facial triad sites are 6C. Comparing a 3 His triad and a facial triad, a 3 His triad is a poorer donor. Thus it is not surprising that the resting site of Dke1 is also found to be 6C (Figure 3.1). When acac binds, the affinity for water is decreased due to the strong \(\pi\) donation from the acac ligand. Indeed, from the calculated structures, the energy required to remove a H\(_2\)O ligand (water affinity) for acac-bound Dke1 is lower than for water bound to resting Dke1 by \(-2.8\)kcal/mol (see Supporting Information, Figure S3.5A-B). So, a 6C to 5C conversion occurs in Dke1 upon acac substrate binding and opens up a coordination position for reaction with O\(_2\).

The parallel comparison can be made for the facial triad and \(\alpha\)KG. Acac is a similar anionic \(\pi\) donor to \(\alpha\)KG, and thus it might be expected that the water affinity in an \(\alpha\)KG-bound facial triad would be low. Indeed, calculations predict loss of H\(_2\)O and the formation of a 5C site with \(\alpha\)KG bound (\(\Delta G = -13\) kcal/mol, see Supporting Information, Figure S3.5C). However, from previous spectroscopic and crystallographic studies on \(\alpha\)KG dependent enzymes, a 6C site is commonly found for the \(\alpha\)KG-bound site. However, in these enzymes, the coordinated water hydrogen bonds to the non-coordinated oxygen of the carboxylate. The calculated water affinity for a hydrogen bonded structure increases 8-9 kcal/mol (\(\Delta G\); Figure S3.5D), in agreement with previously calculated values.\(^{(34)}\) This is important for enzymes such as CS2 where uncoupled reactivity would occur if a 5C site is formed with \(\alpha\)KG bound before substrate is present. Addition of substrate provides the steric to

\[\text{3 The effect of } \delta \text{ versus } \varepsilon \text{ coordination by the His ligand was also examined. No significant geometric, electronic or energetic effects resulted from this change.}\]
eliminate the coordinated water and allow the reaction with O₂ in these substrate plus cofactor dependent enzymes. It should be noted that HPPD is a special case because in HPP the α-keto acid is covalently tethered to the substrate and therefore it binds as both the cofactor and substrate. Interestingly, the resting site of HPPD is already partially 5C and it was suggested that the carboxylate of the facial triad in HPPD is poorly oriented for hydrogen bonding with the coordinated water due to interactions from a conserved phenylalanine residue. Thus in HPPD, the site is prearranged to disrupt hydrogen bonding between the facial triad carboxylate and coordinated water allowing HPP and, in the present study, acac to drive the site to go 5C.

3.4.3 Charge

The dominant difference in the spectra of the 3His relative to the facial triad is in the energy of the MLCT transition, which reflects the charge difference of these two sites. The higher positive charge at the 3His center decreases the energy of the d manifold resulting in the shift to higher energy of its Fe⁺⁺-to-acac MLCT transition (Figure 3.3). Consistent with a higher positive charge on the Fe⁺⁺ of the 3 His triad, studies on the effect of pH on Drk1 showed a depressed pKₐ for bound water (~8.2) relative to the facial triad which does not show deprotonation in the accessible pH range.

Mechanistic implications can be posited from this difference in charge. First, the differences in charge between the 3His and facial triads could affect the nature of substrate binding in particular for α-keto acid where both mono and dianions are accessible. The facial triad found in the α-keto acid enzymes binds this cofactor as the monoanion. Studies on model complexes by Que and coworkers (36) suggest that a dianionic, enolized α-keto acid would react with O₂ to give different cleavage products than the keto form. The proposed mechanism for this reaction would not generate an Fe⁴⁺=O. However this intermediate is observed and is required in the α-keto acid dependent non-heme Fe enzyme reactivity. The lower positive charge at the active site with a facial triad likely helps stabilize the keto form of this cofactor leading to the required Fe⁴⁺=O intermediate and subsequent H atom abstraction reactivity.
In summary, the facial triad, as a ligand set for Fe\textsuperscript{II}, is conserved throughout most of the oxygen activating class of mononuclear non-heme iron enzymes. The 3His triad in Dke1 presents an opportunity to study, not only the role of the Fe\textsuperscript{II}-3His active site in the cleavage of β-diketones, but also to make comparisons to the facial triad ligand set in a functional environment. It has a similar ligand field to the facial triad. While most of the facial triad enzymes require both cofactor and substrate to bind for 6C to 5C conversion, acac binding to the 3His site alone eliminates a coordinated water generating a 5C site and opens a position for O\textsubscript{2} reactivity. Finally, the difference in charge between the 3His and facial triad ligand sets, which leads to the major spectral difference observed, could play a key mechanistic role in promoting the proper form of αKG binding and thus O\textsubscript{2} reactivity in the facial triad. Future studies will explore the effects of this difference in charge on reactivity.

3.5 Acknowledgements
This research was supported by NIH Grant GM40392 (EIS) and FWF Grant P18828 (GDS).

3.6 References


3.7 Supporting Information

References Full Reference #24

![Figure S1.1 VTVH MCD isotherms for (A) Dke1-acac at 11,100 cm⁻¹ and (B) HPPD-acac at 12,300 cm⁻¹](image)

Figure S1.1 VTVH MCD isotherms for (A) Dke1-acac at 11,100 cm⁻¹ and (B) HPPD-acac at 12,300 cm⁻¹
Figure S3.2 CD/MCD of Resting Fe$^{II}$-HPPD. (A) 278K CD spectrum; (B) the 7T, 1.8K MCD spectrum of Resting Fe$^{II}$ HPPD

Figure S3.3 HOMO and LUMO of free acac ligand
Figure S3.4 TD-DFT predicted spectra for the acac-bound complexes. (A) 5C 3His-acac; (B) 6C 3His-acac-H2O; (C) 5C facial triad-acac; (D) 6C facial triad-acac-H2O. The unlabeled peaks in all 4 spectra correspond to computational artifacts predicting metal to histidine ligand transitions which are expected to occur at higher energy. $\pi$ to $\pi^*$ transitions for spectra A and D are calculated to occur outside of the energy window presented.
Figure S3.5 Structures used to calculate water affinity. (A) Resting 3His; (B) acac-bound 3His; (C) pyruvate-bound facial triad (no H bond); (D) pyruvate-bound facial triad (with H bond). $\Delta E_{\text{solv}}$ was calculated by subtracting the energy of the 6C site from the sum of the energies of the 5C site plus H$_2$O. In order to prevent an H bonding interaction between acac and water in (B), an additional constraint was added to the system. This constraint is reflected in the thermal corrections; therefore, solvated electronic energies are reported for comparison. The facial triad structures (C) and (D) did not have any hydrogen bonding interactions allowing for $\Delta H$ and $\Delta G$ energies to be calculated and so they are reported as well. (The $\Delta H$’s are within $\sim$ 1 kcal/mol of $\Delta E_{\text{solv}}$ and the entropy contributes $\sim$ 13 kcal/mol to the free energy.)
Chapter 4

Spectroscopic and Computational Studies of $\alpha$-keto Acid Binding to Dke1: Monoanion Versus Dianion Reactivity
4.1 Introduction

The mononuclear non-heme iron enzymes are an important class with a diverse range of medical and pharmaceutical applications. This class is divided into oxygen and substrate activating enzymes depending on the route taken to overcome the spin forbidden reaction of organic substrates with O\textsubscript{2}. One of the major categories of O\textsubscript{2} activating enzymes are the α-keto acid dependent dioxygenases (1, 2). These enzymes use an α-keto acid cofactor to provide electrons for the reduction of dioxygen. Reaction with dioxygen leads to decarboxylation of the α-keto acid and formation of a reactive Fe\textsuperscript{IV}=O species which is competent for a number of different reactions including hydroxylation, H-atom abstraction, desaturation, and halogenation (1-3). Among the O\textsubscript{2} activating enzymes, a conserved “facial triad” motif of two histidine residues and one carboxylate (Asp or Glu) tethers Fe\textsuperscript{II} to the protein along one face of an octahedron. This facial triad (as it will be referred to in the remainder of this work) is generally found to be functionally important for the oxygen activating subclass (4).

Recently, a number of enzymes with perturbations to the facial triad have been defined. The α-keto acid dependent halogenases CytC3 and SyrB2 lack the carboxylate of the facial triad, and a halogen anion is coordinated in its place (5, 6). In cysteine dioxygenase and the β-diketone cleaving dioxygenase Dke1, the carboxylate is replaced by a histidine residue to form a 3His triad (7, 8).

In the native enzyme reaction, Dke1, from Acinetobacter johnsonii, cleaves acetylacetone (acac) with incorporation of both atoms of dioxygen to form methyl glyoxyl and acetate (Scheme 4.1) (8).

![Scheme 4.1 Native reaction catalyzed by Dke1](image)

Previous work on Dke1 showed that the ligand field at the resting ferrous site was not significantly affected by the change from a facial triad to a 3His triad (9, 10). Two key experimental results, however, suggested that an important difference between the
facial and 3His triads is the net difference in charge at the sites. (The Fe$^{II}$-facial triad has a +1 charge while the Fe$^{II}$-3His triad has a +2 charge.) First, the resting ferrous site of Dke1 was found to have a depressed pKa for water bound at the active site (9). Additionally, the major spectroscopic difference between the acac-bound ferrous complexes of Dke1 and 4-hydroxyphenylpyruvate dioxygenase (a facial triad containing enzyme) was an energy shift in the metal to ligand charge transfer transitions (10). Both were ascribed to the different charges of the complexes. Recently, an atypical cleavage of the α-keto acid phenylpyruvate was reported to be catalyzed by Dke1. Product analysis revealed the benzaldehyde product instead of the expected mandelate product (Scheme 4.2) (11). This new C2-C3 cleavage is unprecedented for α-keto acid reactions with mononuclear non-heme iron enzymes.

Scheme 4.2 Dioxygen-dependent cleavage of PP catalyzed by Dke1 or the facial triad

In this study, we use a combination of spectroscopies to define the bonding of an α-keto acid, HPP, to ferrous Dke1. HPP was chosen for study because it has the α-keto acid moiety and the substrate fused, more closely mimicking the substrate-only reactivity of the native Dke1 reaction (Scheme 4.1). The geometric and electronic structure determined for this site is then used to evaluate the atypical reaction coordinate of the 3His relative to the facial triad.

4.2 Materials and Methods

4.2.1 Sample preparation for spectroscopy

Dke1 was expressed and purified as described previously (8, 12). Apo-Dke1 was exchanged into deuterated buffer (100mM Tris-HCl) at pH 7.0 using an Ultrafree-
4 filter with a 10kDa cutoff membrane (Millipore) to a concentration of 2-4 mM. Sucrose was partially deuterated by dissolving in D₂O (1:10 w/v) and incubated overnight allowing for proton/deuteron exchange, then lyophilized to remove excess water. All other reagents were used as received without further purification. Buffer, 4-hydroxyphenylpyruvate (HPP), ferrous ammonium sulfate, and sucrose were made anaerobic by purging with Ar on a Schlenk line. Dke1 was made anaerobic by alternating cycles of vacuum and purging with Ar at 273K. All samples were prepared in an inert atmosphere, N₂-purged “wet box” to maintain an O₂ free environment. Ferrous ammonium sulfate was dissolved in buffer and added to Dke1 in microliter quantities to a concentration of 90% of the enzyme (monomer) concentration to avoid free iron in the sample. In the “wet box”, HPP was partially dissolved in buffer and then 10M sodium hydroxide was titrated in dropwise until all solid was dissolved. HPP was added in microliter quantities to a concentration of 5-10 times the concentration of Fe. Sucrose (175% w/v) was added to the sample as a glassing agent for MCD measurements, giving a final sample concentration of 1-2 mM.

4.2.2 Spectroscopic Methods

NIR (600-2000 nm) MCD spectra were recorded on a Jasco J730 spectropolarimeter with a liquid N₂-cooled InSb detector (Teledyne Judson Technologies) and an Oxford Instruments SM-4000-7T superconducting magnet. UV-vis (300-900 nm) MCD spectra were recorded on a Jasco J810D spectropolarimeter equipped with an extended S20 photomultiplier tube and a SM-4000-7T superconducting magnet. UV-vis absorption spectra were recorded on an Agilent 8453 diode array spectrometer. Absorption spectra were taken at 278 K in an anaerobic cuvette and were corrected for buffer and protein baseline effects. Natural CD features and baseline effects were eliminated from the MCD spectra by taking the average of the magnitudes of the positive and negative field data. For VTVH MCD, a calibrated Cernox resistor (Lakeshore Cryogenics, calibrated 1.5 – 300 K), inserted into the sample cell, was used for accurate temperature measurement. The VTVH MCD data obtained were normalized to the intensity maximum, and ground state parameters were obtained using previously published procedures (13, 14).
Samples for resonance Raman (rR) were prepared anaerobically as described above and run in an anaerobic NMR tube spinning at 298K. To obtain the O$_2$ exposed spectra, the NMR tubes were exposed to air for 4hrs and the spectra were retaken. The spectra were recorded on an Andor Newton charge coupled device (CCD) detector cooled to -80°C with a triple monochromator (Spex 1877 CP with 1200, 1800, and 2400 grooves/mm holographic spectrograph gratings). Excitation at 363.8 nm was provided by an Ar$^+$ (Innova Sabre 25/7) ion laser with incident power in the 40-80 mW range using an ~ 135° backscattering configuration. Background spectra of charcoal in NMR tubes were used for baseline subtraction.

4.2.3 Computational Methods

The Dke1 active site was modeled using the crystal structure (PDB entry: 3bal) as a starting point. The $\delta$-coordination of one of the histidine residues observed in the crystal structure was preserved. Histidine residues were truncated to methyl imidazole for the model, and constraints imposed by the protein backbone were simulated by fixing the relative positions of the $\beta$-carbons of the backbone. The coordination of the active site was completed with either a keto (monoanion) or enolate (dianion) bidentate coordinated HPP ligand.

Density functional theory (DFT) calculations were performed using Gaussian 03 (15, 16) with the spin unrestricted functional BP86(17) with 10% Hartree-Fock exchange and under tight convergence criteria. For the ferrous ES complexes, the triple-$\zeta$ basis set 6-311G* was used to describe Fe and the OC-CO$_2$ moiety of HPP; the double-$\zeta$ basis set, 6-31G* was used to describe all other atoms. For the reactivity studies, the triple-$\zeta$ basis set was extended to include C3 and O$_2$. The stationary structures were optimized and found to be stable with no imaginary frequencies $>-25$ cm$^{-1}$ (which were associated with the constraints placed on the $\beta$-carbons). Transition state structures were determined using the QST2 transition search function in G03 and were found to have 1 imaginary frequency which correlated to the motion of the bond being broken or formed. Solvation effects were included by applying the polarized continuum model (PCM) (18) with a dielectric constant $\varepsilon = 4.0$. The energies given include thermal and Zero-point corrections. Structures were visualized with
GaussView 3.09 (19); orbital compositions were determined with QMForge (20); and molecular orbitals were visualized with Molden version 4.1 (21). Time-dependent DFT (TD-DFT) calculations were performed to compare to the experimental spectra and SWizard was used to parse the results (22, 23).

4.3 Results

4.3.1 Spectroscopic

NIR MCD spectroscopy of Fe$^{II}$ sites probes the energy splitting of the five d orbitals by the ligand environment. In a symmetric 6C, site the d orbitals split into a ground t$_{2g}$ set and excited e$_g$ set separated by $\sim$ 10,000 cm$^{-1}$ for non-heme ligands. The e$_g$ set is further split in energy by the low symmetry protein environment. 6C Fe$^{II}$ sites exhibit two transitions in the 10,000 cm$^{-1}$ region split by $\sim$ 2,000 cm$^{-1}$. For 5C, the splitting is larger leading to transitions in the $\sim$ 10,000 cm$^{-1}$ and 5,000 cm$^{-1}$ region. For 4C sites, the ligand field (LF) is weak (10Dq$_{Td}$ = -4/9 10Dq$_{Oh}$) leading to transitions in the 5,000 – 7,000 cm$^{-1}$ region. For the high spin d$^6$ center in O$_h$ symmetry, the extra electron in the t$_{2g}$ orbitals gives a $^5$T$_{2g}$ ground state. This is also energy split due to the low symmetry of the protein environment and combined with in state SOC leading to a non-Kramers doublet ground state defined by its zero field splitting (ZFS) $\delta$ and $g_{||}$ values (1). These can be obtained from VTVH MCD data and related to the splitting of the t$_{2g}$ set of d orbitals ($\Delta$, the axial splitting, and $V$, the rhombic splitting) as described in Ref 13.

4.3.1.1 NIR VTVH MCD of Dke1-HPP

The 5K, 7T MCD spectrum of resting (Fe$^{II}$-bound) Dke1 has been published previously (10). Addition of 10 equivalents of HPP to resting Dke1 results in the 5K, 7T MCD spectrum shown in Figure 4.1a (green). Two transitions are observed in this spectrum: a broad transition at $\sim$ 9500 cm$^{-1}$ and the low energy tail of a transition (at less than 5500 cm$^{-1}$). The VTVH MCD isotherms were taken at 9,400 cm$^{-1}$ (arrow in Figure 4.1, green) and are shown in the inset to Figure 4.1 with the best fit to the data.
Figure 4.1 VTVH MCD of Dke1-HPP. The 5K, 7T MCD spectrum of Dke1-HPP (green) and resting ferrous (blue). The arrows indicate the energy at which VTVH MCD isotherms were recorded. Inset – VTVH MCD isotherms taken at 9400 cm\(^{-1}\). The error bars are within the size of the point.

The Dke1-HPP VTVH MCD data fit to a negative zero field split system with ground state splitting parameters of \(\delta = 3.1 \pm 0.2\) cm\(^{-1}\) and \(g_{||} = 9.0 \pm 0.2\) giving \(\Delta = -600 \pm 150\) cm\(^{-1}\) and \(|V| = 260 \pm 60\) cm\(^{-1}\). In both the resting Dke1 and Dke1-HPP complexes, there are transitions in the 8500 – 12,000 cm\(^{-1}\) region (Figure 4.1, green and blue), however, the transition in Dke1-HPP is significantly more intense than the transitions of resting Dke1. This increase in intensity indicates that the transition observed for Dke1-HPP in the 8500 – 12,000 cm\(^{-1}\) region results from the binding of HPP to the Fe\(^{II}\) center and is not residual resting Dke1. Additionally, the ground state parameters from the fit to the VTVH MCD isotherms in Dke1-HPP differ from those of resting Dke1. (VTVH MCD isotherms were taken at 8850 cm\(^{-1}\) and fit to a negative zero field split system with ground state splitting parameters \(\Delta = -300 \pm 100\) cm\(^{-1}\) and \(|V| = 140 \pm 40\) cm\(^{-1}\).) From the energy of the transition and the fit to the VTVH MCD data for Dke1-HPP, the transition at \(~9500\) cm\(^{-1}\) reflects a 6C site with HPP bound. For the < 5500 cm\(^{-1}\) transition, there is no equivalent intensity for the resting Dke1 complex (Figure 4.1, blue in that same energy region). The presence of a low energy (< 5500 cm\(^{-1}\)}
cm\(^{-1}\)) transition reflects a 5C site. Taken together, these data indicate that the active site of Dke1-HPP is a mixture of 5C and 6C sites. This 5C/6C mixture was also seen in the case of acac bound to Dke1. The presence of 5C Fe\(^{II}\) in Dke1-HPP demonstrates that open coordination position at the Fe\(^{II}\) active site is available for reaction with O\(_2\).

**4.3.1.2 UV-vis absorption and MCD of Dke1-HPP**

The UV-visible absorption spectrum of Dke1-HPP is shown in Figure 4.2a (green). It is characterized by transitions at \(\sim 17,500\) cm\(^{-1}\) (\(\varepsilon \sim 700\) M\(^{-1}\) cm\(^{-1}\)), \(\sim 22,500\) cm\(^{-1}\) (\(\varepsilon \sim 1400\) M\(^{-1}\) cm\(^{-1}\)) and \(\sim 30,000\) cm\(^{-1}\) (\(\varepsilon \sim 12,000\) M\(^{-1}\) cm\(^{-1}\)). The transitions in the UV-vis absorption of Dke1-HPP are significantly more intense than those of HPP bound to the facial triad containing enzyme, HPPD (Figure 4.2a, red) (24) which has an \(\varepsilon \sim 350\) M\(^{-1}\) cm\(^{-1}\) for the \(\sim 20,000\) cm\(^{-1}\) transition.

![Figure 4.2 UV-visible absorption and MCD spectra of Dke1-HPP (green). The spectra for HPPD-HPP (red) are shown for reference. (A) The 298K absorption spectra; (B) 5K, 7T MCD spectra.](image-url)
The UV-vis 5K, 7T MCD spectrum of Dke1-HPP (Figure 4.2b, green) shows only a very weak pair of transitions at ~17,000 cm\(^{-1}\) and ~21,500 cm\(^{-1}\). This is in stark contrast to the 5K, 7T MCD spectrum of HPPD-HPP (Figure 4.2b, red) which is an order of magnitude more intense than Dke1-HPP.

4.3.1.3 Resonance Raman

Excitation at 363.8 nm (arrow in Figure 4.2)\(^2\) into the very intense charge transfer of Dke1-HPP resulted in a single resonance enhanced band at 1610 cm\(^{-1}\) (Figure 4.3, blue). Exposure of the Dke1-HPP sample to O\(_2\) as a control resulted in loss of the 1610 cm\(^{-1}\) feature to produce the orange spectrum in Figure 4.3. The \(^{18}\)O-isotope substitution of the HPP ligand and rR of the Dke1-\(^{18}\)O-HPP sample are still underway. Previous rR on the \(\alpha\)-keto acid dependent mononuclear non-heme Fe dioxygenase TauD showed a resonance enhanced (excitation into the Fe\(^{II}\) to \(\alpha\)-ketoglutarate \(\pi^*\) MLCT at 568.2 nm) band at 1686 cm\(^{-1}\) which shifted to 1648 cm\(^{-1}\) on \(^{18}\)O-isotope substitution of \(\alpha\)-ketoglutarate (26). Resonance Raman on well defined Fe\(^{II}\)-model complexes with pyruvate have shown a C=O stretch in the range of 1650-1695 cm\(^{-1}\), enabling the assignment of the band at 1686 cm\(^{-1}\) in TauD-\(\alpha\)-ketoglutarate as a C=O stretch (26). The energy of the vibration observed in Dke1-HPP is significantly shifted down in energy relative to the C=O stretch observed in TauD-\(\alpha\)-ketoglutarate suggesting a different assignment for this vibration.

---

\(^1\) The peak position of the 21,500 cm\(^{-1}\) band is shifted from the absorption intensity maximum due to the negative intensity in the MCD (seen at ~25,000 cm\(^{-1}\)) which overlaps the high energy edge of the transition.

\(^2\) Initial attempts were made to excite into the 22,500 cm\(^{-1}\) band however no features were observed above the intense fluorescence background.
4.3.2 Computational

Due to the atypical cleavage pattern for Dke1 with HPP, the increased intensity in the UV-vis absorption spectrum but decreased intensity in the low temperature MCD spectrum (compared to HPPD-HPP), and the shift in the observed resonance Raman feature (of Dke1-HPP compared to TauD-α-ketoglutarate), two possible binding modes for the Dke1-HPP complex were explored. The carbonyl moiety of an α-keto acid can tautomerize to form the enol (or deprotonated enolate) as seen in Scheme 4.2. Previous work on model complexes has shown that monoanionic enolized derivatives of phenylpyruvate will bind to Fe through the same oxygens as the keto form of phenylpyruvate (27). Both tautomers of HPP are considered in the binding of HPP to Dke1.

Scheme 4.3 Tautomerization of HPP
4.3.2.1 Geometry optimized structures

The keto and enolate forms of HPP were added to the previously optimized structure of resting Dke1 (10) with all three water molecules removed to generate a 5-coordinate Dke1-HPP complex. The geometry optimized structures of Dke1-HPP(keto) and Dke1-HPP(enolate) are shown in Figure 4.4. The molecular orbital energy level diagrams for both complexes are shown in Figure 4.5 with the corresponding contours in Figures 4.6 and 4.7 for Dke1-HPP(keto) and Dke1-HPP(enolate), respectively. In both cases, HPP is bound bidentate through one of the carboxylate oxygens and the oxygen of the carbonyl/alkoxide and the Fe center remains ferrous.

Figure 4.4 Geometry optimized structures of (A) Dke1-HPP(keto) and (B) Dke1-HPP(enolate)

---

3 For these spin unrestricted, open shell systems, occupied orbitals are spin polarized and are very mixed. For the bonding description generated here, we use the unoccupied antibonding orbitals to define the uncompensated occupied orbitals involved in bonding.
Figure 4.5 Molecular orbital energy level diagrams for (A) Dke1-HPP(keto) and (B) Dke1-HPP(enolate)

Figure 4.6 Molecular orbital contours for selected β orbitals of Dke1-HPP(keto)
Figure 4.7 Molecular orbital contours for selected β orbitals of Dke1-HPP(enolate)

4.3.2.2 Electronic structure of enolized HPP

The valence orbitals from the keto and enolate tautomers which are available for bonding to Fe are included in the energy level diagrams in Figure 4.5. The valence orbitals of the keto form of α-keto acids have been described previously (28). They are an occupied α-keto acid n orbital which is primarily a lone pair on the carboxylate and the unoccupied α-keto acid π* ligand orbital which is concentrated on the carbonyl (Figure 4.8, left). In the enolate form, however, there are three potential valence orbitals. The lowest energy is an occupied n type orbital with density primarily on the carboxylate (Figure 4.8, right, bottom). Higher in energy, but still

4 The tautomers of the free HPP ligands are not directly compared because in free enolized HPP, the double bond between C2-C3 is delocalized across the entire ligand, while when bound to Fe, the negative charge on the alkoxide oxygen is stabilized and the double bond stays delocalized on C2-C3.
occupied is a $\pi^*$ type antibonding (with respect to the ligand) orbital with electron density concentrated on the C=C double bond ($\pi^*_{\text{C=C}}$, Figure 4.8, right, middle). Finally, there is an unoccupied $\pi^*$ type antibonding orbital with electron density concentrated on the C-C single bonds ($\pi^*_{\text{C-C}}$, Figure 4.4, right, top), similar to the $\pi^*$ of the keto form, but with additional electron density on the C-C single bond adjacent to the phenyl ring.

Figure 4.8 Comparison of bonding orbitals available for HPP(keto) and HPP(enolate) in complex with Dke1. (The orbital numbers given are in reference to Figures 5-7.)

4.4 Analysis

4.4.1 Spectral Assignments

Time-dependent DFT (TD-DFT) calculations were used to evaluate the predicted transitions for Dke1 with either the keto or enolate bond forms of HPP. These predicted spectra are plotted in Figure 4.9 with Gaussian broadening of the
transitions predicted to involve Fe\textsuperscript{II}-HPP(keto) (red and green) or intraligand (purple) interactions. In Figure 4.9a for the Dke1-HPP(keto) complex, the dominant Fe\textsuperscript{II}-HPP(keto) based transition (at ~ 13,000 cm\textsuperscript{-1}, red) is a metal-to-ligand charge transfer (MLCT) transition from the occupied $\beta$ Fe $d_\pi$ to the HPP(keto) $\pi^*$ (see the energy level diagram in Figure 4.5, left). An intraligand transition from the HPP(keto) n to $\pi^*$ orbitals is predicted at > 32,000 cm\textsuperscript{-1} (purple). This description of the MLCT and intraligand transitions is the same as has been observed for HPP bound to the Fe\textsuperscript{II} facial triad in HPPD (28).

![Figure 4.9 TD-DFT predicted absorption spectra](image)

**Figure 4.9 TD-DFT predicted absorption spectra** for (A) Dke1-HPP(keto) and (B) Dke1-HPP(enolate). The transitions (with Gaussian broadening) for MCLT (red), LMCT (green) and intra-ligand (purple) are shown. Note that the intensity in (A) for the ~20,000 – 27,500 cm\textsuperscript{-1} region is predicted to be dominantly Fe\textsuperscript{II}-His MLCT transitions, which has been seen before with the 3His model (10).

The predicted absorption spectrum for the Dke1-HPP(enolate) complex is very different (Figure 4.9b) from that of the Dke1-HPP(keto) predicted spectrum. For the enolate bound form, the transitions at 18,000 cm\textsuperscript{-1} and ~ 25,000 cm\textsuperscript{-1} (green) arise from interaction of Fe\textsuperscript{II} and HPP(enolate); however, they are now ligand-to-metal charge transfer (LMCT) transitions from the HPP(enolate) $\pi^*$ C=C to the unoccupied Fe $d_\pi$ orbitals (see the energy level diagram in Figure 4.5, right). The predicted transition
at ~31,000 cm$^{-1}$ (Figure 4.9b, purple) is assigned as the intraligand transition from the $\pi^\ast_{\text{C=C}}$ to the $\pi^\ast_{\text{C-C}}$ in Figure 4.8, right.

The different in the origins of the charge transfer transitions (MLCT for keto or LMCT for enolate) lead to a change in the predicted transition intensity. The transitions for Dk€1-HPP(enolate) are significantly more intense than the Dk€1-HPP(keto) charge transfer (note the y-axis scales in Figure 4.9). The previously studied Fe$^{II}$ model complexes with enolized derivatives of phenylpyruvate also experimentally exhibited a large increase in the molar extinction coefficient in the absorption spectrum (27).

Alignment of the $\beta$ manifolds from Figure 4.5 to the occupied Fe $d_\pi$ orbital (Figure 4.10) provides insight into the differences between the predicted transitions. For the Dk€1-HPP(keto) complex (Figure 4.10, left) the $\alpha$-keto acid $\pi^\ast$ unoccupied orbital is low in energy enabling MLCT (red arrow). Enolization of the HPP ligand shifts the ligand manifold up in energy due to the increased negative charge which increases the energy of the unoccupied $\pi^\ast_{\text{C-C}}$ orbital (relative to the occupied Fe $d_\pi$; Figure 4.10, right) which would be an acceptor in the MLCT transition (blue arrow). Additionally, enolization of the HPP ligand produces the new high energy occupied $\pi^\ast$ orbital shown in Figure 4.8, right middle. This orbital is the donor for LMCT to the unoccupied Fe

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{diagram.png}
\caption{Comparison of MLCT and LMCT in the keto and enolate forms of HPP. The black arrows correlate the highest occupied and lowest unoccupied orbitals supplied by HPP. The colored arrows correlate to the potential ML (red and blue) or LM (green) transitions.}
\end{figure}
d_\pi orbitals (Figure 4.10, right, green arrows). From the correlation between the assignments of the transitions in the TD-DFT calculations and the experimentally observed absorption spectrum (Figure 4.2a), the charge transfer transitions observed for Dke1-HPP are assigned as LMCT transitions which arise from an enolized HPP.

Although the experimental absorption intensity of the Dke1-HPP complex is higher than that of HPPD-HPP, the corresponding MCD intensity is much lower. Estimates of the C_0/D_0 ratios for the transitions in HPPD-HPP and Dke1-HPP are given in Table 4.1. Previous studies have shown that C_0/D_0 ratios on the order of ~ 0.01 are typical for charge transfer transitions (29). The C_0/D_0 ratio of ~ 0.029 for the MLCT observed for HPPD-HPP is consistent with a typical charge transfer transition, while the C_0/D_0 ratios for the LMCTs observed in Dke1-HPP are an order of magnitude lower (~ 0.001). Thus for the LMCT transitions of Dke1-HPP the MCD intensity is particularly weak.

Table 4.1 C_0/D_0 ratios for HPPD-HPP and Dke1-HPP

<table>
<thead>
<tr>
<th>transition energy</th>
<th>( \varepsilon ) (Abs)</th>
<th>( \Delta \varepsilon ) (MCD)</th>
<th>C_0/D_0</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPPD-HPP</td>
<td>20,000 cm(^{-1})</td>
<td>350 M(^{-1}) cm(^{-1})</td>
<td>1.35 M(^{-1}) cm(^{-1}) T(^{-1})</td>
</tr>
<tr>
<td>Dke1-HPP</td>
<td>17,500 cm(^{-1})</td>
<td>700 M(^{-1}) cm(^{-1})</td>
<td>0.11 M(^{-1}) cm(^{-1}) T(^{-1})</td>
</tr>
<tr>
<td></td>
<td>22,500 cm(^{-1})</td>
<td>1400 M(^{-1}) cm(^{-1})</td>
<td>0.13 M(^{-1}) cm(^{-1}) T(^{-1})</td>
</tr>
</tbody>
</table>

The MCD intensity mechanism for the MLCT transition in Fe\(^{II}\)-\(\alpha\)-keto acid complexes has not been presented previously. C-term MCD intensity results from either SOC of two perpendicularly polarized transitions close in energy (the pseudo-A mechanism) or a “deviation from sum rule” mechanism in which SOC occurs between the ground state and low-lying excited states. For the Fe d to \(\alpha\)-keto acid \(\pi^*\) CT transition, a second charge transfer for SOC is lacking; therefore, the intensity is all positive and arises from SOC mixing into the ground state (deviation from sum rule).
Figure 4.11 Intensity mechanism for the MLCT transition observed in α-keto acid bound facial triads. The ground state and low-lying first excited state MLCT transitions to the HPP π* orbital. The two transitions are in perpendicular directions (y and x) and couple through $L_z$ acting on the $d_{xz}$ component of the first excited state (which is $d_{xy} + d_{xz}$).

In this mechanism, the acceptor HPP(keto) π* orbital is the same and the donor orbitals are the ground and low-lying excited states. The transition from the ground state (Figure 4.11, top) involves excitation of an e\textsuperscript{-} from an occupied Fe $d_{\pi}$ to the HPP(keto) π* orbital. The low-lying excited state capable of MLCT into the HPP(keto) π* orbital is a d-d transition from the occupied Fe $d_{\pi}$ to the lowest unoccupied Fe $d_{\pi}$ orbital on the Fe\textsuperscript{II}. The polarization direction of this transition is perpendicular to that of the MLCT transition for the ground state. Examination of the orbital overlap between the pair in Figure 4.11, bottom, shows that this is the case. Finally, these two orbitals must SOC in the third mutually perpendicular direction. If we define the open coordination position of the 5C complex as the z-direction, the two transitions are in the y and x directions, thus the SOC must be via $L_z$. In this coordinate system, the ground state Fe $d_{\pi}$ is $d_{yz}$ and the first excited state is primarily $d_{xy}$, but with some $d_{xz}$ contribution. (Note the tilt of this orbital out of the equatorial plane, Figure 4.11, bottom right.) The $d_{yz}$ and $d_{xz}$ components will SOC via $L_z$ providing the mechanism for MCD intensity in the MLCT transitions observed for the keto form of HPP. For the
LMCT of the enolate, the same ligand donor orbital is used and the low-lying excited state mixing with the ground state again results from a d-d transition from the occupied Fe dπ to the lowest unoccupied Fe dπ. These transitions are shown in Figure 4.12. Importantly, in Figure 4.12, the transition from the first excited state, has no orbital overlap between the donor and acceptor in a direction perpendicular to the ground state (Figure 4.12, bottom. Note arrow direction and lack of orbital density in the teal square of the donor.)

![Figure 4.12 Application of the mechanism for intensity from the MLCT transition observed in α-keto acid bound facial triads to the Dke1-HPP enolate LMCT.](image)

As the ground state intensity mechanism seen for the MLCT transition in FeII-α-keto acid is not effective for the LMCT transition in the enolate FeII complex, we must consider the pseudo-A mechanism in which transitions close in energy SOC. For the Dke1-HPP(enolate) complex, there are LMCT transitions from both the π* C=C and n orbitals to the Fe dπ orbitals that could generate the pseudo-A intensity (Figure S4.1).

From the assignment of HPP coordinating to Dke1 as an enolate, the 1610 cm⁻¹ feature in the rR spectrum in Figure 4.3 can now be assigned. Enolization of HPP converts the carbonyl to an alkoxide and adds a C=C bond (Scheme 4.3). Thus the
C=O stretch observed in the 1650-1695 cm\(^{-1}\) range for \(\alpha\)-keto acids should not be observed for Dke1-HPP. This is confirmed by the DFT calculated vibrational modes for the Dke1-HPP(enolate) complex which lack any \(C_{\text{alkoxide}}-O\) motion in the 1500-1700 cm\(^{-1}\) range. From these calculations, however, the C=C stretch is predicted at 1622 cm\(^{-1}\). Thus the 1610 cm\(^{-1}\) feature in the rR is assigned as the enolate C=C stretch.

4.4.2 Reaction Coordinate

Three pathways were evaluated for the reaction of O\(_2\) with the enolized HPP (Figure 4.13): (i) a direct O\(_2\) attack on the substrate; (ii) O\(_2\) activation by the Fe\(^{II}\) followed by decarboxylation (the C1-C2 pathway); and (iii) O\(_2\) activation by the Fe\(^{II}\) followed by O-O bond cleavage (the C2-C3 pathway).

In the study of Fe\(^{II}\)-model complexes with enolized phenylpyruvate derivatives, a mechanism for the reaction with dioxygen was proposed in which the O\(_2\) directly attacks the C3 of the enol double bond (27). Dioxygen approach to C3 to the enolate C=C bond of the Dke1-HPP(enolate) complex (Figure 4.13, right) is dissociative on the S=3 surface, and has a barrier of \(\sim 30\) kcal/mol to bond formation on the S=1 and S=2 surfaces\(^6\) (Figure 4.14, “Substrate attack”). This barrier is prohibitive for reaction, ruling out the substrate mechanism as a potential pathway.

---

\(^5\) The predicted C=O stretch for the TauD-\(\alpha\)-ketoglutarate complex is 1698 cm\(^{-1}\) in good agreement with the experimentally observed value of 1686 cm\(^{-1}\), indicating that these computational models give predicted vibrational data which are comparable with the experimental data.

\(^6\) As the ferrous center contributes S=2 to the total spin state of the calculation, the S=1, S=2, and S=3 total spin states are considered for the substrate attack to allow dioxygen to approach the substrate as the triplet (with either \(\alpha\) or \(\beta\) spin electrons, S=1 and S=3) or as an \(M_s = 0\) (S=2).
Figure 4.13 Three potential mechanistic pathways for the O₂ reaction with Dke1-HPP(enolate). O₂ can either react directly with the C=C bond of the enolate (substrate attack, right) or coordinate to Fe and form a bridge to C2 (top left). From the peroxy-bridged species, the C-C bond can cleave to release CO₂ (left) or the O-O bond can cleave to generate an epoxide (middle). (Structures are geometry optimized and correspond to the equivalently labeled points in Figure 4.14.)
An alternative to the direct attack of dioxygen on the substrate is O₂ coordination to the Fe²⁺ center, utilizing the open coordination position at the Fe²⁺ site that was observed experimentally. Dioxygen binding to the Dke1-HPP(enolate) is approximately thermoneutral in either the S=1 or S=2 spin states (Figure 4.14, “O₂ bridged”). Attack on the C2 carbon gives a peroxy-bridged structure (Figure 4.13, top left and Figure 4.14, “O₂ bridged”) that is best described as an Fe³⁺-HPP•-O₂⁻ (energy level diagram given for S=2 in Figure S4.3, the S=1 electronic structure is similar, but with low spin Fe³⁺). From this structure, either the C-C bond cleaves (Figure 4.13, bottom left) releasing CO₂ and generating an Fe²⁺-peracid (as occurs in the facial triad sites with α-keto acid) or the O-O bond cleaves to form an Fe⁴⁺-epoxide (Figure 4.13, bottom middle, and Figure S4.2). Comparison of the barriers for decarboxylation and epoxide formation (Figure 4.14, TS2, orange for decarboxylation vs blue and black for epoxide formation) shows that the O-O cleavage to form the epoxide is more favorable by 6-13 kcal/mol than decarboxylation.⁷ Thus the most energetically favorable

⁷ Evaluation of decarboxylation on the S=1 surface is ongoing.
pathway involves interaction of O$_2$ with the open coordination position at the Fe site to form a bridged-peroxy species that cleaves O-O to form an Fe$^{IV}$=O-epoxide complex (Scheme 4.4). This reaction can occur on either the S=1 or S=2 spin surfaces with the S=1 slightly lower in energy than S=2. For the remaining part of the reaction, the epoxide opens to form an ether-Fe$^{III}$-oxo structure with a radical on C3 (Figure 4.14 “ether” and Figure S4.2-3). Oxo attack on C2 breaks the ether bond with minimal barrier to form the products benzaldehyde and oxalate with transfer of one electron back to Fe to regenerate the ferrous site (Figure 4.14 and Scheme 4.4).

![Scheme 4.4 Metal-mediated O-O cleavage in Dke1-HPP](image)

4.5 Discussion

The O$_2$-dependent cleavage of HPP catalyzed by Dke1 yields products which are not observed in the typical reaction of $\alpha$-keto acid dependent mononuclear non-heme iron enzymes (Scheme 4.2). The standard $\alpha$-keto acid reactivity involves decarboxylation of the a-keto acid moiety (C1-C2 cleavage), while the products observed for the O$_2$-dependent cleavage of HPP by Dke1 indicate with C2-C3 cleavage. Spectroscopic characterization of the Dke1-HPP complex revealed a new enolate binding mode for the $\alpha$-keto acid. From the UV-vis absorption spectrum of Dke1-HPP (more intense than the corresponding HPPD-HPP spectrum), the MCD spectrum of Dke1-HPP (significantly less intense than the MCD spectrum observed for HPPD-HPP), the C=C rather than C=O stretch observed in the rR, and the corresponding computational studies, HPP is bound to Dke1 as the dianionic enolate form of the $\alpha$-keto acid. Additionally from the NIR MCD spectrum, the Dke1-HPP complex possesses an open coordination site on the Fe$^{II}$.

A previous study on enolized (monoanionic) $\alpha$-keto acids proposed a mechanism in which O$_2$ directly attacks the enolate C=C bond (27). This approach was evaluated for the reaction of O$_2$ with the Dke1-HPP(enolate) complex. The barrier
to form the O$_2$-bound species is prohibitively high (~30kcal/mol) and is not mechanistically relevant.\(^8\) Alternatively, utilization of the open coordination position experimentally observed for the Dke1-HPP allows a reaction of the O$_2$ with the Fe$^{II}$ to form a C2 bridged Fe$^{III}$-HPP$^{•-}$O$_2^{2-}$ species.

From the bridged peroxide species, a lower barrier was found for O-O bond cleavage compared to C-C bond cleavage; the latter would releases CO$_2$ as is typically observed for $\alpha$-keto acids. In the typical $\alpha$-keto acid reactivity of the 2His/1 carboxylate facial triad of the $\alpha$-keto acid dependent dioxygenases, the bridged Fe-$\alpha$-keto acid-O$_2$ adduct has been identified to be an Fe$^{IV}$-peroxide species (see Chapter 5). Cleavage of the O-O bond requires two electron transfer into the O$_2$ $\sigma^*$ orbital. As an Fe$^{IV}$ species, the Fe site is already fully oxidized; therefore, the electrons for cleavage of the O-O bond must come from the high lying, occupied $\alpha$-keto acid n orbital. As this n orbital is primarily a lone pair on the carboxylate of the $\alpha$-keto acid with some density along the C-C bond, transfer of the n orbital pair breaks the C-C bond giving off CO$_2$ (Figure 4.15, left). For the enolized $\alpha$-keto acid which is an Fe$^{III}$-HPP$^{•-}$O$_2^{2-}$, the equivalent of the n orbital is now low in energy and relatively unavailable for transfer to O$_2$. Instead the $\alpha$ radical on C3 of HPP(enolate) and the occupied $\beta$ Fe d orbital are the $\alpha$ and $\beta$ HOMOs, respectively (blue circles in Figure 4.15, right). Transfer of this electron pair to the O$_2$ $\sigma^*$ results in cleavage of the O-O bond and formation of the Fe$^{IV}$-epoxide species. After formation of the Fe$^{IV}$-epoxide species, a low energy pathway was found to cleave the C2-C3 bond and release the products, oxalate and benzaldehyde.

---

\(^8\) A molecular understanding of this is currently being pursued.
This difference in reactivity demonstrates an important role for the facial triad in \(\alpha\)-keto dependent mononuclear non-heme iron enzymes. The pKa of HPP is calculated to decrease by \(\sim 10\) log units in going from coordination to a facial triad to coordination to a 3His triad. Thus the negatively charged carboxylate coordinated to the ferrous site prevents formation of the enolate form of the \(\alpha\)-keto acid which allows the cofactor to further provide the 2 e\(^{-}\)s required for O-O bond cleavage and generation of Fe\(^{IV}\)=O through decarboxylation.

4.6 Acknowledgements

This research was supported by NIH Grant GM40392.

4.7 References


4.8 Supporting Information

References Full Reference #15

Gaussian 03, Revision E.01,
M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R.
Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M.
Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N.
Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J.
E. Knox, H. P. Hratchian, J. B. Cross, V. Bakken, C. Adamo, J. Jaramillo, R.
Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W.
G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A.
D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S.
Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I.
Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A.
Gonzalez, and J. A. Pople,

Figure S4.1 LMCT transitions for pseudo-A MCD intensity in Dke1-LMCT. Note
the lack of d character in the ligand donor orbitals which results in relatively weak
MCD intensity.
Figure S4.2 Molecular orbital energy level diagrams for peroxy-bridged, epoxide, and ether steps along the $O_2$ reaction with Dke1-HPP(enolate) (S=2).

Figure S4.3 Geometry optimized structure of ether-Fe$^{III}$-oxo.
Chapter 5

Understanding the Reactivity of $\alpha$-keto Acid Dependent Dioxygenases: Application of the $\{\text{FeNO}\}^7/\{\text{FeO}_2\}^8$ Methodology to the Initial Steps of $O_2$ Reactivity
5.1 Introduction

The mononuclear non-heme iron enzymes are an important class which catalyze a diverse range of chemical reactions of medical and pharmaceutical importance. These reactions include hydroxylation, ring closure, oxidative desaturation, H-atom abstraction, carbon-carbon and aromatic ring cleavage, and halogenation (1, 2). Within this broad class, the oxygen-activating enzymes use a Fe^{II} center to activate triplet dioxygen for the formally spin forbidden reaction with organic substrates. The α-keto acid dependent dioxygenases fall into this subcategory. These enzymes use an α-keto acid group as a cofactor, providing electrons for dioxygen cleavage (1). A general mechanistic strategy (Scheme 5.1) has been developed in which the binding of both the α-keto acid cofactor and the substrate opens a coordination position for O_2 activation. Reaction with O_2 leads to decarboxylation of the α-keto acid cofactor and generation of a highly reactive Fe^{IV}=O. This Fe^{IV}=O species is the active species for the reaction with the substrate (1, 3). Binding of both the substrate and cofactor and the reactive Fe^{IV}=O species have been extensively studied (2, 4, 5). The initial O_2 binding and activation steps, however, are not observed experimentally and cannot be studied directly.

![Scheme 5.1 Proposed mechanism for α-keto acid dependent dioxygenases. (Ref 1)](image)

As no O_2 intermediates prior to the Fe^{IV}=O species can be experimentally trapped in the α-keto acid dependent dioxygenases, other approaches to probe these initial steps must be taken. NO binds to ferrous sites (6-11); however, it is one electron
deficient with respect to O2 and does not react further. These complexes are described as \{FeNO\}7 following the notation of Enemark and Feltham (12) where the superscript refers to the total number of Fe d plus NO valence electrons (6 electrons from the ferrous site and one from NO). These \{FeNO\}7 complexes are stable and chromophoric with half-integer spin (typically S=3/2) (9), making them a good spectral probe and amenable to study by electron paramagnetic resonance (EPR), UV-visible absorption and magnetic circular dichroism (MCD) spectroscopies. Initial studies on the Fe-EDTA-NO complex showed an S=3/2 site (from EPR) with intense charge transfer transitions in the UV-vis absorption and MCD (Supplementary Figures S5.1, S5.2a). XAS and resonance Raman studies assigned the electronic structure of the \{FeNO\}7 unit to be best described as FeIII antiferromagnetically coupled to NO (13). Computational modeling supported this description giving an FeIII-NO electronic structure characterized by 5 unoccupied \( \beta \) Fe d orbitals and 2 unoccupied \( \alpha \) NO\(^{-}\) orbitals (Figure 5.1) (13, 14).

![Figure 5.1 Molecular orbital energy level diagram for \{FeNO\}7 model complexes.](image)

\[^{1}\text{For these spin unrestricted, open shell systems, occupied orbitals are spin polarized and are very mixed. For the bonding description generated here, we use the unoccupied antibonding orbitals to define the uncompensated occupied orbitals involved in bonding.}\]
The \( \{\text{FeNO}\}^7 \) computational work was then extended to provide experimentally calibrated models for \( \{\text{FeO}_2\}^8 \) (14). This \( \{\text{FeNO}\}^7/\{\text{FeO}_2\}^8 \) methodology was used to characterize the initial steps of the \( \text{O}_2 \) reaction of Isopenicillin \( N \) Synthase (IPNS) (15). In that study, the \( \{\text{FeNO}\}^7 \) complex provided an understanding of the effect of thiolate ligation by the native substrate. Extension of the experimentally calibrated \( \{\text{FeNO}\}^7 \) description to an \( \{\text{FeO}_2\}^8 \) computational model led to an FMO for H-atom abstraction (the first \( \text{O}_2 \)-dependent step of the mechanism). The results for IPNS show the utility of the \( \{\text{FeNO}\}^7/\{\text{FeO}_2\}^8 \) methodology in evaluating the initial steps of the \( \text{O}_2 \) reaction.

This study uses the \( \alpha \)-keto acid dependent dioxygenase, (4-hydroxyphenyl)pyruvate dioxygenase (HPPD). HPPD catalyzes the reaction of (4-hydroxyphenyl)pyruvate (HPP) with dioxygen to release \( \text{CO}_2 \) and yield homogentisate (Scheme 5.2) (16).

\[ \begin{align*}
\text{HPP} & \quad \text{O}_2 \quad \text{CO}_2 \\
\text{HG} & \quad \text{OH} \\
\end{align*} \]

\textbf{Scheme 5.2 Reaction catalyzed by HPPD}

HPPD has been chosen for this study because the resting ferrous and ES complexes have been studied previously (17-19). Additionally, in HPP the \( \alpha \)-keto acid is moiety fused to the substrate facilitating formation of the catalytically relevant ES complex. From our previous study of the ferrous ES complex (19), there is less ferrous d-d transition intensity at low energy relative to other \( \alpha \)-keto acid dependent dioxygenases which will be important in the assignment of transitions in the HPPD-HPP-NO complex. This study examines the effect of the \( \alpha \)-keto acid moiety on the \( \{\text{FeNO}\}^7 \) unit. The NO-bound complexes of both resting HPPD and HPPD-HPP are studied here where the HPPD-NO complex provides a reference to explain the new features which arise from \( \alpha \)-keto acid binding. The experimental study of the HPPD-NO and
HPPD-HPP-NO complexes is then extended to computational models. In order to obtain a general picture of α-keto acid reactivity, the computational model used in this study is based on the crystal structure of the α-keto acid dependent dioxygenase Clavaminate synthase (CS2) in complex with α-ketoglutarate and NO (20). To ensure that this choice of model is appropriate, key computational steps have also been done with HPP as the substrate and are presented in the supporting information. Analysis of the \{FeNO\}_7 computational models in conjunction with the experimental HPPD-(HPP)-NO data leads to an understanding of the effects of the α-keto acid moiety on the \{FeNO\}_7 unit. Insight gained from the \{FeNO\}_7 complex is extended to the mechanistically relevant \{FeO_2\}_8 complex. Evaluation of the \{FeO_2\}_8 complex and the subsequent reaction coordinate allows an evaluation of proposed mechanisms of reactivity and provides insight into the initial steps of the O₂ reaction in α-keto acid dependent dioxygenases leading to the formation of the Fe^{IV}=O for reaction with substrate.

5.2 Materials and Methods

HPPD was purified according to previously published procedures (16). Apo-HPPD was exchanged into 50mM HEPES buffer in D₂O at a pD of 7.1 using an Ultrafree-4 filter with a 10kDa cutoff membrane (Millipore) to a concentration of 2-4 mM. All other reagents were used as received without further purification. Buffer, HPP, ferrous ammonium sulfate, MamaNONOate and glycerol were made anaerobic by purging on with Ar on a Schlenk line. HPPD was made anaerobic by alternating cycles of vacuum and purging with Ar at 273K. All samples for spectroscopy were prepared in an inert atmosphere, N₂-purged “wet box” to maintain an O₂ free environment. Ferrous ammonium sulfate was dissolved in buffer and added to HPPD in microliter quantities to a concentration of 90% of the enzyme (monomer) concentration to avoid free iron in the sample. HPP was added to buffer to form a saturated solution. Residual solid HPP was filtered out of the solution and the concentration of the HPP solution was determined from the intensity of the 270nm maximum in the absorption spectrum. For the substrate-bound samples, HPP was
added in microliter quantities to a concentration of 5-10 times the concentration of the protein. Solid MamaNONOate was dissolved in 220 mM NaOH for a final concentration of 90mM and was added to the HPPD-Fe and HPPD-Fe-HPP solutions in microliter quantities to a concentration of ~2 times the concentration of the protein. Glycerol (~50-60% v/v) was added as a glassing agent to samples for MCD.

NIR (600-2000 nm) MCD spectra were taken on either a Jasco J-200D or Jasco J-730 spectropolarimeter with a liquid N\textsubscript{2} cooled InSb detector (Teledyne Judson Technologies) and an Oxford Instruments SM-4000-7T superconducting magnet. UV-vis (300-900 nm) MCD spectra were taken on a Jasco J-810D spectropolarimeter equipped with an extended S20 photomultiplier tube and a SM-4000-7T superconducting magnet. UV-vis Abs spectra were taken on an Agilent 8453 diode array spectrometer. Abs spectra were taken at 278K in an anaerobic cuvette and were corrected for buffer and protein baseline effects by subtraction. Natural CD features and baseline effects were excluded from the MCD spectra by taking the average of the magnitudes of the positive and negative field data. For VTVH MCD, a calibrated Cernox resistor (Lakeshore Cryogenics, calibrated 1.5 – 300 K), inserted into the sample cell, was used for accurate temperature measurement. The VTVH MCD data obtained were normalized to the intensity maximum. X-band EPR spectra were taken on a Bruker EMX spectrometer with a Bruker ER 041XG/ER microwave bridge and ER 4102ST/ER 5106QT cavity. Spectra were taken at temperatures between 3.8 and 50K using an Oxford ITC503 temperature controller with an ESR 900 continuous flow cryostat.

The computational models for the resting enzyme plus NO (E-NO) and the α-keto acid bound enzyme plus NO (E-αKA-NO) were modeled from the CS2-α-ketoglutarate-NO crystal structure (PDB ID: 1gvg). Histidine residues were truncated to methyl imidazole and glutamate was truncated to propionate for the models. Constraints imposed by the protein backbone were simulated in both computational models by fixing the relative positions of the β-carbons of the backbone.

Density functional theory (DFT) calculations were performed with the Gaussian 03 program(21, 22) with the unrestricted BP86 functional(23) with 10%
Hartree-Fock Exchange under tight convergence criteria. The Pople triple-ζ basis set, 6-311G*, was used to describe Fe, NO/O2, and the α-keto acid moiety (OCCO2). The double-ζ basis set, 6-31G* was used to describe all other atoms. This method has been calibrated previously for {FeNO}7 complexes. Calculations for comparison with literature results were performed with the unrestricted B3LYP functional under tight convergence criteria. The LANL2DZ effective core potential basis set was used to describe Fe and the Pople double-ζ basis set, 6-31G, was used to describe all other atoms. All structures were optimized and found to be stable with no imaginary frequencies > -15 cm⁻¹ (which are associated with the constraints placed on the β-carbons). Effects of the protein environment were included by applying the polarized continuum model (PCM) (24) with a dielectric constant, ε = 4.0, to calculate solvated energies for the complexes. These solvated single point calculations were done with the 6-311+G(2d,p) basis set. (For the B3LYP models, the 6-311G** basis set was used, consistent with literature.) The energies given include thermal and Zero-point corrections. ZFS parameters were calculated in ORCA for both models with the CP(PPP) basis set on Fe, TZVP on N and O, and SVP on all other atoms (25). Structures were visualized with GaussView 3.09 (26); orbital compositions were determined with QMForge (27); and molecular orbitals were visualized with Molden version 4.1 (28). Time-dependent DFT (TD-DFT) calculations were performed to compare to the experimental spectra and SWizard was used to parse the results (29, 30).

5.3 Results

5.3.1 Spectroscopic

Addition of NO to the resting, FeII-bound form of HPPD results in the EPR spectrum shown in Figure 5.2a. The HPPD-NO complex exhibits effective g’ values of 3.96 and 2.00 indicating that it is a close to axial S=3/2 species with an E/D = 0.008. A plot of the temperature dependence of the EPR signal intensity under non-saturating conditions is shown in Figure 5.3 (red). Addition of NO to HPPD in complex with the native substrate HPP results in the EPR spectrum shown in Figure
5.2b. The EPR signal of the HPPD-HPP-NO complex indicates that the spin state is still \( S=3/2 \) but with \( g_x' = 4.14 \) and \( g_y' = 3.90 \) giving an \( E/D \) of 0.02. The inverse dependence of the EPR intensity with increasing temperature for the HPPD-HPP-NO spectrum is also shown in Figure 5.3 (blue).

![Figure 5.2 EPR of \{FeNO\}7 enzyme complexes. EPR of \( S=3/2 \) HPPD-NO (A) and HPPD-HPP-NO (B) showing the inverse temperature dependence of the signal intensity.](image)

![Figure 5.3 Temperature dependence of \{FeNO\}7 \( S=3/2 \) EPR signal. The inverse temperature dependence of the EPR signal intensity for HPPD-NO (red) and HPPD-HPP-NO (blue). The best fit to the data for the axial ZFS parameter, \( D \), is shown in gray. \( D = 16 \pm 2 \) cm\(^{-1}\) for HPPD-NO and \( 8.4 \pm 0.7 \) cm\(^{-1}\) for HPPPD-HPP-NO.](image)

These EPR spectra are described by the spin Hamiltonian given in Equation 1, where \( g_0 = 2.0 \) and \( D \) and \( E \) are the axial and rhombic ZFS parameters, respectively (13).

\[
\hat{H}_{\text{spin}} = D[S_z^2 - 5/4 + E / D(S_x^2 - S_y^2)] + g_0 \beta \vec{S} \cdot \vec{H}
\]  

(1)
This splits the $S=3/2$ $M_s = \pm 1/2, 3/2$ by an amount $2D$. The inverse temperature dependence of the EPR signal intensity indicates that $D$ is positive with $M_s = \pm 1/2$ lowest and is fit to a Boltzmann population of the Curie law dependence (Equation 2 where $C$ is the Curie constant, $T$ is the temperature in Kelvin and $k$ is the Boltzmann constant).

$$\text{Intensity} = \frac{C}{T} \left[ \frac{1}{1 + \exp(-2D/kT)} \right]$$  \hspace{1cm} (2)

For the HPPD-NO EPR sample, this gives $D = 16 \pm 2 \text{ cm}^{-1}$ while for the HPPD-HPP-NO sample, the value of the axial ZFS parameter has decreased to $D = 8.4 \pm 0.7 \text{ cm}^{-1}$.

![Figure 5.4 Room temperature UV-vis absorption spectra of HPPD-NO (red) and HPPD-HPP-NO (blue).](image)

The room temperature UV-vis absorption spectra of the HPPD-NO and HPPD-HPP-NO complexes are shown in Figure 5.4. The two spectra are very similar up to 25,000 cm$^{-1}$. However, a new transition resulting from HPP binding is observed above 25,000 cm$^{-1}$. The Fe-EDTA-NO complex has been extensively studied and provides a point of reference for this study (13). The HPPD-NO UV-vis absorption spectrum is very similar to that of Fe-EDTA-NO (Supplementary Figure S5.1). For the Fe-EDTA-NO complex, the two bands observed are assigned as the ligand-to-metal charge transfer transitions from NO$^-$ to Fe$^{III}$ d (at 20,000 – 25,000 cm$^{-1}$) and the Fe$^{III}$ ligand field, d-d transitions (at ~15,000 cm$^{-1}$).

The 5K, 7T MCD spectra of the HPPD-NO and HPPD-HPP-NO complexes are shown in Figure 5.5. The MCD spectra of these two complexes are very similar with only small differences in energy and intensity of the major features. The starred
feature in the HPPD-NO MCD spectrum is unreacted resting HPPD. The major difference between the resting and substrate-bound NO complexes is the new band at low energy (< 5000 cm\(^{-1}\)) in the HPPD-HPP-NO complex.

![Figure 5.5 VTVH MCD of \{FeNO\}\(^7\). (A,B) The 5K, 7T MCD spectra of HPPD-NO (A) and HPPD-HPP-NO (B). The Gaussian fits to the spectra are shown. For HPPD-NO, the star indicated unreacted resting HPPD. VTVH MCD isotherms were taken at the points indicated with arrows on the spectra of HPPD-NO and HPPD-HPP-NO. (C-E) The VTVH MCD isotherms and best fits to the data. VTVH MCD taken for HPPD-NO at 21,700 cm\(^{-1}\) (C) and HPPD-HPP-NO at 21,400 cm\(^{-1}\) (D) and 5650 cm\(^{-1}\) (E).](image)

The Gaussian resolutions of the bands in the two MCD spectra are also shown in Figure 5.5. The MCD spectrum of HPPD-NO is very similar to that observed for the Fe-EDTA-NO complex (Supplementary Figure S5.2). From this comparison, the three transitions at ~18,000-24,000 cm\(^{-1}\) of the HPPD-NO complex are assigned as NO\(^-\) 2\(\pi^*\) to Fe\(^{III}\) d\(\pi\) charge transfer transitions while the two lower energy transitions are assigned as two of the Fe\(^{III}\) d-d ligand field transitions (which are formally spin forbidden, but gain intensity through mixing with the nearby charge transfer transitions, \textit{vide infra}). For the MCD spectrum of the HPPD-HPP-NO complex, the three transitions at ~17,000-25,000 cm\(^{-1}\) are also assigned as the NO\(^-\) to Fe\(^{III}\) d charge transfers and the two lower energy transitions at 11,500 cm\(^{-1}\) and 15,000 cm\(^{-1}\) are assigned as Fe\(^{III}\) d-d, ligand field transitions. Assignment of the < 5000 cm\(^{-1}\) transition in the HPPD-HPP-NO complex will be considered in the analysis. To higher energy,
the new feature observed in the UV-vis absorption spectrum is not accessible in the MCD spectrum due to the light limiting conditions of the sample.\(^2\)

VTVH MCD gives the polarizations of the transitions observed in Abs/MCD spectra. The arrows in Figure 5.5 indicate the energies where VTVH MCD isotherms were taken to evaluate the polarizations of the corresponding transitions (where the Gaussians show little overlap). The VTVH MCD isotherms for the 21,700 cm\(^{-1}\) transition in HPPD-NO are shown in Figure 5.5c. The VTVH MCD for the ligand field transitions taken at 15,100 cm\(^{-1}\) is shown in Supplementary Figure 5.3a. These isotherms were taken on the less intense ligand field transition to avoid any contribution to the VTVH MCD isotherms from the residual resting HPPD (which has negative MCD intensity in this region). For the HPPD-HPP-NO complex, VTVH MCD isotherms were taken at 21,400 cm\(^{-1}\) (Figure 5.5d), at 15,000 cm\(^{-1}\) for the ligand field transition (Supplementary Figure S5.3b), and at 5,650 cm\(^{-1}\) for the low energy transition (Figure 5.5e, the large error bars reflect the noisy band at this limit of our accessible spectral region). These VTVH MCD data were modeled by Equation 3 (31).

\[
\frac{\Delta \epsilon}{E} = \frac{\gamma}{4\pi \alpha} \int_0^\pi \left[ \sum_i N_i (l_z \langle S_z \rangle_i M_{xy}^{eff} + l_y \langle S_y \rangle_i M_{xz}^{eff} + l_x \langle S_x \rangle_i M_{yz}^{eff}) \right] \sin \theta \, d\theta \, d\phi
\]

A fit of the MCD intensity to Equation 3 using the spin Hamiltonian parameters \(g_0\), \(D\) and \(E\) allowed for the \(M_i^{eff}\) transition moments to be obtained for the transition being probed. These transition moments were used to obtain the \% polarization in the x, y and z directions using Equation 4, shown for % x polarization. The % polarizations in the y and z directions are obtained from cyclic permutations of the indices of Equation 4 (31).

\[
%_x = 100 \times \left[ \frac{(M_{xy}^{eff} M_{xz}^{eff})^2}{(M_{xy}^{eff} M_{xz}^{eff})^2 + (M_{xy}^{eff} M_{yz}^{eff})^2 + (M_{yz}^{eff} M_{xz}^{eff})^2} \right]
\]

From this analysis, the VTVH MCD data for HPPD-NO at 21,700 cm\(^{-1}\) are 85% z-polarized (fit to the isotherms is included in Figure 5.5c). This is consistent with the

\(^2\) Further characterization of this species is in progress.
assignment of that transition as NO\textsuperscript{–} to Fe\textsuperscript{III} d CT. The z polarization refers to the axial tensor of the ZFS that is collinear with the Fe-N\textsuperscript{NO} bond and NO\textsuperscript{–} to Fe\textsuperscript{III} d CT is polarized along this bond. For the HPPD-HPP-NO complex, the fit of the VTVH MCD data at 21,400 cm\textsuperscript{-1} (fit shown in Figure 5.5d) is 49% z-polarized and 51% x,y-polarized reflecting a new interaction resulting from the binding of the α-keto acid substrate. Fits of the VTVH isotherms of the ligand field transitions give the same polarizations as their respective NO\textsuperscript{–} to Fe\textsuperscript{III} d charge transfer transitions (predominantly z polarized for HPPD-NO and mixed polarization for HPPD-HPP-NO, Supplementary Figure S5.3). The < 5000 cm\textsuperscript{-1} transition in HPPD-HPP-NO can be fit with a range of polarizations between 88% and 47% z-polarized (Supplementary Figure S5.4). A more definitive fit cannot be obtained due to the noise in the data, however, the lowest energy transition is no more x,y polarized than the NO\textsuperscript{–} to Fe\textsuperscript{III} d CT transition at 21,400 cm\textsuperscript{-1}.

5.3.2 Computational – \{FeNO\}\textsuperscript{7}

In order to further evaluate the geometric and electronic structure of the \{FeNO\}\textsuperscript{7} complexes, computational models have been studied. A previous study evaluated a number of different functional and basis set combinations to provide an experimentally correlated rational for the choice of computational parameters (14). From this study, the BP86 functional with 10% Hartree-Fock exchange included, in combination with a split basis set consisting of triple-\(\zeta\) on the Fe-N-O unit and double-\(\zeta\) on the remaining atoms (for computational efficiency, hereafter referred to as Gen) proved to be the most consistent with experiment. Previous computational studies on αKA by other groups have used a combination of B3LYP and the LACVP basis set (32-34). Further rational for the current choice of functional and basis set will be presented below. There are a number of structural choices for the computational model of α-keto acid reactivity. The binding of HPP to HPPD has not been observed crystallographically and there are two different orientations which have been proposed for the coordination of the α-keto acid moiety to the active site which differ in the orientation of the carboxylate – axial or equatorial with respect to NO. The equatorial coordination was used in previous studies (17, 18) and the axial coordination was
proposed from molecular modeling of the HPP substrate in the active site pocket (35). (See the NO complexes in Supplementary Figure S5.5 for these orientations.) In the text, the results for a-ketoglutarate bound to a facial triad (abbreviated as E-αKA) are shown. The results for the two orientations of HPP bound to HPPD are indicated throughout the text and presented in the supporting information.

The HPPD-NO complex can be modeled by the three protein derived residues making up the facial triad (2His/1 carboxylate), 2 water ligands and NO coordinated to Fe. The geometry optimized computational model (E-NO) is shown in Figure 5.6a and key geometric parameters are given in Table 5.1. The geometric structure shows a strong Fe-N\textsuperscript{NO} bond at 1.76Å (with an Fe-N-O angle of 147°) which defines the z-axis of the molecule. The geometric structure description of the FeNO unit in E-NO is similar to both the experimental EXAFS and geometry optimized structure of Fe-EDTA-NO (Table 5.1). Examination of the molecular orbital diagram (Figure 5.7, left) and orbital contours (Figure 5.8) shows two unoccupied α NO π* and 5 unoccupied β Fe d orbitals. This electronic structure is consistent with the Fe\textsuperscript{III}-NO\textsuperscript{-} description determined for Fe-EDTA-NO.

![Figure 5.6 Computational models for S=3/2 \{FeNO\}\textsuperscript{7}. Geometry optimized structures for E-NO (A) and E-αKA-NO (B).](image)

| Table 5.1 Key geometric and electronic structure parameters for E-NO and E-αKA-NO |
|-----------------|-----------------|-----------------|-----------------|
|                 | E-NO            | E-αKA-NO        | Fe-EDTA-NO      |
| Fe-N\textsuperscript{NO} | 1.76Å           | 1.76Å           | 1.78Å\textsuperscript{a}/1.76Å |
| N-O             | 1.17Å           | 1.17Å           | 1.10Å/1.18Å      |
| Fe-N-O          | 147°            | 149°            | 156°/149°        |
| Charge on NO    | -0.220          | -0.244          |                 |

\textsuperscript{a} XAFS parameters  
\textsuperscript{b} computational parameters
To model the HPPD-HPP-NO complex, the two water ligands of the E-NO complex are replaced by α-ketoglutarate (αKA) as described above. This geometry optimized computational model (E-αKA-NO) also shows a strong Fe-N\(^\text{NO}\) bond (1.76Å, Fe-N-O angle of 149\(^\circ\)) which again defines the z-axis of the molecule (Figure 5.6b and Table
The electronic structure of the Fe-NO unit of E-αKA-NO is very similar to that of E-NO (molecular orbital diagram in Figure 5.7, right and contours in Figure 5.9).

Figure 5.9 Molecular orbital contours for geometry optimized structure of S=3/2 E-αKA-NO.

The αKA moiety has two molecular orbitals (the HOMO and LUMO) that interact with the Fe center (Figure 5.10). The occupied αKA n orbital (the αKA HOMO) is primarily a lone pair on the carboxylate with some in-plane character on the carbonyl and will have a σ bonding interaction with the d_{xy} and d_{x^2-y^2} orbitals. (Note that in this coordinate system, y is in the Fe-N-O plane that bisects equatorial ligand-metal bonds therefore d_{x^2-y^2} is now a d_π orbital with electron density bisecting the ligand bonds and

---

3 The geometry optimized structures and key geometric parameters for the 2 HPPD-derived models (HPPD-HPP-NO 1 and HPPD-HPP-NO 2) are shown in Supplementary Figures S5.5-S5.6 and Supplementary Table S5.1.
\( d_{xy} \) is the \( d_\sigma \) orbital with electron density along the ligand-metal bonds.) The unoccupied \( \alpha \text{KA} \pi^* \) orbital is mostly on the carbonyl with conjugation onto the carboxylate and will have a \( \pi \) interaction with the Fe \( d_{yz} \) and \( d_{xz} \) orbitals. In the E-\( \alpha \text{KA-NO} \) complex, these \( \alpha \text{KA} \) orbitals are seen in the MO diagram (Figure 5.7, right) and in the orbital contours \( \beta 117 \) (n) and \( \beta 122 \) (\( \pi^* \)).

![Figure 5.10 Molecular orbital contours of \( \alpha \text{KA} \) available for bonding to Fe.](image)

### 5.4 Analysis

#### 5.4.1 Correlation of calculations to experiment

Using the geometry optimized computational models E-NO and E-\( \alpha \text{KA-NO} \) presented above, the effects of \( \alpha \text{KA} \) binding on the experimental spectra were evaluated.

Time-dependent density functional theory (TD-DFT) was used to generate a predicted absorption spectrum for each computational model. The TD-DFT predicted absorption spectrum for the E-NO complex is shown in Figure 5.11a with Gaussian broadening of for the transitions with significant NO⁻ to Fe d character. The primary transitions and their corresponding transition dipole moment directions are given in Table 5.2. The TD-DFT predicted absorption spectrum for the E-\( \alpha \text{KA-NO} \) complex is shown in Figure 5.11b.4

---

4 The overlaid TD-DFT predicted absorption spectra for E-\( \alpha \text{KA-NO} \) and the two \text{HPPD-HPP-NO} derived models are shown in Supplementary Figure S5.7.
Table 5.2 TD-DFT transition energies and directions of transition moment dipoles

<table>
<thead>
<tr>
<th>Transition</th>
<th>Experiment HPPD-NO</th>
<th>HPPD-HPP-NO</th>
<th>Calculated E-NO</th>
<th>E-aKA-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>NO$^-$ $\rightarrow$ Fe d</td>
<td>19,500cm$^{-1}$</td>
<td>17,600cm$^{-1}$</td>
<td>13,900cm$^{-1}$; z</td>
<td>13,500cm$^{-1}$; z</td>
</tr>
<tr>
<td></td>
<td>20,800cm$^{-1}$</td>
<td>19,800cm$^{-1}$</td>
<td>16,600cm$^{-1}$; x,y/z(^a)</td>
<td>17,500cm$^{-1}$; x,y/z</td>
</tr>
<tr>
<td></td>
<td>22,100cm$^{-1}$</td>
<td>22,000cm$^{-1}$</td>
<td>23,700cm$^{-1}$; z</td>
<td>22,000cm$^{-1}$; x,y/z</td>
</tr>
<tr>
<td>$\alpha$KA n $\rightarrow$ Fe d</td>
<td>$\sim$30,000cm$^{-1}$</td>
<td></td>
<td></td>
<td>25,500cm$^{-1}$; x,y</td>
</tr>
</tbody>
</table>

\(^a\) Transition is mixed with a His-Fe d transition.

Binding of the $\alpha$KA ligand introduces new bands in the predicted spectrum with significant contributions from the $\alpha$KA n to Fe $d_{x^2-y^2}$ ($d_z$) transition. These are included in Figure 5.11b (teal). The TD-DFT calculation predicts these transitions to be higher in energy than the NO$^-$ to Fe d transitions (Table 5.2). From this, the highest energy transition in the UV-vis absorption of HPPD-HPP-NO is assigned as an $\alpha$KA n to Fe $d_{x^2-y^2}$ CT transition. The predicted CT transitions are shown schematically in Figure 5.12 (red for NO$^-$ and teal for $\alpha$KA CT).
Figure 5.12 Transitions in E-NO and E-αKA-NO. The transitions of the E-NO and E-αKA-NO complex are shown with the NO’ to Fe d CT transitions in red, the αKA n to Fe d CT transition in teal, and ligand field transitions in blue.

The assignment of the new high energy transition in the absorption spectrum to an αKA n to Fe d CT transition provides insight into the mixed polarization observed in the VTVH MCD data of the HPPD-HPP-NO. From the transition dipole moment directions given in Table 5.2, the NO’ to Fe d CT transitions in E-NO are predominantly polarized in the z direction. This polarization direction refers to the projected axis of the ZFS and is consistent with the strong Fe-N\(^{\text{NO}}\) bond which defines the z-axis of the system. For the E-αKA-NO complex predicted NO’ to Fe d CT transitions, however, there is an x,y component of the transition dipole moment leading to an off axis transition moment direction. This reflects mixing with the αKA n to Fe d transition that has a transition dipole moment dominantly in the x,y direction (Table 5.2) consistent with αKA binding in the equatorial plane. The two charge transfer transitions are close in energy and mix to generate an off axis transition dipole moment, i.e. the mixed polarization observed in the VTVH MCD of HPPD-HPP-NO complex.

The lower energy transitions in the MCD spectra of \{FeNO\}\(^7\) complexes are assigned as the formally spin forbidden Fe\(^{\text{III}}\), ligand field d-d transitions (shown schematically in Figure 5.12, blue). As TD-DFT calculations only calculate the spin-allowed transitions, ΔSCF calculations were undertaken to probe the energies of the specific Fe\(^{\text{III}}\) ligand field, d-d transitions.\(^5\) As promotion of a full electron resulted in

\(^5\) ΔSCF energies for HPPD-HPP-NO 1 are given in Supplementary Table S5.2.
wavefunctions which would not converge, these transitions were calculated as Slater transition states. Table 5.3 gives the calculated transition energies for the Fe$^{III}$ $\alpha d_{\sigma}$ to $\beta d_{\pi}$ ligand field transitions. From Table 5.3 for most of the ligand field transitions, the energies change only slightly between the E-NO and E-$\alpha$KA-NO complexes.

<table>
<thead>
<tr>
<th>Transition</th>
<th>E-NO (cm$^{-1}$)</th>
<th>E-$\alpha$KA-NO (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$d_{z^2} - d_{x^2-y^2}$</td>
<td>3600</td>
<td>7400</td>
</tr>
<tr>
<td>$d_{xy} - d_{x^2-y^2}$</td>
<td>8000</td>
<td>8700</td>
</tr>
<tr>
<td>$d_{xy} - d_{yz}$</td>
<td>12,500</td>
<td>12,500</td>
</tr>
<tr>
<td>$d_{z^2} - d_{xz}$</td>
<td>14,100</td>
<td>14,800</td>
</tr>
<tr>
<td>$d_{xy} - d_{x^2-y^2}$</td>
<td>16,100</td>
<td>19,100</td>
</tr>
</tbody>
</table>

The most notable exception lies in the lowest energy, Fe$^{III}$ $\alpha d_{z^2}$ to $\beta d_{x^2-y^2}$, transition which increases by $\sim$3800 cm$^{-1}$ upon binding of $\alpha$KA. This change is consistent with the new band at low energy in the HPPD-HPP-NO spectrum having moved up in energy with respect to HPPD-NO. As mentioned previously, the Fe$^{III}$ ligand field transitions are formally spin-forbidden. These transitions become allowed through metal-based SOC with the nearby spin allowed charge transfer transitions. To SOC, the two transitions must differ by one electron and the metal character of those orbitals must rotate into each other via one of the SOC operators. For this analysis, we use a coordinate system that is rotated by 45° in the x,y plane to align x and y with the bonds. In this primed coordinate system, the lowest energy LF transition, $d_{z^2}$ to $d_{x^2-y^2}$, is now $d'_{z^2}$ to $d'_{x'y'}$. The NO$^{\prime}_{(ip)}$ to Fe $d'_{x'y'}$ CT transition and the $d'_{x^2}$ to $d'_{x'y'}$ ligand field transition differ by the electron in NO$^{\prime}_{(ip)}$ or $d'_{x^2}$. The predominant metal character in the NO$^{\prime}_{(ip)}$ orbital is $d_{y'z'+x'z'}$. The $d_{y'z'}$ component of this will SOC with $d_{x^2}$ via $L_{x'}$, thus providing an intensity mechanism for this ligand field transition.

The increase in energy of the lowest energy Fe$^{III}$ $d_{z^2}$ to $d_{x^2-y^2}$ transition upon binding of $\alpha$KA reflects either a decrease in the energy of Fe $d_{z^2}$ or an increase in the energy of $d_{x^2-y^2}$. Alignment of the MO energy levels to the highest occupied NO$^{\prime}$ orbital (Supplementary Figure S5.8) shows that $d_{z^2}$ does not significantly change in

---

$^6$ Attempts to obtain a Slater transition state for the $d_{z^2}$ to $d_{yz}$ transition were not successful for either the E-NO or E-$\alpha$KA-NO systems.
energy between the E-NO and E-αKA-NO complexes (in fact, it increases in energy). This is consistent with the similar Fe-N\(^{\text{NO}}\) bond lengths in the geometry optimized structures, and reflects the strong \(\sigma\) interaction of Fe \(d_{z^2}\) with NO. However, \(d_{x^2-y^2}\) increases in energy with addition of αKA which reflects addition of a much stronger donor ligand in the equatorial plane than the two water ligands present in the E-NO complex. Assignment of the αKA moiety as a strong donor ligand has implications for \(O_2\) reactivity.

The ZFS parameter, \(D\), obtained from fitting the temperature dependence of the \(S=3/2\) EPR signal decreases by half with the addition of αKA (16.0 cm\(^{-1}\) to 8.4 cm\(^{-1}\)). Using the optimized geometries for E-NO and E-αKA-NO, the ZFS parameters were calculated. The calculated value of \(D\) decreases from 10 cm\(^{-1}\) to 6.6 cm\(^{-1}\). This is consistent with a significant decrease in the value of \(D\) upon addition of αKA to the \{FeNO\}_7 unit. Rodriguez, et. al. have evaluated at the origin of the large ZFS in \(S=3/2\) \{FeNO\}_7 complexes (for Fe-[Me\(_3\)TACN]-\((N_3)_2\)-NO where \(D=22\text{cm}^{-1}\)) (36). They attribute the large value of the ZFS to delocalization of electron density from NO\(^{-}\) to the Fe \(d_{yz}\) and \(d_{xz}\) orbitals which strongly overlap in the \{FeNO\}_7 unit. From Table 5.4, the amount of NO\(^{-}\) character in the \(\beta\) Fe \(d_{yz}\) and \(d_{xz}\) molecular orbitals does in fact decrease from 72% to 54% upon binding of αKA. This reflects competition from the strong donation of the αKA unit. Thus the high energy CT in absorption, the mixed polarization in VTVH MCD, the new low energy LF band and the decreased value of \(D\) all reflect the strong donation of the αKA ligand to the Fe\(^{III}\).

<table>
<thead>
<tr>
<th>Orbital</th>
<th>E-NO</th>
<th>E-αKA-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>(d_{yz})</td>
<td>24%</td>
<td>22%</td>
</tr>
<tr>
<td>(d_{xz})</td>
<td>48%</td>
<td>32%</td>
</tr>
<tr>
<td>Total</td>
<td>72%</td>
<td>54%</td>
</tr>
</tbody>
</table>

5.4.2 Comparison to B3LYP/LACVP

Most calculations published on the reactivity of \(\alpha\)-keto acid dependent mononuclear non-heme Fe complexes use the B3LYP/LACVP combination of functional and basis set (32-34). Therefore, it was useful to evaluate its performance
with respect to these experimental data. Geometry optimization of the E-NO and E-αKA-NO complexes with B3LYP/LACVP results in structures with Fe-N\(^{\text{NO}}\) bond lengths ~ 0.3 Å longer than the structures optimized with BP86+10%HF/Gen (Figure 5.13 and Supplementary Table S5.3).

![Figure 5.13 Comparison of geometry optimized structures of E-NO and E-αKA-NO for the BP86+10%HF/Gen and B3LYP/LACVP functional and basis set combinations.](image)

This long Fe-N\(^{\text{NO}}\) bond results in less electron density transferred to NO giving electronic structures which are best described as Fe\(^{\text{II}}\)-NO’ (4 unoccupied \(\beta\) Fe d orbitals, 2 unoccupied \(\alpha\) NO orbitals and 1 unoccupied \(\beta\) NO orbital shown in the energy level diagram in Supplementary Figure S5.9). The TD-DFT predicted absorption spectra of these complexes show a large change upon binding αKA (Supplementary Figure S5.10), and the TD-DFT calculations also predict transitions which are predominantly Fe d to NO in character, consistent with the initial electronic structure description as Fe\(^{\text{II}}\)-NO’. This calculated large change in the predicted absorption spectrum of the TD-DFT upon binding αKA is not consistent with the observed experimental data. Using the B3LYP/LACVP optimized structures and the “spectroscopic parameters” basis set (CP(PPP) on Fe) with the B3LYP functional to calculate the ZFS gives values of D that are negative and E/D values that are rhombic.
Supplementary Table S5.3). These are also not consistent with the experimentally observed strongly axial EPR signal with positive values of D.

The N-O stretch of Fe-TACN-(N_3)_2-NO obtained from rR was used in the original validation of functional/basis set (13). The B3LYP/LACVP computational approach gives an N-O stretching frequency of 1614 cm\(^{-1}\) for this complex which is significantly lower than the experimental value (1712 cm\(^{-1}\)) (13). (The BP86+10%HF/Gen method gives 1758 cm\(^{-1}\) for the N-O stretching frequency.) Finally, the free energy of NO binding to a resting, facial triad enzyme complex can be extracted from the known dissociation constant of NO with metapyrochatecase (37). This free energy of binding can be compared to the energies for the E-NO computational models. (In metapyrocatechase, the Fe center is also ligated by the facial triad, 2 water ligands, and NO and the resulting complex has an S=3/2 ground state.) The experimentally derived free energy of NO binding is ~-4.4 kcal/mol while the calculated values for the two model systems are -4.9kcal/mol for BP86+10%/Gen and +9.4 kcal/mol for B3LYP/LACVP. The calculated NO binding energy for B3LYP/LACVP predicts that the E-NO complex is not stable which is inconsistent with experiment.

Thus two different computational methods produce different electronic structures. The data support the choice of BP86+10%/Gen as an experimentally calibrated computational model to best evaluate the NO complex and by extension the O_2 complex. The difference between these two electronic structures reflects the extent of charge donation from Fe to NO which will have implications on the interactions of O_2 with the Fe center in the evaluation of the O_2 reaction coordinate to form the Fe^{IV}=O species.

5.4.3 Extension to \(\{FeO_2\}_{8}^8\)

From the analysis above, the BP86+10%HF/Gen computational approach was used to evaluate the \(\{FeO_2\}_{8}^8\) complex and the reaction coordinate of \(\alpha\)KA dependent dioxygenases. The free energy of O_2 binding to the E computational model in this study is 17 kcal/mol, consistent with the previous computational study and the \(\Delta G^8\) of 22 kcal/mol for the resting form of metapyrocathechase and the fact that the facial triad
does not react with O₂ in the absence of αKA (or substrate for metapyrocatechase) (38). To evaluate the {FeO₂}₈ complex, NO from the E-αKA-NO complex was replaced with O₂ and the structures for the different possible spin states were reoptimized.

The complex of O₂ (S=1) bound to Feᵢᵢ (S=2) can have a total spin of S=3, S=2, or S=1. Dioxygen binding in each of these spin states was evaluated. The geometry optimized structures and MO diagrams for the E-αKA-O₂ complexes are shown in Figures 5.14 and 5.15, respectively, with key geometric parameters given in Table 5.5.

![Figure 5.14](image)

**Figure 5.14 Computational models for {FeO₂}₈.** Geometry optimized structures for S=3 (left), S=2 (middle) and S=1 (right).

<table>
<thead>
<tr>
<th></th>
<th>S=3</th>
<th>S=2</th>
<th>S=1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-O</td>
<td>2.18Å</td>
<td>2.06Å</td>
<td>1.80Å</td>
</tr>
<tr>
<td>O-O</td>
<td>1.28Å</td>
<td>1.28Å</td>
<td>1.41Å</td>
</tr>
<tr>
<td>Fe-O-O</td>
<td>118.0°</td>
<td>118.5°</td>
<td>110.3°</td>
</tr>
<tr>
<td>electronic structure</td>
<td>Feᵢᵢ-O₂⁻</td>
<td>Feᵢᵢ-O₂⁻</td>
<td>Feᵢᵣ-O₂⁻²⁻</td>
</tr>
<tr>
<td>ΔG O₂ binding</td>
<td>12.0 kcal/mol</td>
<td>12.5 kcal/mol</td>
<td>5.8 kcal/mol</td>
</tr>
</tbody>
</table>
The S=3 E-αKA-O₂ optimized structure is best described as a Fe^{III} ferromagnetically coupled to O₂ (5 unoccupied β Fe d orbitals and 1 unoccupied β O₂ π* orbitals, Figure 5.15, left). The optimized E-αKA-O₂ structure in the S=2 spin state is best described as a high spin Fe^{III} antiferromagnetically coupled to O₂•- (5 unoccupied β Fe d orbitals and 1 unoccupied α O₂ π* orbital, Figure 5.15, middle, and Supplementary Figure S5.9 for contours). For the S=1 E-αKA-O₂ complex, the lowest energy structure is a new bridged binding mode to the αKA ligand (Figure 5.15, right). This structure is best described as a low spin Fe^{IV}-O₂^{2-} (4 unoccupied β Fe d orbitals and 2 unoccupied α Fe d orbitals, Figure 5.15, right, with molecular orbital contours in Figure 5.16).\(^7\) Comparison of the free energy of O₂ binding for each spin state (Table 5.5) reveals the S=1 bridged binding mode is significantly lower in energy than the S=2 and S=3 spin states.\(^8\)

\(^7\) HPPD-HPP-O₂ also shows the S=1 Fe^{IV}-O₂^{2-} bridged binding mode lowest in energy. Structures and MO diagrams are given in Supplementary Figures S5.12 and S5.13.

\(^8\) This is in contrast to the energy ordering with B3LYP/LACVP where an S=1 O₂ end on geometry is lowest in energy and an S=1 bridged binding mode is higher in energy by 22 kcal/mol. These E-αKA-O₂ optimized structures in B3LYP/LACVP have a very long Fe-O^{O₂} interaction also found for the E-αKA-NO complexes. The geometry optimized structures for
A linear transit calculation was used to evaluate O$_2$ binding to form the new bridged binding mode (Figure 5.17 and Table 5.6). At long Fe—O$_2$ distances, an S=1 description of the E-αKA-O$_2$ complex starts as an antiferromagnetic coupled E-αKA (Fe$^{II}$, S=2) and O$_2$ (S=1) system (Figure 5.17, left).
Figure 5.17 Formation of S=1 Fe$^{IV}$-O$_2$$^{2-}$ bridge. Three points along formation of S=1 Fe$^{IV}$-O$_2$$^{2-}$ bridge structure: initial Fe$^{II}$-O$_2$ (left), intermediate low spin Fe$^{III}$-O$_2$$^-$(middle) and bridged Fe$^{IV}$-O$_2$$^{2-}$(right).

Table 5.6 Steps to S=1 peroxo-bridged

<table>
<thead>
<tr>
<th></th>
<th>approach</th>
<th>intermediate</th>
<th>bridged structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-O</td>
<td>2.50Å</td>
<td>2.10Å</td>
<td>1.80Å</td>
</tr>
<tr>
<td>O-O</td>
<td>1.23Å</td>
<td>1.30Å</td>
<td>1.41Å</td>
</tr>
<tr>
<td>O-C</td>
<td>3.15Å</td>
<td>1.90Å</td>
<td>1.47Å</td>
</tr>
<tr>
<td>Fe-O$^{CO}$</td>
<td>2.31Å</td>
<td>1.91Å</td>
<td>1.84Å</td>
</tr>
<tr>
<td>Fe-O$^{CO2}$</td>
<td>2.02Å</td>
<td>1.90Å</td>
<td>1.89Å</td>
</tr>
<tr>
<td>Fe-O-O</td>
<td>120.0°</td>
<td>111.7°</td>
<td>110.3°</td>
</tr>
<tr>
<td>Fe-O-CO-C-C</td>
<td>5.2°</td>
<td>39.4°</td>
<td>55.8°</td>
</tr>
<tr>
<td>relative energy</td>
<td>0.0kcal/mol</td>
<td>-9.5kcal/mol</td>
<td>-19.4kcal/mol</td>
</tr>
<tr>
<td>electronic structure</td>
<td>Fe$^{II}$-O$_2$</td>
<td>Fe$^{III}$-O$_2$$^-$</td>
<td>Fe$^{IV}$-O$_2$$^{2-}$</td>
</tr>
</tbody>
</table>

As O$_2$ approaches the Fe$^{II}$ E-αKA complex, charge is transferred from Fe to O$_2$, giving the Fe center more ferric character. This in turn increases the interaction with αKA resulting in shorter Fe-O$^{αKA}$ bonds and strengthens the ligand field at the iron center towards low spin. These αKA interactions lead to further e$^-\$ transfer to O$_2$ resulting in an E-αKA-O$_2$ complex that is a low spin, ferromagnetically coupled Fe$^{III}$-O$_2$$^-$ (Figure 5.17, middle). Further interaction of the superoxide with the Fe center leads to transfer of the second electron to form the Fe$^{IV}$-O$_2$$^{2-}$ (Figure 5.17, right). To finish forming the bridge, the bond between peroxide and the carbonyl carbon of αKA must form. The occupied O$_2$ $\pi^*$(ip) orbital has a nucleophilic interaction with the unoccupied αKA $\pi^*$ orbital (Scheme 5.3) forming a σ bond to the carbonyl carbon of αKA and completing the bridge.
Scheme 5.3 Nucleophilic interaction between the occupied O\textsubscript{2} \pi^*(ip) orbital and the unoccupied \alpha KA \pi^* orbital.

5.4.4 Completion of \(\alpha\)-keto acid dependent non-heme iron reaction coordinate

The first experimentally observed intermediate for the \(\alpha\)-keto acid dependent dioxygenases is an Fe\textsuperscript{IV}=O which is an S=2 (39-41). From the low spin Fe\textsuperscript{IV}-O\textsubscript{2}\textsuperscript{2-} S=1 structure in Figure 5.14 right, a spin crossover point must be found to allow the reaction coordinate to intersystem cross onto the S=2 surface. Cleavage of both the C-C (to release CO\textsubscript{2}) and O-O bonds occurs before formation of the Fe\textsuperscript{IV}=O species. From Figure S5.14, changing the O-O coordinate increases the energy of both spin states. The two spin states on the C-C coordinate, however, cross in energy and this coordinate was pursued. Spin crossover on the C-C coordinate requires that the two surfaces have a similar energy and geometry (minimum energy crossing point, MECP), and their electronic structures should differ by one electron, such that the two states can spin orbit couple. The optimizations of the bridged O\textsubscript{2} structure in both the S=1 and S=2 spin states show that the geometries were very similar with the exception of the Fe-O CO\textsubscript{2} distance (which is 1.93Å in S=1 and 2.30Å in S=2). Thus a 2-D PES approach was taken to find the spin crossover point by elongation of the C-C bond while decreasing the difference between the Fe-O CO\textsubscript{2} bond lengths (Figure 5.18). A crossing point in the energies was found at a C-C bond length of ~2.1Å. The electronic structures of the S=1 and S=2 structures are both Fe\textsuperscript{III}-O\textsubscript{2}\textsuperscript{2-} where as the C-C was elongated, one e\textsuperscript{-} transferred from the \(\alpha\)KA n orbital to the Fe (Supplementary Figure
S5.15). The $S=1$ and $S=2$ states at this MECP differ by one $e^{-}$: occupied $\beta$ Fe $d_{xz}$ and $\alpha$ Fe $d_{z^2}$. Thus, these two states SOC via $L_y$ (Supplementary Figure S5.16).\(^9\)\(^10\)

**Figure 5.18 2-D spin crossover surface.** Energies for elongation of C-C while lessening the difference in Fe-$O^{\text{CO}_2}$ bond length. The energies of the two spin states cross at an Fe-$O^{\text{CO}_2}$ distance of 2.15Å and a C-C bond length of 2.10Å. The energies are relative to the optimized $S=1$ bridged structure.

Once on the $S=2$ surface, the C-C bond cleaves without any additional barrier and transfers the second $\alpha$ KA n electron to Fe forming an Fe$^{\text{II}}$-peracid (Figure 5.19, 3rd structure). The transfer of 2 $e^{-}$s from Fe$^{\text{II}}$ into the peracid $\sigma^*$ orbital to break the O-O bond is accomplished with a minimal barrier (calculated $<$1kcal/mol) to form the experimentally observed $S=2$ Fe$^{\text{IV}}$=O. The free energies for these steps of the $O_2$ reaction are given in Figure 5.20. The reaction of the Fe$^{\text{IV}}$-oxo with substrate in HPPD has been evaluated in Ref 18.

\(^9\) Movement towards the crossing point results in a weaker interaction along $N^{\text{eq}}$-Fe-$O^{\text{CO}_2}$ and a tetragonal elongation of the site which rotates the coordinate system such that the z-axis is along $N^{\text{eq}}$-Fe-$O^{\text{CO}_2}$.

\(^{10}\) Φυρτηρ ελονγατιον ωφ τηε $X-X$ βονδ ων τηε $S=1$ συρβάχε χοντινυες το βε ενεργετιχάλλψ υ νψαραβε και δ ωιτη τηε $S=1$ συρβάχε υναβε το τρανσφερ τηε σεχονδ ελεχτρον φροι τηε $\alpha$ KA n orbital (Supplementary Figure S5.15).
Figure 5.19 Electron flow for initial steps of E-αKA-O$_2$ reaction. Dioxygen binding occurs on the S=1 surface with formation of a bridge to the α-keto acid moiety. Two electrons are transferred to the O$_2$ $\pi^*$ orbital generating a low spin Fe$^{IV}$-peroxide. Elongation of the C-C bond results in transfer of $1e^-$ from the αKA n orbital to form an Fe$^{III}$-peroxide which crosses over to the S=2 surface. Once on the S=2 surface, the second electron of the αKA n orbital is transferred to Fe, generating an Fe$^{II}$-peracid and free CO$_2$. Finally, cleavage of the O-O bond leads to formation of an Fe$^{IV}$-oxo.

Figure 5.20 Free energy diagram for initial steps of E-αKA-O$_2$ reaction.
5.5 Discussion

The comparison of the NO complexes of HPPD and HPPD-HPP provides a probe of the new spectral features which arise from the α-keto acid binding to the ferric site. Four new spectral features are observed with HPP binding to the HPPD-NO complex which reflect the new electronic structure: a new αKA to FeIII charge transfer transition above 25,000 cm⁻¹ in the UV-vis absorption spectra, mixed polarization of the NO⁻ to FeIII charge transfer bands, a new low energy ligand field MCD transition < 5000 cm⁻¹, and a decrease by half in the ZFS. The new charge transfer transition reflects the donor interaction of the α-keto acid ligand to the FeIII. The new < 5000 cm⁻¹ ligand field band in the HPPD-HPP-NO complex also reflects the strong equatorial donor interaction of the α-keto acid with the {FeNO}⁷ unit which further leads to the decrease in the ZFS. Taken together, these spectroscopic changes indicate that the α-keto acid ligand is a strong donor that promotes increased charge transfer from FeIII to NO⁻.

Replacement of NO with O₂ in calculations that reproduce the α-keto acid-{FeNO}⁷ spectral features results in a new, low energy S=1 peroxy-bridged structure. Charge donation from iron to O₂ results in increased ferric character that increases the bonding and donation of the α-keto acid ligand which promotes formation of this low spin FeIV peroxy bridge (Figure 5.17). As the experimentally observed FeIV=O product of O-O cleavage has an S=2 ground state, the S=1 bridged peroxy must crossover to the S=2 surface to form the FeIV=O species. A minimum energy crossing point from the S=1 to S=2 surface along the C-C coordinate (C-C = 2.10Å) was found. The free energy to this step is ~ 17 kcal/mol (starting from E-αKA plus dioxygen) which is in reasonable agreement with experiment (∆G‡ ~ 14 kcal/mol) (39-41). Thus a bridged FeIV peroxy binding mode is a viable step along the α-keto acid dependent mononuclear non-heme Fe O₂ reaction coordinate. From this S=2 peroxy species, decarboxylation occurs and the resultant FeII-peracid undergoes O-O cleavage to give the FeIV=O species with a minimal (< 1 kcal/mol) barrier.

Previous studies, using the B3LYP/LACVP approach, have found that an S=2 FeIII-O₂⁻ species initiates the reaction with α-keto acids. However, this
functional/basis set combination generates structures with long Fe-O\textsuperscript{O2} bonds and less charge transfer from Fe to O\textsubscript{2} (i.e. less Fe\textsuperscript{III} character) which in turn results in less donor bonding by the αKA and prevents the formation of a low energy peroxo bridged structure. In this study, the experimentally validated computational methods lead to αKA binding as a strong donor ligand that drives the formation of the S=1, low spin bridged Fe\textsuperscript{IV}-peroxide intermediate.

As triplet O\textsubscript{2} approaches the Fe\textsuperscript{II}, orbital overlap would lead to antiferromagnetic coupling to give the S=1 surface (rather than the interaction of singlet dioxygen with Fe\textsuperscript{II} to give the S=2 surface). Continuing along the S=1 surface leads to the S=1 Fe\textsuperscript{IV}-peroxide intermediate that can nucleophilically attack the α-keto acid π* LUMO on the carbonyl. This intermediate provides an appealing target for mechanism-based drug design. The herbicide NTBC is already used as a substrate inhibitor of HPPD, but it does not form O\textsubscript{2} adducts. A more potent mechanism-based inhibitor for the general class of α-keto acid dependent non-heme iron enzymes could be imagined in which the inhibitor is a derivative of αKA such that the peroxo-bridged species would still form but not decarboxylate. (In contrast, the previous computational studies, which did not account for the strong donation of αKA to Fe\textsuperscript{III} and as a result did not predict a stable peroxo bridge, would not predict this as a target for inhibition. (32-34)) Finally, the effect, detailed in this study, associated with the binding of a strong donor ligand (i.e. αKA) on the initial dioxygen activation steps likely has implications for the mechanisms of other non-heme Fe oxygen activating enzymes in which the substrate or cofactor binds directly to the metal center.

5.6 Acknowledgements
This research was supported by NIH Grant GM40392.
5.7 References


avermitilis: The basis for ordered substrate addition, *Biochemistry* 42, 2072-2080.


5.7 Supporting Information

Table S5.1 Key geometric and electronic structure parameters for HPPD-HPP-NO 1 and 2

<table>
<thead>
<tr>
<th></th>
<th>HPPD-HPP-NO 1</th>
<th>HPPD-HPP-NO 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fe-N&lt;sub&gt;NO&lt;/sub&gt;</td>
<td>1.78Å</td>
<td>1.78Å</td>
</tr>
<tr>
<td>N-O</td>
<td>1.18Å</td>
<td>1.18Å</td>
</tr>
<tr>
<td>Fe-N-O</td>
<td>143°</td>
<td>143°</td>
</tr>
<tr>
<td>Charge on NO</td>
<td>-0.305</td>
<td>-0.305</td>
</tr>
</tbody>
</table>

Table S5.2 ΔSCF Fe<sup>III</sup> d-d ligand field transition energies for HPPD-HPP-NO 1

<table>
<thead>
<tr>
<th>Transition Energy</th>
<th>d&lt;sub&gt;z²&lt;/sub&gt; – d&lt;sub&gt;x²-y²&lt;/sub&gt;</th>
<th>7,500cm&lt;sup&gt;-1&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d&lt;sub&gt;xy&lt;/sub&gt; – d&lt;sub&gt;x²-y²&lt;/sub&gt;</td>
<td>12,200cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>d&lt;sub&gt;xy&lt;/sub&gt; – d&lt;sub&gt;yz&lt;/sub&gt;</td>
<td>14,000cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>d&lt;sub&gt;z²&lt;/sub&gt; – d&lt;sub&gt;xz&lt;/sub&gt;</td>
<td>13,600cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Table S5.3 Key geometric and electronic structure parameters for E-NO and E-αKA-NO with B3LYP/LACVP and BP86+10%HF/Gen

<table>
<thead>
<tr>
<th></th>
<th>E-NO</th>
<th>E-αKA-NO</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BP86+10%HF/Gen</td>
<td>B3LYP/LACVP</td>
</tr>
<tr>
<td>Fe-N&lt;sub&gt;NO&lt;/sub&gt;</td>
<td>1.76Å</td>
<td>2.03Å</td>
</tr>
<tr>
<td>N-O</td>
<td>1.17Å</td>
<td>1.19Å</td>
</tr>
<tr>
<td>Fe-N-O</td>
<td>147°</td>
<td>132.1°</td>
</tr>
<tr>
<td>Charge on NO</td>
<td>-0.219</td>
<td>-0.049</td>
</tr>
<tr>
<td>electronic structure</td>
<td>Fe&lt;sup&gt;III&lt;/sup&gt;-NO&lt;sup&gt;-&lt;/sup&gt;</td>
<td>Fe&lt;sup&gt;II&lt;/sup&gt;-NO&lt;sup&gt;-&lt;/sup&gt;</td>
</tr>
<tr>
<td>calculated value of D</td>
<td>10 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
<td>-7.9 cm&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>calculated value of E/D</td>
<td>0.035</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Figure S5.1 Fe-EDTA-NO room temperature UV-vis absorption spectrum. (Ref. 13)
Figure S5.2 Fe-EDTA-NO (A) and HPPD-NO (B) 5K, 7T MCD spectra with Gaussian resolution.

Figure S5.3 VTVH MCD isotherms for ligand field transitions. (A) HPPD-NO VTVH MCD isotherms at 15,100 cm\(^{-1}\) and (B) HPPD-HPP-NO VTVH MCD isotherms at 15,000 cm\(^{-1}\) with best fits to the data shown and polarizations indicated.

Figure S5.4 Simulated VTVH MCD for HPPD-HPP-NO 5,650 cm\(^{-1}\) band. The simulated VTVH MCD fits for a 88\% z polarized (A) and a 47\% z polarized (B) transition. Both of these fits agree within error to the experimental data (C).
Figure S5.5 Computational models for HPPD-HPP-NO. Geometry optimized structures for S=3/2 HPPD-HPP-NO 1 (from molecular modeling studies) and HPPD-HPP-NO 2 (from standard orientation of α-ketoglutarate in αKA dependent dioxygenases).

Figure S5.6 Molecular orbital diagrams for three computational models of S=3/2 α-keto acid bound {FeNO}^7. Comparison of the molecular orbital diagrams for E-αKA-NO, HPPD-HPP-NO 1 and HPPD-HPP-NO 2.
Figure S5.7 TD-DFT predicted absorption spectra for computational models of $S=3/2$ $\alpha$-keto acid bound {FeNO}$^7$. The predicted absorption spectrum for E-\(\alpha\)KA-NO, HPPD-HPP-NO 1 and HPPD-HPP-NO 2. Note that the increased intensity in the HPPD-HPP-NO 1 predicted absorption spectrum results from protein derived Glu transitions which have shifted down in energy with respect to the other models.

Figure S5.8 Comparison of the $d_{z^2}$ and $d_{x^2-y^2}$ orbitals between E-NO and E-\(\alpha\)KA-NO. Molecular orbitals were aligned by setting the energy of the highest occupied b NO$^-$ orbital to 0.
Figure S5.9 Molecular orbital diagrams for E-NO and E-αKA-NO using B3LYP/LACVP.

Figure S5.10 Comparison of TD-DFT predicted absorption spectra for BP86+10%HF/Gen and B3LYP/LACVP for both E-NO and E-αKA-NO.
Figure S5.11 Molecular orbital contours for S=2 E-aKA-O_{2}.

Figure S5.12 HPPD-HPP-O_{2} 1 S=1 bridged structure.
Figure S5.13 Molecular orbital diagram for S=1 bridged HPPD-HPP-O₂ 1.

Figure S5.14 Choice of O-O or C-C coordinate for spin crossover. The relative energies (to the optimized S=1 bridged structure) for elongation of C-C (blue) or O-O (green) in the S=1 and S=2 spin states.
Figure S5.15 Molecular orbital diagram for S=1 and S=2 E-aKA-O2 at the crossing point

occupied b121 (S=1) becomes occupied a123 (S=2)

Figure S5.16 Spin crossover. Relevant molecular orbital contours for S=1 and S=2 at the crossing point and axes for the rotated coordinate system.
Chapter 6

Reaction Coordinate of Isopenicillin N Synthase: Oxidase versus Oxygenase Activity

6.1 Introduction

Isopenicillin N-synthase (IPNS) is a mononuclear non-heme iron enzyme found in fungi and bacteria that catalyzes the formation of isopenicillin N, a bicyclic precursor to the β-lactam antibiotics including the penicillins and cephalosporins. (1,2) IPNS binds a tripeptide substrate δ-(L-α-aminoadipoyl)-L-cysteinyld-valine (ACV) and performs a four electron oxidative double ring closure, fully reducing one equivalent of O₂ to H₂O and closing the β-lactam and thiazolidine rings of isopenicillin N. (3-6) (Scheme 1) This oxidase reactivity is unusual as most non-heme iron enzymes catalyze oxygenation reactions.

Scheme 6.1 The four electron oxidative double ring closure of ACV to form Isopenicillin N

Previous studies of the IPNS-ACV {FeNO} complex revealed that a major factor contributing to the oxidase reactivity of IPNS is charge donation from the ACV thiolate ligand, which renders the formation of the Fe³⁺-superoxide complex energetically favorable and drives the reaction only at the Fe center. (7) This single center, one electron reaction allows IPNS to avoid the bridged binding of O₂ between the Fe²⁺ and the substrate/cofactor required for its two electron reduction; a reaction generally invoked for the non-heme Fe enzymes that leads to oxygen insertion. The thiolate coordination of the IPNS ACV substrate further activates the reactive Fe³⁺-superoxide complex of the enzyme through a configuration interaction with the bound superoxide π* orbital which creates a Frontier Molecular Orbital (FMO) with correct orientation for H-atom abstraction from the ACV substrate. (7)

Density Functional Theory (DFT) studies of the reaction coordinate of IPNS reveal that the Fe³⁺-superoxide FMO will carry out the H-atom abstraction from the
cysteiny1 β carbon of ACV with a low barrier. H-atom abstraction from ACV is accompanied by an additional electron transfer from the ACV substrate to yield an FeII-hydroperoxide and a double bond between the thiol sulfur and adjacent carbon of ACV. (Box in Scheme 2) (8-11) This IPNS-FeII-hydroperoxide is proposed to deprotonate either the amide nitrogen of ACV (10,11) or the iron-bound water (9) and cleave the O-O bond heterolytically, resulting in the formation of H2O and an FeIV-oxo species. An SN2-type reaction occurs between the lone pair on the ACV amide nitrogen and the carbon of the C-S double bond, leading to ring closure and formation of the beta-lactam ring of isopenicillin N. (Center reaction, Scheme 2)

Scheme 6.2 Three Reaction Pathways of FeII-Hydroperoxide

The heterolytic cleavage of the O-O bond of an FeII-hydroperoxide is unusual, as FeII-hydroperoxide is generally thought to undergo Fenton chemistry to cleave the O-O bond homolytically, resulting in the production of a hydroxyl radical and an FeIII-
oxo(OH−) species. (12-14) Baldwin et. al. have studied a series of ACV substrate analogues and have proposed mechanisms for their reactivity with dioxygen based upon crystal structures of the product complexes of these IPNS-FeII-analogue complexes after exposure to dioxygen. (15-19) In one such analogue ACOV1, the amide nitrogen of the ACV valine is replaced with an oxygen atom as an ester, removing one of the proposed sources of the proton that could assist in O-O bond cleavage. Upon exposure to dioxygen, this analogue results in the hydroxylation of the cysteine carbon, effectively modifying the reactivity of IPNS from an oxidase to an oxygenase through a proposed nucleophilic attack of the FeII-hydroperoxide. (19) In this study, DFT calculations calibrated by our experimental studies of the IPNS-ACV-{FeNO}7 complex (7) were performed to explore how substrate interactions with the FeII-hydroperoxide moiety can avoid Fenton chemistry (homolytic cleavage) and modify the reactivity of this species, from heterolytic O-O bond cleavage to nucleophilic attack (Scheme 2). The exploration of factors governing reactivity (substrate direction of oxidase vs. oxygenase activity) adds a new facet of understanding to the body of knowledge on this important enzyme that has not been addressed in previous theoretical studies.

6.2 Methods

The starting geometry for the Fe-IPNS-ACV-O2 complex was taken from the crystal structure of Fe-IPNS-ACV-NO from Aspergillus nidulans. (11) Protein-derived ligands were truncated with methyl imidizoles modeling histidines and propionate modeling aspartate. The ACV substrate was truncated to remove the 6 carbon aminoacidipoyl chain but was otherwise left intact. The β-carbons of the protein ligands were frozen relative to each other to impose the constraints of the protein backbone. The cysteine nitrogen was also frozen relative to the alpha-carbons of the protein ligands to mimic hydrogen bonding to the substrate in the protein pocket.

All complexes were geometry optimized using the Gaussian 03 software package (20), with the spin unrestricted BP86 functional (21,22) with 10% Hartree-
Fock exchange under tight convergence criteria. This functional was previously calibrated for mononuclear non-heme iron \{FeNO\} \(^7\) complexes. (23) Geometry optimizations were carried out using the Pople 6-311G* basis set on Fe, S, and the O\(_2\) unit with the 6-31G* basis set on the remaining atoms. Single point calculations were performed on the optimized structures to generate molecular orbitals using the functional and basis set above. Orbital compositions were calculated using QMForge (24) and optimized structures and molecular orbitals were visualized using Molden version 4.1. (25) Frequencies and thermodynamic parameters were calculated using the split 6-311G*/6-31G* basis set. In order best describe the effects of the ACV sulfur interaction with the Fe-IPNS models, single point energies were calculated for the Fe-IPNS-ACV complexes using the 6-311+G(2d,p) basis set. Solvation effects on the energy of the optimized structure were included using the Polarized Continuum Model (PCM) (26) with a dielectric constant \(\varepsilon = 4.0\) to model the protein environment.

Cartesian coordinates for all models are given in the supplementary information.

6.3 Results

The Potential Energy Surfaces (PES) for homolytic O-O bond cleavage, heterolytic O-O bond cleavage and nucleophilic attack of three models of the IPNS active site were obtained as follows. The starting geometry for the Fe-IPNS-HO\(_2\) complexes was taken from previously optimized Fe-IPNS-ACV-O\(_2\) structure (7) with subsequent ACV H-atom abstraction. After H-atom abstraction from the ACV carbon, the Fe\(^{II}\)-IPNS-ACV-HO\(_2\) complex relaxes through reorientation of the hydroperoxide moiety to optimize hydrogen bonding interactions with the ACV amide and carboxylate residues (Figure 6.1, Model 1).\(^2\) In order to model the nucleophilic attack by hydroperoxide seen for the ACOV substrate, the IPNS-Fe-ACV-HO\(_2\) was truncated between the \(\alpha\)-carbon and carbonyl carbon of the cysteine (Figure 6.1, Model 2), thus removing the amide nitrogen. Finally, this truncated ACOV model was used to explore the homolytic cleavage reaction by introducing a constraint on the model to fix the distance between the distal peroxide oxygen atom and the carbon atom of the

\(^2\) The reorientation of the hydroperoxide was accomplished through contraction of the distance between the ACV amide nitrogen and cysteinyl carbon to a distance of approximately 2.8Å.
substrate, preventing the interaction of the distal oxygen with the ACOV carbon (Figure 6.1, Model 3).

Figure 6.1 Models Used to Calculate Three Reaction Pathways of Fe$^{II}$-Hydroperoxide The arrow in Model 3 indicates the extra constraint added to prohibit interaction of the distal oxygen with the carbon of the substrate.

Figure 6.2 Molecular Orbital Diagram of IPNS-ACV-Fe$^{II}$-Hydroperoxide spin unrestricted contours given for representative molecular orbitals. Note the 4 unoccupied $\beta$ d orbitals indicating Fe$^{II}$, and the unoccupied $\alpha$, $\beta$ pair of the $\sigma^*$ orbital
of peroxide with the $\alpha$, $\beta$ unoccupied pair of C-S $\pi^*$ orbitals of the substrate $\pi$ bond. The observed $d_{xy}$ character (24%) in the C-S $\pi^*$ bond reflects backbonding.

For all three models studied, the electronic structure of the starting Fe$^{II}$-hydroperoxide complex is similar and is shown in Figure 6.2. Both the in-plane (ip) and out-of-plane (op) $2\pi^*$ orbitals of the hydroperoxide are fully occupied and the unoccupied $\sigma^*$ orbital of the hydroperoxide has come down in energy, in preparation for O-O bond cleavage. (27) As previously described, (9) the Fe $d_{xy}$ orbital in the $\beta$ manifold is occupied, and a double bond has formed between the cysteine carbon and sulfur atoms, evidenced by the unoccupied C-S $\pi^*$ orbital in both the $\alpha$ and $\beta$ manifolds. At the optimized O-O bond length, there is a mixing of the occupied $\beta$ Fe $d_{xy}$ orbital and the C-S $\pi^*$ orbital, which is indicative of backbonding of the occupied Fe$^{II}$ d orbital into the C-S $\pi^*$ double bond. As the O-O bond is elongated, this backbonding is alleviated, localizing the electron on the Fe and allowing the occupied $\beta$ Fe $d_{xy}$ orbital to rotate to interact with the descending hydroperoxide $\sigma^*$ orbital. (Figure S6.1)

![Figure 6.3 Geometric Structures of IPNS-Fe$^{II}$-Hydroperoxide Complexes with O-O Bond Elongation](image)

The three models undergo homolytic cleavage (top, 3), heterolytic O-O bond cleavage (middle, 1) and nucleophilic attack by the peroxide (bottom, 2).
Figure 6.4 Donor and Acceptor Orbitals for Homolytic Cleavage, Heterolytic Cleavage and Nucleophilic Attack. In the homolytic cleavage reaction, one β electron is transferred from the Fe d to the hydroperoxide σ* orbital. Heterolytic cleavage and nucleophilic attack involve the transfer of an α/β electron pair. For clarity, only the alpha donor and acceptor orbitals of these electron pairs are shown.

Starting with the optimized Fe$^{II}$-hydroperoxide structures, the PES of each model was explored by optimizing each structure with incrementally increasing O-O bond distance until the O-O bond was fully cleaved. Optimized geometries for the O-O elongation along the PES’s of 1, 2 and 3 are shown in Figure 6.3 and key computational results are summarized in Table S6.1. The PES of 3 corresponds to the expected Fenton chemistry with homolytic cleavage of the O-O bond. This homolytic cleavage is accomplished by transfer of one β electron from the Fe d$_{xy}$ orbital to the O-O σ* orbital (Figure 6.4, left), resulting in the formation of an Fe$^{III}$-oxo complex and a hydroxyl radical. This is evidenced by the accumulation of negative spin density on the distal oxygen (-0.48 at O-O 2.20Å. See Table S6.1). The PES of 1 (Figure 6.3, middle) proceeds as a heterolytic cleavage of the O-O bond, with one electron in each spin manifold transferred from the β Fe d$_{xy}$ orbital and the α Fe d$_{z^2}$ into the O-O σ* orbital (Figure 6.4, middle). This produces an Fe$^{IV}$-oxo complex and hydroxide, which
remains within hydrogen-bonding distance of the amide of the ACV valine (Figure 6.3, middle right). The PES of model 2 involves a nucleophilic attack of the hydroperoxide moiety at the ACOV carbon followed by heterolytic O-O bond cleavage (Figure 6.3, bottom). Nucleophilic attack is accomplished by the transfer of an electron pair from the peroxide $\pi^*$ out-of-plane orbital to the C-S $\pi^*$ orbital (Figure 6.4, right), followed by two electron transfer from the Fe$^{II}$ to the O-O $\sigma^*$ orbital to give an Fe$^{IV}$-oxo complex with a hydroxylated substrate carbon.

These calculated model reactions demonstrate that the same Fe$^{II}$-hydroperoxide unit can undergo different reactions modes. The factors that govern the choice of reaction pathways were evaluated further. Table S6.1 shows that at short distances, models 1 (heterolytic) and 3 (homolytic) show similar electronic structure up to 1.9Å for the transfer of the first electron from Fe$^{II}$ to the hydroperoxide $\sigma^*$ orbital. A plot of the energies along the reaction coordinate for models 1 (heterolytic) and 3 (homolytic) (Figure 6.5) shows an initial stabilization of this electron transfer, and this stabilization is expected to be similar for the second electron in the heterolytic O-O bond cleavage pathway as opposed to the homolytic pathway.

![Figure 6.5 Energetics of O-O Bond Cleavage](image)

**Figure 6.5 Energetics of O-O Bond Cleavage** The relative electronic energies are plotted for the homolytic cleavage (3, red circles), heterolytic cleavage (1, blue triangles) and nucleophilic attack (2, black squares) models as the O-O bond is elongated. Note that the nucleophilic attack model is only valid for O-O bond cleavage in ACOV and that for the ACV substrate, an additional barrier to break the amide-hydroperoxide hydrogen bond is present.
Comparing the structures, the distal oxygen of the heterolytically cleaving 1 is within hydrogen bonding distance of the amide proton of the ACV valine, while the distal oxygen of the homolytic coordinate 3 has no such interaction. This hydrogen bond in 1 stabilizes the transfer of both electrons to the distal oxygen.

Importantly, 1 has an unoccupied \( \sigma^* \) orbital that is polarized toward the proximal oxygen (\( O_p \), Figure 6.6, left), while the \( \sigma^* \) orbital of 3 is polarized toward the distal oxygen (\( O_d \), Figure 6.6, middle). When the \( \sigma^* \) orbital is polarized toward the proximal oxygen, its bonding \( \sigma \) counterpart is polarized toward the distal oxygen. As this peroxide \( \sigma \) orbital is fully occupied, this polarization will lead to an electron pair being transferred to the distal oxygen upon O-O bond cleavage thus the heterolytic cleavage of the O-O bond. Polarization of the \( \sigma^* \) orbital toward the distal oxygen allows only for the transfer of a single electron to the distal oxygen and results in homolytic cleavage. The hydrogen bonding interaction of 1 can be modeled by adding an equivalent point dipole to 3, which reverses the polarization of the \( \sigma^* \) orbital (Figure 6.6, right). Elongation of the O-O bond in the presence of a point dipole also leads to heterolytic cleavage (Table S6.2). Therefore, it is the interaction of the dipole of the amide N-H bond with the distal oxygen in 1 that directs the ACV complex toward heterolytic cleavage of the O-O bond.

\[ \alpha \text{ O-O } \sigma^* \text{ Orbitals} \]

**Figure 6.6 Alpha Hydroperoxide \( \sigma^* \) Orbitals** This orbital polarizes toward the proximal oxygen in Model 1, directing the reaction to heterolytic O-O bond cleavage, and toward the distal oxygen in Model 3, directing its reaction to homolytic O-O bond cleavage. By modeling the dipole interaction of the ACV amide N-H, the polarization of the \( \sigma^* \) bond in Model 3 is reversed.
In comparing the nucleophilic attack relative to the heterolytic O-O bond cleavage (2 vs. 1), both of these pathways proceed with similarly small reaction barriers (Figure 6.5). From a more detailed transition state analysis on model 1, the ΔG‡ is 4.1 kcal/mol. (The coordinates are given in Table S6.5; the transition state occurs at an O-O bond length of approximately 1.6 Å.) In order for 1 to undergo a nucleophilic attack, the hydrogen bond between the distal oxygen and the amide proton would have to be broken to allow the peroxide to rotate to the proper orientation (Scheme 3).

![Scheme 6.3 Rotation of the Peroxide for Nucleophilic Attack](image)

The strength of the hydrogen bonding interaction that must be broken in order for the hydroperoxide to undergo this rotation is calculated to be 5.6 kcal/mol, which is already larger than the barrier for heterolytic cleavage (Figure 6.5). This hydrogen bonding interaction with the amide proton in 1 would thus bias the reaction of the Fe^{II}-hydroperoxide toward heterolytic cleavage over nucleophilic attack. In this way, the hydrogen bonding interaction of the Fe^{II}-hydroperoxide helps distinguish between reactions possible for the IPNS-Fe^{II}-hydroperoxide-ACV intermediate. As the iron-bound water (the alternate proposed source for the proton in the reaction with ACV (9)) would be present in both the IPNS-Fe^{II}-hydroperoxide-ACV and IPNS-Fe^{II}-hydroperoxide-ACOV complexes, hydrogen bonding to the water would direct both complexes toward heterolytic cleavage of the O-O bond. Thus the observed nucleophilic reactivity of the ACOV complex argues strongly against the coordinated water being the source of the proton in either reaction.
The coordinates were extended beyond O-O bond cleavage to complete the reaction cycle for the different substrates. For 1, the hydroxide produced upon O-O bond cleavage is within hydrogen-bonding distance of the amide proton of the ACV valine and can abstract this proton with a negligible barrier (< 1 kcal/mol) compared to the previous intermediate. This leaves a lone pair of electrons on the valine amide with appropriate overlap for a S_N2-type reaction with the C-S π* orbital to close the β-lactam ring of isopenicillin N (Figure 6.7).

![Image](image.png)

**Figure 6.7 Donor and Acceptor Orbitals and Product for Beta-Lactam Ring Closure** Deprotonation of the ACV amide leaves a lone pair on the amide N with the correct orientation for SN2 nucleophilic attack at the C-S double bond.

The orientation of the lone pair on the ACV amide is mechanistically significant, thus the ACV amide must be deprotonated before β-lactam ring closure. This reaction pathway is similar to that of the previous literature. For 2, the transfer of two protons, one from the hydroxyl group and one from the hydroxylated carbon of ACOV to the Fe-oxo would lead to the Fe-IPNS-Thiocarboxylate structure observed for the oxygen-exposed Fe-IPNS-ACOV complex (Scheme 2, bottom).

**6.4 Discussion**

In the first stage of the reaction of the IPNS- Fe^{II} -ACV complex with dioxygen, the ACV thiolate bond activates the one electron reduction of dioxygen to form an Fe^{III}-superoxide complex with good FMO overlap for H-atom abstraction from the substrate. This is atypical for most mononuclear non-heme Fe enzymes as
seen in, for example, the \( \alpha \)-ketoglutarate dependent and extradiol dioxygenases where the unfavorable one electron reduction of \( O_2 \) is circumvented by adopting a bridged binding mode allowing \( 2e^- \) reduction that results in oxygenase activity. This mechanism of a one \( e^- \) reduction of \( O_2 \) avoids its bridged binding to substrate and opens up a pathway for oxidase activity in IPNS not energetically available for other mononuclear non-heme Fe enzymes.

The initial H-atom abstraction in IPNS is calculated to produce an Fe\( \text{II} \)-hydroperoxide complex that can undergo a variety of reaction channels, dependent upon its interaction with the substrate. While Fe\( \text{II} \)-hydroperoxide species are generally thought to undergo Fenton chemistry (i.e. homolytic O-O bond cleavage in solution), the experimental evidence from reactions of IPNS-Fe\( \text{II} \)-ACV and analogue complexes suggests that an Fe\( \text{II} \)-hydroperoxide can follow alternative reaction pathways including heterolytic O-O bond cleavage and nucleophilic attack by the peroxide at a substrate double bond. DFT calculations on three IPNS-Fe\( \text{II} \)-HO\( \text{2} \) models show that all three of the above reaction pathways are energetically accessible.

A fourth reaction pathway, not invoked in previous IPNS mechanisms but proposed for hydroxyethylphosphonate dioxygenase (HEPD), involves attack via the proximal oxygen of the hydroperoxide (with respect to the Fe center). This reaction is different from HEPD in that, for IPNS, it would involve the nucleophilic attack by the hydroperoxide but in HEPD, a hydroperoxylolation is invoked.(28) To evaluate this pathway, the \( \text{C}…\text{O}_p \) reaction coordinate was examined. Starting from the optimized Fe\( \text{II} \)-hydroperoxide structure (Figure 6.1, model 2), the attack by the proximal oxygen occurs with a barrier of 2-3 kcal/mol, comparable to that observed in Figure 6.4 for the nucleophilic attack by the distal oxygen.
Scheme 6.4 Attack by the distal and proximal oxygen of the hydroperoxide

Upon formation of the C-O_p bond, the C-S thioaldehyde is reduced while the Fe center remains Fe^{II} (Scheme 4, bottom). This intermediate is lower in energy than the starting structure by ~21 kcal/mol (compared to -67 kcal/mol for the Fe^{IV}-oxo intermediate from the nucleophilic attack by the distal oxygen, Scheme 4, top). From this intermediate, the O-O bond can be cleaved heterolytically and a proton lost from the C center to give the crystallographically observed thio carboxylate intermediate. Although along a different reaction coordinate and with less driving force, attack by the proximal oxygen appears to be an additional, viable pathway that will lead to the product observed in the crystallography.

The DFT calculations show that the substrate interactions with the Fe^{II}-hydroperoxide moiety can determine its reactivity. For the ACV substrate, hydrogen bonding interactions with the amide hydrogen polarize the peroxide \( \sigma^* \) orbital to make the heterolytic cleavage of the O-O bond energetically favorable. In the absence of this hydrogen bonding interaction, nucleophilic attack of either the distal or proximal O of the hydroperoxide at the carbon of the ACV C-S double bond occurs, leading to the substrate hydroxylation observed experimentally for ACOV. In this way the hydrogen bonding interaction with the ACV substrate directs the Fe^{II}-hydroperoxide to heterolytic cleavage and enables the unusual oxidase activity of this enzyme.
6.5 Acknowledgements
This research was supported by NIH Grant GM40392. We thank Dr. Marcus Lundberg for insightful discussions.

6.6 References


### 6.7 Supporting Information

**References Full Reference #20**


**Figure S6.1 Alleviation of backbonding with O-O bond elongation.** As the O-O bond is elongated mixing with the C-S π* orbital is reduced and the dxy orbital rotates and interacts with the hydroperoxide σ* orbital.
### Table S6.1 Key Computational Results

<table>
<thead>
<tr>
<th>Bond Length (Å)</th>
<th>Spin Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>OO</td>
<td>Fe-S</td>
</tr>
<tr>
<td>Model 1</td>
<td></td>
</tr>
<tr>
<td>1.46</td>
<td>2.50</td>
</tr>
<tr>
<td>1.60</td>
<td>2.54</td>
</tr>
<tr>
<td>1.70</td>
<td>2.89</td>
</tr>
<tr>
<td>1.90</td>
<td>2.83</td>
</tr>
<tr>
<td>2.20</td>
<td>2.76</td>
</tr>
<tr>
<td>2.50</td>
<td>2.64</td>
</tr>
<tr>
<td>Model 2</td>
<td></td>
</tr>
<tr>
<td>1.42</td>
<td>2.44</td>
</tr>
<tr>
<td>1.60</td>
<td>2.54</td>
</tr>
<tr>
<td>1.75</td>
<td>2.53</td>
</tr>
<tr>
<td>1.90</td>
<td>2.37</td>
</tr>
<tr>
<td>2.05</td>
<td>2.32</td>
</tr>
<tr>
<td>Model 3</td>
<td></td>
</tr>
<tr>
<td>1.42</td>
<td>2.44</td>
</tr>
<tr>
<td>1.60</td>
<td>2.50</td>
</tr>
<tr>
<td>1.75</td>
<td>2.68</td>
</tr>
<tr>
<td>1.90</td>
<td>2.70</td>
</tr>
<tr>
<td>2.05</td>
<td>2.71</td>
</tr>
<tr>
<td>Free(2.20)</td>
<td></td>
</tr>
<tr>
<td>Bond Length (Å)</td>
<td>Spin Density</td>
</tr>
<tr>
<td>OO</td>
<td>Fe-S</td>
</tr>
<tr>
<td>1.42</td>
<td>2.44</td>
</tr>
<tr>
<td>1.60</td>
<td>2.50</td>
</tr>
<tr>
<td>1.75</td>
<td>2.68</td>
</tr>
<tr>
<td>1.90</td>
<td>2.70</td>
</tr>
<tr>
<td>2.05</td>
<td>2.71</td>
</tr>
<tr>
<td>Free(2.20)</td>
<td></td>
</tr>
</tbody>
</table>

### Table S6.2 Spin Densities from Elongation of the O-O bond with the dipole present

<table>
<thead>
<tr>
<th>Bond Length (Å)</th>
<th>Spin Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>O-O (Å)</td>
<td>Fe</td>
</tr>
<tr>
<td>1.75</td>
<td>3.76</td>
</tr>
<tr>
<td>1.90</td>
<td>3.77</td>
</tr>
<tr>
<td>2.05</td>
<td>3.73</td>
</tr>
<tr>
<td>2.20</td>
<td>3.59</td>
</tr>
</tbody>
</table>

### Table S6.3 Cartesian Coordinates of Optimized IPNS-ACV-FeII-Peroxide (Model 1)

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.394007</td>
<td>-2.264633</td>
<td>1.330973</td>
</tr>
<tr>
<td>N</td>
<td>1.605436</td>
<td>-1.133044</td>
<td>1.445520</td>
</tr>
<tr>
<td>C</td>
<td>1.188543</td>
<td>-1.093504</td>
<td>2.704090</td>
</tr>
<tr>
<td>N</td>
<td>1.680047</td>
<td>-2.159461</td>
<td>3.395994</td>
</tr>
<tr>
<td>C</td>
<td>2.456378</td>
<td>-2.927673</td>
<td>2.535435</td>
</tr>
<tr>
<td>Fe</td>
<td>1.025291</td>
<td>0.177297</td>
<td>-0.158453</td>
</tr>
<tr>
<td>O</td>
<td>2.607274</td>
<td>-0.581714</td>
<td>-1.263451</td>
</tr>
<tr>
<td>C</td>
<td>3.035883</td>
<td>-0.297749</td>
<td>-2.444075</td>
</tr>
</tbody>
</table>
C      4.244234    -1.093783    -2.945894
C      4.703662    -2.216254    -2.017996
C      3.134931    -4.191713     2.950798
N      2.403460     1.762493     0.373586
C      3.724547     1.682557     0.391177
N      4.270423     2.909490     0.614839
C      3.479314     5.287521     0.960925
S     -0.320710    -1.603954    -1.265666
C     -1.368124    -2.155431    -0.110721
C     -2.714716    -2.740130    -0.402081
N     -3.597239    -3.597239    -1.583714
O     -0.444280     1.031647     0.770263
O     -1.258529     0.260804     1.710500
O      0.573119     1.494784    -1.845448
C     -3.837009    -1.633115    -0.500893
O     -4.884949    -1.908636    -1.085056
N     -5.588031    -0.434687     0.090782
C     -4.584107     0.622350     0.062677
C     -4.697617     1.274777     1.430095
O     -3.826692     1.335011     2.288145
C     -4.325131     1.728675    -1.018127
C     -4.184382     1.087981    -2.405230
C     -3.119723     2.620508    -0.686030
O     -2.560975     0.577264    -3.213042
O     -5.901449     1.866712     1.596769
H     -5.862307     2.314570     2.469937
H     -5.543126     0.140491    -0.180256
H     -5.237389     2.352244    -1.014860
H     -4.139723     1.871845    -3.177813
H     -3.257186     0.496624    -2.470521
H     -5.024802     0.416249    -2.633663
H     -2.941078     3.331000    -1.508979
H     -3.285778     3.215405     0.226830
H     -2.195537     2.037359    -0.532763
H     -2.687210    -0.271183     0.568344
H     -3.708463    -3.627036    -1.898399
H     -2.209593    -3.117592    -2.327220
H     -1.113421    -2.007441     0.942820
H     -1.913845     0.930264     2.008939
H     3.686940     4.610267     2.098226
H     3.857160     4.030207     3.769163
H     2.414757     4.956977     3.286584
H     1.489301    -2.369080     4.370307
H     0.501379    -0.355869     3.106095
H     2.855787    -2.515717     0.381607
H     5.580079    -2.737624    -2.431350
H     4.970736    -1.828325    -1.023975
H     3.901636    -2.958949    -1.877321
H     3.983390    -1.483929    -3.943438
H     5.058288    -0.368989    -3.123264
H     -0.243724     1.241439    -2.310163
H     1.339651     1.239752    -2.480808
H     2.517693     5.814783     1.027035
Table S6.4 Cartesian Coordinates of Optimized IPNS-ACOV-Fe\textsuperscript{II}-Peroxide (Models 2 & 3)

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>-2.150520</td>
<td>-2.714020</td>
<td>-0.476916</td>
</tr>
<tr>
<td>N</td>
<td>-1.358521</td>
<td>-1.651373</td>
<td>-0.861528</td>
</tr>
<tr>
<td>C</td>
<td>-1.237284</td>
<td>-1.727642</td>
<td>-2.177515</td>
</tr>
<tr>
<td>N</td>
<td>-1.922399</td>
<td>-2.804003</td>
<td>-2.652288</td>
</tr>
<tr>
<td>C</td>
<td>-2.516986</td>
<td>-3.453331</td>
<td>-1.577012</td>
</tr>
<tr>
<td>Fe</td>
<td>-0.453657</td>
<td>-0.180903</td>
<td>0.418424</td>
</tr>
<tr>
<td>O</td>
<td>-0.935272</td>
<td>-1.662339</td>
<td>1.965554</td>
</tr>
<tr>
<td>C</td>
<td>-3.343414</td>
<td>-4.685890</td>
<td>-1.714623</td>
</tr>
<tr>
<td>N</td>
<td>0.139727</td>
<td>1.145638</td>
<td>-1.191211</td>
</tr>
<tr>
<td>C</td>
<td>1.358532</td>
<td>1.262194</td>
<td>-1.836339</td>
</tr>
<tr>
<td>C</td>
<td>1.337199</td>
<td>2.333917</td>
<td>-2.701194</td>
</tr>
<tr>
<td>N</td>
<td>0.060381</td>
<td>2.867361</td>
<td>-2.562764</td>
</tr>
<tr>
<td>C</td>
<td>-0.627622</td>
<td>2.123829</td>
<td>-1.650081</td>
</tr>
<tr>
<td>C</td>
<td>2.367730</td>
<td>2.911816</td>
<td>-3.614638</td>
</tr>
<tr>
<td>O</td>
<td>1.342447</td>
<td>-1.121786</td>
<td>0.130370</td>
</tr>
<tr>
<td>C</td>
<td>1.931596</td>
<td>-2.012084</td>
<td>0.847296</td>
</tr>
<tr>
<td>O</td>
<td>1.427342</td>
<td>-2.629441</td>
<td>1.823496</td>
</tr>
<tr>
<td>S</td>
<td>0.529331</td>
<td>1.022150</td>
<td>2.296991</td>
</tr>
<tr>
<td>C</td>
<td>0.145728</td>
<td>2.637856</td>
<td>2.082932</td>
</tr>
<tr>
<td>C</td>
<td>-0.162744</td>
<td>3.550324</td>
<td>3.241273</td>
</tr>
<tr>
<td>O</td>
<td>-2.091320</td>
<td>0.747161</td>
<td>0.609554</td>
</tr>
<tr>
<td>C</td>
<td>-2.297944</td>
<td>2.137959</td>
<td>0.804492</td>
</tr>
<tr>
<td>S</td>
<td>3.373691</td>
<td>-2.364083</td>
<td>0.473571</td>
</tr>
<tr>
<td>C</td>
<td>4.037171</td>
<td>-1.403496</td>
<td>-0.510745</td>
</tr>
<tr>
<td>N</td>
<td>1.090692</td>
<td>4.237120</td>
<td>3.591698</td>
</tr>
<tr>
<td>H</td>
<td>0.915490</td>
<td>4.912753</td>
<td>4.342586</td>
</tr>
<tr>
<td>H</td>
<td>1.742642</td>
<td>3.542569</td>
<td>3.968440</td>
</tr>
<tr>
<td>H</td>
<td>0.467551</td>
<td>3.134979</td>
<td>1.158387</td>
</tr>
<tr>
<td>H</td>
<td>-1.524660</td>
<td>2.408468</td>
<td>1.398952</td>
</tr>
<tr>
<td>H</td>
<td>3.292412</td>
<td>2.322396</td>
<td>-3.548375</td>
</tr>
<tr>
<td>H</td>
<td>2.043135</td>
<td>2.905531</td>
<td>-4.669221</td>
</tr>
<tr>
<td>H</td>
<td>2.618882</td>
<td>3.952606</td>
<td>-3.348938</td>
</tr>
<tr>
<td>H</td>
<td>-0.294720</td>
<td>3.688501</td>
<td>-3.042080</td>
</tr>
<tr>
<td>H</td>
<td>-1.627194</td>
<td>2.352423</td>
<td>-1.284620</td>
</tr>
<tr>
<td>H</td>
<td>2.162868</td>
<td>0.568832</td>
<td>-1.613330</td>
</tr>
<tr>
<td>H</td>
<td>5.082037</td>
<td>-1.693685</td>
<td>-0.704563</td>
</tr>
<tr>
<td>H</td>
<td>3.502111</td>
<td>-1.395922</td>
<td>-1.472257</td>
</tr>
<tr>
<td>H</td>
<td>4.036887</td>
<td>-0.375710</td>
<td>-0.117348</td>
</tr>
<tr>
<td>H</td>
<td>3.941441</td>
<td>-2.427119</td>
<td>1.415743</td>
</tr>
<tr>
<td>H</td>
<td>3.361981</td>
<td>-3.391907</td>
<td>0.069416</td>
</tr>
<tr>
<td>H</td>
<td>-1.060949</td>
<td>-1.222754</td>
<td>2.825995</td>
</tr>
<tr>
<td>H</td>
<td>-0.021753</td>
<td>-2.146635</td>
<td>2.017249</td>
</tr>
<tr>
<td>H</td>
<td>-3.694080</td>
<td>-5.003132</td>
<td>-0.723686</td>
</tr>
</tbody>
</table>
Table S6.5 Cartesian Coordinates from the Transition State of IPNS-ACV-Peroxide O-O Bond Cleavage (Model 1)

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.438642</td>
<td>-2.211706</td>
<td>1.413955</td>
</tr>
<tr>
<td>N</td>
<td>1.610804</td>
<td>-1.104783</td>
<td>1.470836</td>
</tr>
<tr>
<td>C</td>
<td>1.083642</td>
<td>-1.090551</td>
<td>2.688499</td>
</tr>
<tr>
<td>N</td>
<td>1.543948</td>
<td>-2.150558</td>
<td>3.409626</td>
</tr>
<tr>
<td>C</td>
<td>2.414947</td>
<td>-2.886615</td>
<td>2.613240</td>
</tr>
<tr>
<td>Fe</td>
<td>1.087480</td>
<td>0.218682</td>
<td>-0.131081</td>
</tr>
<tr>
<td>O</td>
<td>2.666048</td>
<td>-0.580781</td>
<td>-1.236210</td>
</tr>
<tr>
<td>C</td>
<td>2.990600</td>
<td>-0.473027</td>
<td>-2.476023</td>
</tr>
<tr>
<td>C</td>
<td>4.206605</td>
<td>-1.279723</td>
<td>-2.940368</td>
</tr>
<tr>
<td>C</td>
<td>4.730663</td>
<td>-2.283733</td>
<td>-1.916657</td>
</tr>
<tr>
<td>C</td>
<td>3.090840</td>
<td>-4.135883</td>
<td>3.075699</td>
</tr>
<tr>
<td>N</td>
<td>2.475092</td>
<td>1.790608</td>
<td>0.376898</td>
</tr>
<tr>
<td>C</td>
<td>3.792791</td>
<td>1.697964</td>
<td>0.286570</td>
</tr>
<tr>
<td>N</td>
<td>4.366317</td>
<td>2.916141</td>
<td>0.479636</td>
</tr>
<tr>
<td>C</td>
<td>3.357897</td>
<td>3.846669</td>
<td>0.701293</td>
</tr>
<tr>
<td>C</td>
<td>2.192067</td>
<td>3.116315</td>
<td>0.636286</td>
</tr>
<tr>
<td>C</td>
<td>3.634225</td>
<td>5.296323</td>
<td>0.914142</td>
</tr>
<tr>
<td>S</td>
<td>-0.337483</td>
<td>-1.575163</td>
<td>-1.237173</td>
</tr>
<tr>
<td>C</td>
<td>-1.393514</td>
<td>-2.089860</td>
<td>-0.083799</td>
</tr>
<tr>
<td>C</td>
<td>-2.718372</td>
<td>-2.717763</td>
<td>-0.367813</td>
</tr>
<tr>
<td>N</td>
<td>-2.718116</td>
<td>-3.587416</td>
<td>-1.536650</td>
</tr>
<tr>
<td>O</td>
<td>-0.314790</td>
<td>1.112244</td>
<td>0.794807</td>
</tr>
<tr>
<td>O</td>
<td>-1.357578</td>
<td>0.311973</td>
<td>1.707034</td>
</tr>
<tr>
<td>O</td>
<td>0.646137</td>
<td>1.434045</td>
<td>-1.887229</td>
</tr>
<tr>
<td>C</td>
<td>-3.855360</td>
<td>-1.618941</td>
<td>-0.482834</td>
</tr>
<tr>
<td>O</td>
<td>-4.893131</td>
<td>-1.917776</td>
<td>-1.078962</td>
</tr>
<tr>
<td>N</td>
<td>-3.629142</td>
<td>-0.413301</td>
<td>0.100503</td>
</tr>
<tr>
<td>C</td>
<td>-4.631729</td>
<td>0.636716</td>
<td>0.026595</td>
</tr>
<tr>
<td>C</td>
<td>-4.799267</td>
<td>1.304193</td>
<td>1.382414</td>
</tr>
<tr>
<td>O</td>
<td>-3.954967</td>
<td>1.396128</td>
<td>2.261649</td>
</tr>
<tr>
<td>C</td>
<td>-4.330828</td>
<td>1.731131</td>
<td>-1.054677</td>
</tr>
<tr>
<td>C</td>
<td>-4.161682</td>
<td>1.079489</td>
<td>-2.433692</td>
</tr>
<tr>
<td>C</td>
<td>-3.122657</td>
<td>2.609480</td>
<td>-0.696885</td>
</tr>
<tr>
<td>O</td>
<td>2.410854</td>
<td>0.244545</td>
<td>-3.333193</td>
</tr>
<tr>
<td>O</td>
<td>-6.022982</td>
<td>1.869972</td>
<td>1.503160</td>
</tr>
<tr>
<td>H</td>
<td>-6.020281</td>
<td>2.330227</td>
<td>2.370676</td>
</tr>
<tr>
<td>H</td>
<td>-5.579197</td>
<td>0.149454</td>
<td>-0.246816</td>
</tr>
<tr>
<td>H</td>
<td>-5.233861</td>
<td>2.367442</td>
<td>-1.080265</td>
</tr>
<tr>
<td>H</td>
<td>-4.088724</td>
<td>1.856693</td>
<td>-3.210822</td>
</tr>
<tr>
<td>H</td>
<td>-3.240014</td>
<td>0.477102</td>
<td>-2.472331</td>
</tr>
<tr>
<td>H</td>
<td>-5.003796</td>
<td>0.415806</td>
<td>-2.679149</td>
</tr>
</tbody>
</table>
Table S6.6 Cartesian Coordinates of Optimized IPNS-ACV-β Lactam Fe^IV-oxo (Model 1)

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.158068</td>
<td>2.733272</td>
<td>0.831306</td>
</tr>
<tr>
<td>N</td>
<td>2.390237</td>
<td>1.435938</td>
<td>0.418168</td>
</tr>
<tr>
<td>C</td>
<td>3.702131</td>
<td>1.304893</td>
<td>0.296733</td>
</tr>
<tr>
<td>N</td>
<td>4.320096</td>
<td>2.472457</td>
<td>0.621195</td>
</tr>
<tr>
<td>C</td>
<td>3.349593</td>
<td>3.410532</td>
<td>0.961338</td>
</tr>
<tr>
<td>Fe</td>
<td>0.808783</td>
<td>0.152976</td>
<td>-0.221781</td>
</tr>
<tr>
<td>O</td>
<td>0.759280</td>
<td>1.680152</td>
<td>-1.788855</td>
</tr>
<tr>
<td>C</td>
<td>3.670091</td>
<td>4.825097</td>
<td>1.331263</td>
</tr>
<tr>
<td>N</td>
<td>1.144589</td>
<td>-1.356994</td>
<td>1.290177</td>
</tr>
<tr>
<td>C</td>
<td>1.916935</td>
<td>-2.502097</td>
<td>1.253112</td>
</tr>
<tr>
<td>C</td>
<td>1.633291</td>
<td>-3.302664</td>
<td>2.337453</td>
</tr>
<tr>
<td>N</td>
<td>0.660292</td>
<td>-2.599845</td>
<td>3.040153</td>
</tr>
<tr>
<td>O</td>
<td>0.391301</td>
<td>-1.440676</td>
<td>2.381073</td>
</tr>
<tr>
<td>C</td>
<td>2.151715</td>
<td>-4.638712</td>
<td>2.768253</td>
</tr>
<tr>
<td>O</td>
<td>2.202805</td>
<td>-0.841584</td>
<td>-1.267677</td>
</tr>
<tr>
<td>C</td>
<td>2.715415</td>
<td>-0.626563</td>
<td>-2.438308</td>
</tr>
<tr>
<td>O</td>
<td>2.475674</td>
<td>0.354432</td>
<td>-3.180186</td>
</tr>
<tr>
<td>S</td>
<td>-0.877654</td>
<td>-0.953793</td>
<td>-1.377404</td>
</tr>
<tr>
<td>C</td>
<td>-2.280024</td>
<td>-1.186963</td>
<td>-0.241430</td>
</tr>
<tr>
<td>C</td>
<td>-3.366016</td>
<td>-2.238635</td>
<td>-0.703421</td>
</tr>
<tr>
<td>C</td>
<td>-4.390283</td>
<td>-1.111184</td>
<td>-0.448760</td>
</tr>
</tbody>
</table>
### Table S6.7 Cartesian Coordinates of Optimized IPNS-Thiocarboxylate FeII (Model 2)

<table>
<thead>
<tr>
<th>Atom</th>
<th>X</th>
<th>Y</th>
<th>Z</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>2.871525</td>
<td>1.194494</td>
<td>-0.940189</td>
</tr>
<tr>
<td>N</td>
<td>2.323899</td>
<td>-0.000448</td>
<td>-0.783955</td>
</tr>
<tr>
<td>C</td>
<td>3.370577</td>
<td>-0.905435</td>
<td>-0.787215</td>
</tr>
<tr>
<td>C</td>
<td>4.573502</td>
<td>-0.251983</td>
<td>-0.945968</td>
</tr>
<tr>
<td>Element</td>
<td>X</td>
<td>Y</td>
<td>Z</td>
</tr>
<tr>
<td>---------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------</td>
</tr>
<tr>
<td>N</td>
<td>4.226032</td>
<td>1.090789</td>
<td>-1.042910</td>
</tr>
<tr>
<td>Fe</td>
<td>0.224534</td>
<td>-0.399929</td>
<td>-0.346320</td>
</tr>
<tr>
<td>O</td>
<td>0.565833</td>
<td>-0.353316</td>
<td>1.636583</td>
</tr>
<tr>
<td>C</td>
<td>1.013474</td>
<td>-1.292335</td>
<td>2.397663</td>
</tr>
<tr>
<td>O</td>
<td>1.407235</td>
<td>-2.424244</td>
<td>2.012172</td>
</tr>
<tr>
<td>C</td>
<td>5.987596</td>
<td>-0.735806</td>
<td>-1.009894</td>
</tr>
<tr>
<td>N</td>
<td>-0.430895</td>
<td>1.646926</td>
<td>-0.307872</td>
</tr>
<tr>
<td>C</td>
<td>-1.101451</td>
<td>2.308653</td>
<td>-1.243065</td>
</tr>
<tr>
<td>N</td>
<td>-1.652639</td>
<td>3.441068</td>
<td>-0.721149</td>
</tr>
<tr>
<td>C</td>
<td>-1.324902</td>
<td>3.511900</td>
<td>0.626554</td>
</tr>
<tr>
<td>C</td>
<td>-0.561135</td>
<td>2.389069</td>
<td>0.854192</td>
</tr>
<tr>
<td>C</td>
<td>-1.787065</td>
<td>4.608984</td>
<td>1.529788</td>
</tr>
<tr>
<td>O</td>
<td>-0.162661</td>
<td>-0.417730</td>
<td>-2.440683</td>
</tr>
<tr>
<td>O</td>
<td>0.710407</td>
<td>-2.511787</td>
<td>-0.472268</td>
</tr>
<tr>
<td>C</td>
<td>1.078169</td>
<td>-0.970525</td>
<td>3.891006</td>
</tr>
<tr>
<td>C</td>
<td>-0.088777</td>
<td>-0.108463</td>
<td>4.384348</td>
</tr>
<tr>
<td>S</td>
<td>-2.293254</td>
<td>-1.229637</td>
<td>0.007107</td>
</tr>
<tr>
<td>C</td>
<td>-3.197160</td>
<td>-0.908293</td>
<td>-1.433414</td>
</tr>
<tr>
<td>C</td>
<td>-4.736874</td>
<td>-1.037429</td>
<td>-1.379233</td>
</tr>
<tr>
<td>N</td>
<td>-5.395400</td>
<td>-1.412551</td>
<td>-0.137750</td>
</tr>
<tr>
<td>O</td>
<td>-2.722205</td>
<td>-0.568836</td>
<td>-2.543514</td>
</tr>
<tr>
<td>H</td>
<td>-5.021917</td>
<td>-2.318388</td>
<td>0.163814</td>
</tr>
<tr>
<td>H</td>
<td>-5.087905</td>
<td>-0.765865</td>
<td>0.596203</td>
</tr>
<tr>
<td>H</td>
<td>-1.175488</td>
<td>-0.521320</td>
<td>-2.583798</td>
</tr>
<tr>
<td>H</td>
<td>-1.400870</td>
<td>4.437320</td>
<td>2.543784</td>
</tr>
<tr>
<td>H</td>
<td>-1.433260</td>
<td>5.601078</td>
<td>1.201053</td>
</tr>
<tr>
<td>H</td>
<td>-2.887173</td>
<td>4.651654</td>
<td>1.597565</td>
</tr>
<tr>
<td>H</td>
<td>-2.236718</td>
<td>4.099200</td>
<td>-1.226540</td>
</tr>
<tr>
<td>H</td>
<td>-1.229228</td>
<td>1.988825</td>
<td>-2.272162</td>
</tr>
<tr>
<td>H</td>
<td>-0.117981</td>
<td>2.033148</td>
<td>1.778527</td>
</tr>
<tr>
<td>H</td>
<td>-0.006761</td>
<td>0.080491</td>
<td>5.466776</td>
</tr>
<tr>
<td>H</td>
<td>-0.108051</td>
<td>0.859390</td>
<td>3.864840</td>
</tr>
<tr>
<td>H</td>
<td>-1.054001</td>
<td>-0.602987</td>
<td>4.196686</td>
</tr>
<tr>
<td>H</td>
<td>1.135146</td>
<td>-1.923720</td>
<td>4.436830</td>
</tr>
<tr>
<td>H</td>
<td>2.033836</td>
<td>-0.445558</td>
<td>4.073358</td>
</tr>
<tr>
<td>H</td>
<td>-0.147375</td>
<td>-2.980482</td>
<td>-0.471959</td>
</tr>
<tr>
<td>H</td>
<td>1.048764</td>
<td>-2.601201</td>
<td>0.497998</td>
</tr>
<tr>
<td>H</td>
<td>6.007114</td>
<td>-1.829330</td>
<td>-0.907561</td>
</tr>
<tr>
<td>H</td>
<td>6.471500</td>
<td>-0.481371</td>
<td>-1.968005</td>
</tr>
<tr>
<td>H</td>
<td>6.606040</td>
<td>-0.315720</td>
<td>-0.198883</td>
</tr>
<tr>
<td>H</td>
<td>4.873812</td>
<td>1.862982</td>
<td>-1.161682</td>
</tr>
<tr>
<td>H</td>
<td>2.330454</td>
<td>2.134899</td>
<td>-0.976503</td>
</tr>
<tr>
<td>H</td>
<td>3.184392</td>
<td>-1.968703</td>
<td>-0.672631</td>
</tr>
<tr>
<td>H</td>
<td>-5.132276</td>
<td>-0.064422</td>
<td>-1.720874</td>
</tr>
<tr>
<td>H</td>
<td>-5.010126</td>
<td>-1.758905</td>
<td>-2.168575</td>
</tr>
<tr>
<td>H</td>
<td>0.301263</td>
<td>-1.118306</td>
<td>-2.925607</td>
</tr>
</tbody>
</table>