MECHANISTIC INVESTIGATIONS OF ENZYMES
IN PHOSPHONATE METABOLISM

BY

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DISSERTATION

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Abstract

Synthetic and naturally-occurring phosphonates have found widespread use in both agriculture and medicine. A program at the Institute for Genomic Biology at the University of Illinois at Urbana-Champaign was established to discover novel phosphonate natural products. In addition to this effort, the metabolic pathways for the biosynthesis of these natural products as well as phosphonate catabolism were examined for interesting biochemical reactions. Herein are detailed my contributions toward this enterprise.

2-Hydroxyethylphosphonate dioxygenase (HEPD) is a non-heme iron enzyme that catalyzes the cleavage of the carbon-carbon bond of 2-hydroxyethylphosphonate (2-HEP) during the biosynthesis of the herbicide phosphinothricin. Mechanistic studies were undertaken to elucidate the mechanism of catalysis by HEPD. These studies demonstrated that an unusual iron(IV)-oxo intermediate is at the heart of the catalytic cycle of HEPD. Also reported in this dissertation are studies on an enzyme with distant homology to HEPD, methylphosphonate synthase (MPnS), which likewise breaks the carbon-carbon bond of 2-HEP. These results strongly suggested the possibility of a consensus mechanism between HEPD and MPnS. This hypothesis was further evaluated through a combination of substrate analog incubations, site-directed mutants, and $^{18}$O KIE studies. Additionally, this thesis presents a study on an alcohol dehydrogenase that reduces phosphonoacetaldehyde (PnAA) to 2-HEP; the implications for the biosynthetic pathway for fosfomycin are discussed. Finally, observations made while studying an aldehyde dehydrogenase that oxidizes PnAA to phosphonoacetate are reported.
Sing in me, Muse, and through me tell the story
of that man skilled in all ways of contending,
the wanderer, harried for years on end,
after he plundered the stronghold
on the proud height of Troy.

He saw the townlands
and learned the minds of many distant men,
and weathered many bitter nights and days
in his deep heart at sea, while he fought only
to save his life, to bring his shipmates home.

-The Odyssey by Homer, translated by Robert Fitzgerald
Acknowledgments

I am grateful to so many people who made this odyssey possible.

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I also wish to thank all my friends, both those in the van der Donk group and those outside of it. In particular, Tyler Takeshita, Kyle Dunbar, Joel Melby, Tucker Maxson, Chris Schwalen, and the Mitchell lab as a whole gave me a group to hang out with (as an adjunct Mitchell-group member). Thanks to Noah Bindman for his friendship and editing this thesis. I also owe so much to Emily Ulrich, who has tolerated me for longer than I had any right to expect, particularly during the periods when I was stressed.

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There are so many others to whom I owe thanks that I have not mentioned here explicitly. If I have seen further, truly, it has been by standing on the shoulders of giants.
Table of Contents

List of Figures..................................................................................................................xi
List of Tables.....................................................................................................................xv

Chapter 1: Phosphonate Biosynthesis and Enzymology.............................................1
  1.1: Phosphonate Natural Products.............................................................................1
  1.2: Catabolism of Phosphonates .............................................................................4
  1.3: HEPD Is a Non-heme Iron-Dependent Enzyme Involved in Phosphinothricin
        Biosynthesis........................................................................................................6
  1.4: MPnS Is an Enzyme with Homology to HEPD..................................................11
  1.5: References..........................................................................................................14

Chapter 2: Stereochemical Outcome of the HEPD Reaction with 2-HEP .............25
  2.1: Introduction...........................................................................................................25
    2.1.1: Divergent Hypothetical Mechanisms with Differing Stereochemical
            Outcomes..........................................................................................................25
    2.1.2: Hydrogen Atom Abstraction by HEPD Is Stereo- and Regiospecific ......28
    2.1.3: Mosher’s Ester Analysis of the Stereochemical Outcome at C1.....29
    2.1.4: Analysis of Formate Produced Is Consistent with Racemization ......30
  2.2: Results and Discussion......................................................................................31
    2.2.1: Experiments to Corroborate Racemization..............................................31
    2.2.2: Racemization Does Not Occur After the Reaction.................................33
    2.2.3: A Methylphosphonate Intermediate Does Not Cause Racemization ..34
  2.3: Conclusions........................................................................................................37
  2.4: Materials and Methods....................................................................................38
    2.4.1: General Experimental..................................................................................38
2.4.2: Steady-State Oxidation Kinetics by HEPD………………………………38
2.4.3: Testing the Possibility of HMP Breakdown After the Reaction..........40
2.4.4: Assaying the Possibility of a Methylphosphonate Intermediate………..40
2.5: References.........................................................................................41

Chapter 3: Characterization of an Unexpected Ferryl Intermediate in HEPD Catalysis...43
3.1: Introduction.......................................................................................43
3.2: Results..............................................................................................43
  3.2.1: Kinetics of Steady-State Oxidation of 2-HEP by WT HEPD………43
  3.2.2: Construction and Characterization of an Active-Site Mutant of HEPD..45
  3.2.3: Pre-Steady State Kinetics Assessed by Stopped-Flow UV-Visible Spectroscopy………………………………………………………………………………………………47
  3.2.4: Characterization of the Intermediate by Rapid-Freeze Quench Mössbauer Spectroscopy…………………………………………………………………………49
  3.2.5: Kinetic Fitting of the Spectroscopic Data………………………………53
  3.2.6: Chemical Quench Experiments Demonstrate Competence of the Intermediate………………………………………………………………………………54
  3.2.7: Reacting HEPD-E176A with 2-[2-²H₂]-HEP in Deuterated Buffer Yields Greater Amounts of the Ferryl Species………………………………………55
  3.2.8: Solvent Wash-In Decreases with Increasing Ferryl Accumulation……56
  3.2.9: Kinetics of Steady-State Oxidation of 2-HEP by WT HEPD and HEPD-E176A in Deuterated Buffer………………………………………………57
3.3: Discussion..........................................................................................59
3.4: Materials and Methods.................................................................65
  3.4.1: Fe(II) Titrations for WT HEPD and HEPD-E176A.......................65
  3.4.2: Steady-State Kinetic Assays with WT HEPD and HEPD-E176A.......65
3.4.3: Confirming the Products of HEPD-E176A Catalysis………………...66
3.4.4: Stopped-Flow UV-Visible Experiments……………………………67
3.4.5: Mössbauer Time Course of HEPD-E176A and 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP………..68
3.4.6: Generating the Ferryl Intermediate in WT HEPD…………………………69
3.4.7: Quantifying Solvent-Exchange in HEPD-E176A…………………………69

3.5: References……………………………………………………………………………72

Chapter 4: Stereochemical Outcome of the MPnS Reaction………………………78

4.1: Introduction……………………………………………………………………………78
4.2: Results…………………………………………………………………………………79
4.2.1: Steady-State Oxidation Kinetics of 2-HEP Isotopologues………………79
4.2.2: Stereochemistry of Initial Hydrogen Atom Abstraction from 2-HEP…..81
4.2.3: Origin of the Incipient Hydrogen in MPn…………………………………...84
4.3: Discussion………………………………………………………………………………86
4.4: Materials and Methods……………………………………………………………88
4.4.1: Kinetic Assays……………………………………………………………………88
4.4.2: Stereochemistry of Hydrogen Atom Abstraction…………………………89
4.4.3: Determining the Origin of the Incipient Hydrogen Atom in MPn………89

4.5: References……………………………………………………………………………..90

Chapter 5: Evidence of a Consensus Mechanism for HEPD and MPnS………………….93

5.1: Introduction…………………………………………………………………………..93
5.2: Results and Discussion…………………………………………………………...95
5.2.1: A Bifunctional Mutant of HEPD……………………………………………….95
5.2.2: Pre-Steady-State Reactivity of the HEPD-E176H Mutant………………..100
5.2.3: Tuning the Product Distribution of the HEPD-E176H Mutant…………101
5.2.4: Enzymes with Homology to HEPD and MPnS……………………..105
5.2.5: Incubation of HEPD and MPnS with Cyclopropyl Substrate Analogs…110
5.2.6: Halogenated Substrate Analog Studies with WT HEPD………………116
5.2.7: Halogenated Substrate Analog Studies with WT MPnS………………123
5.2.8: $^{18}$O KIE Studies………………………………………………………129
5.3: Conclusions………………………………………………………………131
5.4: Materials and Methods…………………………………………………132
5.4.1: HEPD-E176H Construction and Kinetic Characterization…………132
5.4.2: Identification of Products Formed by HEPD-E176H………………133
5.4.3: Analysis of HEPD-E176H by Stopped-Flow UV-Vis Experiments……134
5.4.4: HEPD-E176H/S196D and –E176H/S196N Double Mutant Construction and Characterization………………………………………………135
5.4.5: Attempting to Skew the HEPD-E176H Product Distribution by Addition of Exogenous Formate………………………………………………136
5.4.6: Cloning and Characterization of HEPD/MPnS Homologs…………136
5.4.7: Syntheses of Cyclopropyl Substrate Analogs………………………139
5.4.8: Enzymatic Assays with the Cyclopropyl Substrate Analogs………..140
5.4.9: Syntheses of Halogenated Substrate Analogs………………………142
5.4.10: Assays of HEPD and MPnS with Halogenated Substrate Analogs…146
5.4.11: $^{18}$O KIE Studies…………………………………………………………148
5.5: References………………………………………………………………149
Chapter 6: Enzymatic Transformations Acting on Phosphonoacetaldehyde……154
6.1: Introduction………………………………………………………………154
<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>A panel of bioactive phosphonates</td>
</tr>
<tr>
<td>1.2</td>
<td>A simplified overview of phosphonate biosynthesis</td>
</tr>
<tr>
<td>1.3</td>
<td>Catabolic pathways of phosphonates</td>
</tr>
<tr>
<td>1.4</td>
<td>Unusual transformations in phosphinothricin biosynthesis</td>
</tr>
<tr>
<td>1.5</td>
<td>O₂ activation by non-heme iron enzymes with use of a cosubstrate</td>
</tr>
<tr>
<td>1.6</td>
<td>O₂ activation by non-heme iron enzymes without the use of a cosubstrate</td>
</tr>
<tr>
<td>1.7</td>
<td>Gene cluster of methylphosphonate synthase</td>
</tr>
<tr>
<td>1.8</td>
<td>Alignment of three related non-heme iron enzymes</td>
</tr>
<tr>
<td>1.9</td>
<td>Related activities catalyzed by HEPD, MPns, and HppE</td>
</tr>
<tr>
<td>2.1</td>
<td>Initial hypotheses for the mechanism of HEPD with 2-HEP</td>
</tr>
<tr>
<td>2.2</td>
<td>The reaction and mechanism of HEPD with 1-HEP reaction</td>
</tr>
<tr>
<td>2.3</td>
<td>¹H NMR spectra of the Mosher's acid analysis of standards of HMP as well as enzymatically generated HMP</td>
</tr>
<tr>
<td>2.4</td>
<td>Explanation of the stereochemical outcome of the HEPD reaction</td>
</tr>
<tr>
<td>2.5</td>
<td>Steady-state oxidation kinetics of HMP by HEPD</td>
</tr>
<tr>
<td>2.6</td>
<td>Racemization could occur by transient breakdown of HMP</td>
</tr>
<tr>
<td>2.7</td>
<td>LC-MS analysis of (R)-[²H₁]-HMP anaerobically incubated with HEPD</td>
</tr>
<tr>
<td>2.8</td>
<td>Racemization could occur via an intermediate methylphosphonate molecule</td>
</tr>
<tr>
<td>2.9</td>
<td>LC-FTMS analysis of possible solvent incorporation into HMP by HEPD</td>
</tr>
<tr>
<td>3.1</td>
<td>Michaelis-Menten oxidation kinetics by WT HEPD with 2-HEP and O₂</td>
</tr>
<tr>
<td>3.2</td>
<td>Products of HEPD-E176A catalysis with 2-HEP</td>
</tr>
</tbody>
</table>
5.1 Strategies to detect intermediates in the HEPD and MPnS catalytic cycles........93
5.2 Fe(II)-dependence of HEPD-E176H activity........................................95
5.3 Steady-state Michaelis-Menten kinetics of HEPD-E176H.........................96
5.4 $^{31}$P NMR spectrum demonstrating the bifunctionality of HEPD-E176H........98
5.5 Energy landscape of the partitioning step of HEPD-E176H......................98
5.6 $^{31}$P NMR spectrum of the reaction of HEPD-E176H with 2-[2-$^2$H$_2$]-HEP.....100
5.7 Stopped-flow UV-Visible experiments with HEPD-E176H.........................101
5.8 Overlay of the HEPD-E176H crystal structure with WT HEPD....................102
5.9 Product distribution of HEPD-E176H with exogenous formate.....................103
5.10 Characterization of HEPD-E176H double mutants................................104
5.11 Phylogenetic analysis of HEPD and MPnS homologs..............................106
5.12 Phosphonate biosynthetic gene cluster from S. monomycini.......................107
5.13 Characterization of the homolog from S. monomycini..............................108
5.14 Clustal alignment of the characterized HEPD/MPnS homologs..................108
5.15 $^{31}$P NMR spectroscopic characterization of additional HEPD homologs......110
5.16 Synthetic scheme and mechanism of cyclopropyl substrate analogs...........111
5.17 The reaction between WT HEPD or WT MPnS and 2-cyc-2-HEP................113
5.18 WT HEPD and WT MPnS do not consume O$_2$ with 2-cyc-2-HEP................113
5.19 A possible steric clash between 2-cyc-2-HEP and residue Y184 in HEPD.....114
5.20 HEPD-Y184L does not consume O$_2$ when incubated with 2-cyc-2-HEP.......115
5.21 WT HEPD and WT MPnS do not consume O$_2$ with 1-cyc-1-HEP.................116
5.22 Scheme for synthesis of halogenated substrate analogs and possible explanations for their inactivation of HEPD and MPnS.................................117
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.23</td>
<td>Assays with an O₂ electrode of incubations of WT HEPD with 1-Cl-2-HEP.</td>
</tr>
<tr>
<td>5.24</td>
<td>Assays with an O₂ electrode of incubations of WT HEPD with 1-F-2-HEP.</td>
</tr>
<tr>
<td>5.25</td>
<td>Assays using a fluoride electrode to detect fluoride release.</td>
</tr>
<tr>
<td>5.26</td>
<td>$^{31}$P NMR characterization of the reaction of WT HEPD with 1-Cl-2-HEP.</td>
</tr>
<tr>
<td>5.27</td>
<td>$^{31}$P NMR characterization of the reaction of WT HEPD with 1-F-2-HEP.</td>
</tr>
<tr>
<td>5.28</td>
<td>Assays with an O₂ electrode of incubations of WT MPnS with 1-Cl-2-HEP.</td>
</tr>
<tr>
<td>5.29</td>
<td>Steady-state kinetics of WT MPnS oxidation of 1-F-2-HEP.</td>
</tr>
<tr>
<td>5.30</td>
<td>$^{31}$P NMR characterization of the reaction of WT MPnS with 1-Cl-2-HEP.</td>
</tr>
<tr>
<td>5.31</td>
<td>$^{31}$P NMR characterization of the reaction of WT MPnS with 1-F-2-HEP.</td>
</tr>
<tr>
<td>6.1</td>
<td>Overall apo-structure of PhnY.</td>
</tr>
<tr>
<td>6.2</td>
<td>Primary sequence alignment of characterized aldehyde dehydrogenases.</td>
</tr>
<tr>
<td>6.3</td>
<td>PhnY in complex with PnAA and NAD$^+$.</td>
</tr>
<tr>
<td>6.4</td>
<td>Proposed biosynthetic pathways for fosfomycin biosynthesis.</td>
</tr>
<tr>
<td>6.5</td>
<td>Primary sequence alignment of group III-ADs.</td>
</tr>
<tr>
<td>6.6</td>
<td>A strategy to determine the stereochemistry of DhpG reduction.</td>
</tr>
<tr>
<td>6.7</td>
<td>An enzymatic cascade produces HMP from PnAA.</td>
</tr>
<tr>
<td>6.8</td>
<td>LC-MS analysis of the formate produced in the enzymatic cascade.</td>
</tr>
<tr>
<td>6.9</td>
<td>Michaelis-Menten curves for PhnY and site-directed mutants.</td>
</tr>
<tr>
<td>6.10</td>
<td>Covalent adduct formation between PhnY-E254A and PnAA.</td>
</tr>
<tr>
<td>6.11</td>
<td>Substrate analogs tested with PhnY.</td>
</tr>
<tr>
<td>6.12</td>
<td>G3P binding to PhnY and GAPDH.</td>
</tr>
<tr>
<td>6.13</td>
<td>Concentration-dependence of the activity of GAPDH with various substrates.</td>
</tr>
<tr>
<td>6.14</td>
<td>A mechanism for PhnY.</td>
</tr>
</tbody>
</table>
## List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1</td>
<td>Michaelis-Menten parameters for WT HEPD and HEPD-E176A Oxidation of 2-HEP in H$_2$O</td>
</tr>
<tr>
<td>3.2</td>
<td>Michaelis-Menten parameters for WT HEPD and HEPD-E176A Oxidation of 2-HEP in D$_2$O</td>
</tr>
<tr>
<td>4.1</td>
<td>Michaelis-Menten parameters for WT MPnS oxidation of 2-HEP isotopologues</td>
</tr>
<tr>
<td>5.1</td>
<td>Michaelis-Menten parameters for HEPD-E176H oxidation of 2-HEP</td>
</tr>
<tr>
<td>6.1</td>
<td>Kinetic parameters for PhnY and GAPDH oxidation of various substrates</td>
</tr>
</tbody>
</table>
1.1: Phosphonate Natural Products

Both synthetic and naturally-produced phosphonates/phosphinates have found widespread usage in fields ranging from agriculture to medicine.\textsuperscript{1,2} The defining structural motifs in these compounds are their carbon-phosphorus (C-P) bonds; phosphonates contain one C-P bond whereas phosphinates contain either two C-P bonds or one C-P bond and one P-H bond to the same phosphorus atom. Organisms ranging from animals such as freshwater snails\textsuperscript{3} to bacteria such as actinomycetes\textsuperscript{4} are able to employ phosphonate natural products as polar headgroups for lipids or exopolysaccharides.\textsuperscript{5} Actinomycetes in particular, along with some species of Pseudomonads and Bacillus, also have the capability to produce phosphonates and phosphinates with potent bioactivities.\textsuperscript{1} These compounds are able to structurally mimic molecules that are ubiquitous in metabolic and signaling pathways, such as phosphate esters, carboxylic acids, and tetrahedral intermediates formed during transformations of carbonyl groups.\textsuperscript{6} Thus, phosphonates and phosphinates can serve as small-molecule inhibitors of many different processes.

Many naturally produced phosphonates and phosphinates are found in short peptides. Examples of these peptides include the antibacterial agent dehydrophos as well

\textsuperscript{a} Reprinted in part from Methods in Enzymology, Vol. 516 Spencer C. Peck, Jiangtao Gao, and Wilfred A. van der Donk, "Mechanistic Investigations of Enzymes Involved in Phosphonate Biosynthesis," 101-123, Copyright (2012), with permission from Elsevier. Figure 1 was adapted from Current Opinion in Chemical Biology, Vol. 17, Spencer C. Peck and Wilfred A. van der Donk, "Phosphonate biosynthesis and catabolism: a treasure trove of unusual enzymology," 580-588, Copyright (2013), with permission from Elsevier.
as bialaphos, a herbicidal peptide produced by *Streptomyces viridochromogenes* and *Streptomyces hygroscopicus*, that contains the nonproteinogenic amino acid phosphinothricin (glufosinate) (Figure 1.1). The phosphinate moiety of phosphinothricin mimics the tetrahedral intermediate of glutamate formed during glutamine biosynthesis, resulting in inhibition of glutamine synthase. This inhibition causes build-up of ammonia, ultimately killing the plant; phosphinothricin and its derivatives have therefore been widely used as herbicides. The self-resistance gene from the biosynthetic cluster, the *bar* gene in *S. hygroscopicus* and the *pat* gene in *S. viridochromogenes*, has been used as a selectable marker for genetic engineering of crops and fungi. The gene encodes phosphinothricin acetyl-transferase, which *N*-acetylates phosphinothricin using acetyl-CoA. Phosphinothricin itself is not an effective antibiotic because of its inability to be readily transported across the cell membrane of bacteria. To improve uptake by target organisms, the producer strains incorporate phosphinothricin into short peptides, often tripeptides. Once transported into the cell by oligopeptide permeases, the peptide undergoes proteolysis by peptidases to release the active form. Because the compound is taken up in an inactive form and only unveiled as the active form inside the cell, phosphinothricin-containing peptides belong to the class of antibiotics known as "Trojan horses."
**Figure 1.1:** A panel of bioactive phosphonates and one phosphinate, with the bioactivity of and the protein(s) inhibited by each molecule detailed below. MurA, also known as UDP-N-acetylglucosamine 1-carboxyvinyltransferase, catalyzes the committed step in bacterial cell wall biosynthesis. Molecules are the same color as in Figure 1.2.

With two exceptions, all known phosphonate biosynthesis proceeds through the unfavorable rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (PnPy) catalyzed by PEP mutase (Figure 1.2); due to the conservation of this mechanism across various biosynthetic pathways, this enzyme has been used as a genetic handle to identify the phosphonate biosynthetic gene clusters responsible for making phosphinothricin, dehydrophos, FR-900098, and the rhizoticin peptides. In some cases, the driving force for PnPy formation after the initial unfavorable isomerization is an aldol-like condensation to drive flux, as in the biosynthetic pathways to produce fosfomycin from *Pseudomonads* and FR-900098 (blue and red, respectively, in Figure 1.2). More commonly, the initial isomerization is coupled to an irreversible decarboxylation step to afford phosphonoacetaldehyde (PnAA), as in the biosyntheses of phosphinothricin, the antibacterial tripeptide dehydrophos, and the antibiotic fosfomycin (in *Streptomyces* species) (green, purple, and blue, respectively, in Figure 1.2). In the dehydrophos, fosfomycin, and phosphinothricin pathways, PnAA produced by the action of the decarboxylase is subsequently reduced by a Group III Fe-dependent alcohol dehydrogenase (AD) to afford 2-hydroxyethylphosphonate (2-HEP) in a
transformation with stereochemistry that was undefined prior to this work.\textsuperscript{30,31} The stereochemistry associated with this NADH-dependent reduction has certain implications for the unresolved fosfomycin pathway in \textit{Streptomyces} species, and in determining that the hydride from NADH was transferred to the \textit{Re} face of PnAA by the AD, certain hypothetical pathways could be excluded (Chapter 6).\textsuperscript{31}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure1_2.png}
\caption{A simplified overview of phosphonate biosynthesis. Virtually all known pathways begin with an unfavorable isomerization followed by an irreversible second step. PnAA and 2-HEP are early intermediates that are common to a number of different pathways in secondary metabolism. These molecules also play a role in the formation of various phosphonolipids and phosphonoglycans. The colors correspond to those in Figure 1.1.}
\end{figure}

\section*{1.2: Catabolism of Phosphonates}

Although phosphonates are often resistant to enzymes and harsh chemical treatments\textsuperscript{32} that cleave the P-O bonds in phosphate esters and anhydrides, their C-P bonds are readily broken by specialized enzymes, and pathways have evolved that allow certain organisms to use phosphonates as a phosphorus source.\textsuperscript{33-40} In many of these pathways for phosphorus mobilization, PnAA is a central intermediate. These pathways include the hydrolysis of PnAA by phosphonatase (Figure 1.3, black) to yield phosphate
and acetaldehyde, the transamination of PnAA to 2-AEP followed by its hydroxylation and oxidative cleavage (Figure 1.3, blue) to afford phosphate and glycine, and the NADH-dependent oxidation of PnAA to phosphonoacetate (PnA) with subsequent hydrolysis by the metalloenzyme PhnA to yield phosphate and acetate. PhnY, the protein that catalyzes the formation of PnA, exhibits structural homology with non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase and was demonstrated to have relaxed substrate specificity in vitro (Chapter 6) (note that Martinez et al. have designated the protein that hydroxylates 2-AEP PhnY as well). Additionally, a kinetic assay was developed to characterize site-directed mutants of PhnY important for activity. This study expanded our understanding of the critical role that PnAA plays in phosphorus mobilization.

**Figure 1.3:** Several catabolic pathways exist in which PnAA is a common intermediate. In each pathway, PnAA is processed to ultimately liberate phosphate from the phosphonate precursors.
1.3: HEPD Is a Non-heme Iron-Dependent Enzyme Involved in Phosphinothricin Biosynthesis

Though the phosphinothricin pathway remains incompletely elucidated after formation of 2-HEP, a number of noteworthy transformations occur in the later steps. Among these reactions are the methyl-insertion carried out by PhpK, a methylcobalamin (MeCbl)- and S-adenosylmethionine (SAM)-dependent enzyme, the CMP ligation reaction catalyzed by PhpF, and the carbon-carbon bond cleavage of 2-HEP performed by PhpD (Figure 1.4). In vitro characterization of PhpD demonstrated that it was a non-heme iron-dependent dioxygenase that produced hydroxymethylphosphonate (HMP) and formate. The protein was thus renamed 2-hydroxyethylphosphonate dioxygenase (HEPD).

![Chemical Diagram]

**Figure 1.4:** A number of unusual reactions take place in the phosphinothricin biosynthetic pathway.

A crystal structure of HEPD was solved in collaboration with the Nair group at the University of Illinois Urbana-Champaign. NHI enzymes are most commonly ligated by a pair of endogenous histidine ligands and a carboxylate (either a glutamate or aspartate); these proteins belong to the class known as "facial triad" enzymes. Several
facets of catalysis by HEPD are unusual compared to catalysis by other NHI enzymes. Dr. Rob Cicchillo, a former post-doctoral associate in the van der Donk group, demonstrated that HEPD required only Fe(II) and O$_2$ for catalysis, which is in stark contrast with many NHI enzymes that require an additional cofactor or cosubstrate for activity. The cosubstrate, such as α-ketoglutarate (α-KG), is capable of donating two electrons to assist in the complete cleavage of the oxygen-oxygen double bond, a process that requires four electrons (Figure 1.5A). This scission is hypothesized to occur with concomitant formation of a reactive intermediate with sufficient oxidizing power to cleave a C-H bond. Many NHI α-ketoglutarate-dependent enzymes that catalyze reactions including hydroxylation, halogenation, desaturation, and epimerization are hypothesized to undergo two single electron reductions of the O$_2$ to the peroxo species by the Fe(II) center followed by a further two-electron reduction by α-KG to completely cleave the oxygen-oxygen bond and form an Fe(IV)-oxo species. This ferryl species is thought to be the active oxidant that cleaves the C-H bond to form a carbon-centered radical; the fate of this carbon-centered radical determines the reaction that takes place (Figure 1.5A). By contrast, NHI enzymes such as tyrosine hydroxylase require the cofactor tetrahydrobiopterin (THB) for activity. This cofactor plays an analogous role to α-KG in donating two electrons in concert with the Fe(II) center to generate the reactive Fe(IV)-oxo intermediate that then activates substrate for downstream reactivity (Figure 1.5B).$^{50}$ Though the timing of the electrons transferred from the cosubstrate to O$_2$ is slightly different in THB-dependent enzymes than in α-KG-dependent enzymes, the critical role played by the different cosubstrates is the same.
Figure 1.5: Activation of O$_2$ by non-heme iron enzymes with the aid of a cosubstrate.  
(A) α-KG-dependent enzymes use two electrons from the Fe center and two electrons from the cosubstrate to completely cleave the oxygen-oxygen bond. The resulting Fe(IV)-oxo species is responsible for substrate activation to form a carbon-centered radical. Rebound of an iron-bound halide (in halogenases such as CytC3) yields halogenated products (blue, 1).$^{51}$ Rebound instead of the ferric-hydroxide (brown, 2) yields hydroxylated products in taurine dioxygenase and prolyl-4-hydroxylase.$^{52-54}$ Hydrogen atom donation from a donor such as a tyrosine to the opposite face of the carbon radical (red, 3) yields epimerized products in CarC.$^{55}$ An electron transfer to the Fe center followed by deprotonation would yield desaturated products (purple, 4). (B) THB-dependent enzymes such as tyrosine hydroxylase instead use a cofactor to initially reduce O$_2$ to the peroxide state. The adjacent Fe(II) center can then cleave the peroxide to yield hydroxy-THB and an Fe(IV)-oxo intermediate that can then catalyze downstream chemistry.$^{50}$

Oxidative scission of carbon-carbon bonds is well-precedented in the biochemical literature, but the bond targeted for cleavage is usually activated by a proximal functional group such as an alkene,$^{56}$ vicinal diol,$^{57}$ or aromatic ring.$^{58}$ Many of these cleavage reactions are catalyzed by non-heme iron enzymes that can activate O$_2$ even in the absence of a cofactor or cosubstrate. For example, 4-hydroxyphenylpyruvate (4-HPP)
dioxygenase is a NHI enzyme that oxidizes (4-HPP) to homogentisate and CO₂ in tyrosine catabolism (Figure 1.6A). However, it is thought that the α-ketoacid moiety in 4-HPP functionally mimics the role of α-KG in other non-heme iron dioxygenases in generating the active Fe(IV)-oxo complex.⁵⁹ Other examples of NHI enzymes activating O₂ without the need for cosubstrates include the extradiol-cleaving dioxygenases, such as homoprotocatechuate dioxygenase (HPCD). HPCD effects the four-electron oxidation of homoprotocatechuate to 5-carboxymethyl-2-hydroxymuconic semialdehyde (Figure 1.6B). In this case, it is thought that the electron-rich nature of the substrate facilitates oxygen activation via electron transfer to the reactive oxygen species mediated by the iron center.⁶⁰ By contrast, HEPD breaks a relatively unactivated sp³-sp³ carbon-carbon bond without an electron-rich moiety and is able to extract all four electrons for complete reduction of O₂ from its relatively electron-deficient substrate. A corollary to this aspect of catalysis is that the 2-HEP substrate, and the 2-HEP substrate alone, enables formation of a carbon-hydrogen cleaving complex. This latter facet of catalysis by HEPD is in stark contrast with the fashion in which most non-heme iron enzymes operate.
Figure 1.6: NHI enzymes capable of activating O\textsubscript{2} without the need for a cosubstrate. (A) Similar to \(\alpha\)-KG-dependent enzymes, 4-HPP dioxygenase acts on a substrate that has an \(\alpha\)-ketoacid moiety that binds directly to the Fe center and assists in O\textsubscript{2} activation. (B) Extradiol-cleaving dioxygenases such as HPCD can use their electron-rich substrates to form a substrate radical and initiate O\textsubscript{2} activation.

For these reasons, HEPD has been the subject of a number of mechanistic and computational studies\textsuperscript{48,61-67}. These investigations enabled the formulation of a pair of mechanistic hypotheses, each of which made distinct predictions regarding the stereochemical outcome of the reaction. In collaboration with Dr. John Whitteck, then a graduate student in the van der Donk group, and Petra Malova, a graduate student in the Hammerschmidt laboratory at the University of Vienna, I helped determine the stereochemistry of the HMP product using 2-HEP stereospecifically deuterated at C1 as starting material as described in Chapter 2. Contrary to the expectation of net inversion during the reaction catalyzed by HEPD, which would have been consistent with a hydroperoxylolation mechanism that we favored at the time, the product afforded was racemic\textsuperscript{62}, indicating that neither of the previously postulated mechanisms was correct\textsuperscript{63}. I therefore set out to obtain direct spectroscopic evidence for intermediates in the
catalytic cycle of HEPD with the help of the Bollinger and Krebs group at the Pennsylvania State University (Chapter 3). Despite employing a substrate that could have enabled trapping the initial reactive oxygen species, I instead detected an unexpected and unusual Fe(IV)-oxo species.

1.4: MPnS Is an Enzyme with Homology to HEPD

Chapter 4 describes the characterization of a protein homologous to HEPD\(^6^8\) that was discovered while attempting to understand why parts of the aerobic ocean are supersaturated with CH\(_4\) despite the absence of obligate-anaerobe methanogens, a situation known as the "oceanic methane paradox."\(^6^9\) Several years ago, researchers postulated that the high levels of oceanic CH\(_4\) could be explained by C-P lyase catabolism of methylphosphonate (MPn) by bacteria to yield CH\(_4\) and liberate phosphate.\(^7^0\) However, this proposed solution did not adequately explain how MPn was biosynthesized. One possible source of MPn was as a product of a gene cluster from *Nitrosopumilus maritimus* (Figure 1.7), an extraordinarily abundant aquatic archaeon.\(^7^1,7^2\) This cluster contained the canonical phosphonate biosynthetic genes required to make 2-HEP as well as an enzyme with distant homology to HEPD. Dr. Benjamin Griffin, then a postdoctoral associate in the Metcalf group at the University of Illinois Urbana-Champaign, reasoned that the first four genes might afford HMP, and that the sulfatases in the cluster might reductively cleave the C-O bond in a novel fashion to afford methylphosphonate.
Figure 1.7: The cluster in which MPnS was located. The action of the first four genes yields MPn, which could then be ligated to polysaccharides by the action of the sulfatases or nucleotidyl transferase (nct) in the cluster. Bacteria might liberate the MPn from the exopolysaccharides so as to convert it to phosphate under phosphorus-limited conditions.

The HEPD homolog was cloned, expressed, and purified by Dr. Cicchillo. He found that this protein accepted 2-HEP as a substrate in an Fe(II)- and O₂-dependent manner. However, surprisingly, the enzyme directly produced MPn without the need for a sulfatase to convert HMP to MPn, indicating that *N. maritimus* was at least in part responsible for MPn (and therefore indirectly CH₄) in the ocean. Based on two-dimensional nuclear magnetic resonance (NMR) spectroscopic experiments, *N. maritimus* probably decorates its exopolysaccharides with MPn as a novel headgroup (hence the sulfatases might act as group transfer enzymes). This observation underscores that phosphonates, such as MPn, 2-HEP, and 2-aminoethylphosphonate, can fulfill key roles not just in secondary metabolism or catabolism but also in structural capacities (Figure 1.2). Biochemical investigation of this HEPD homolog (termed methylphosphonate synthase, MPnS) indicated that despite low overall sequence similarity to HEPD and the enzyme that catalyzes epoxide ring formation in fosfomycin biosynthesis (2-hydroxypropylphosphonate epoxidase, HppE), key residues known to be critical for either Fe or substrate binding were retained (Figure 1.8). MPnS also exhibited kinetic parameters that were similar to HEPD and likewise abstracted the pro-S C2 hydrogen of
2-HEP (Chapter 4). However, whereas the pro-$R$ C2 hydrogen is incorporated into formate during HEPD catalysis, the hydrogen atom from this position is transferred into the MPn product (Figure 1.9).\(^7\)

**Figure 1.8.** An alignment of three related mononuclear non-heme iron enzymes involved in phosphonate biosynthesis demonstrates that key residues responsible for binding either phosphonate (blue) or Fe(II) (red) are mostly conserved. MPnS does not have an aspartate or glutamate at the positions where HEPD and HppE have glutamate residues, and therefore it is unclear if MPnS functions with only two histidines ligating its active site Fe(II).

**Figure 1.9:** The related dioxygenases (A) HEPD and (B) MPnS use $O_2$ as the cosubstrate to oxidize the same phosphonate to different products. The hydrogen atom originating from the pro-$R$ position at C2 of 2-HEP (red, "$H_R$") is transferred to the formate by HEPD and incorporated into MPn by MPnS. (C) HppE, by contrast, oxidizes (S)-2-hydroxypropylphosphonate ((S)-2-HPP) to fosfomycin by using $H_2O_2$ as the cosubstrate.\(^7\) This reactivity makes HppE the first known NHI peroxidase.
This unusual labeling pattern led us to propose a consensus mechanism between HEPD and MPnS in which product identity is governed by the partitioning of a late-stage intermediate. Additional evidence for a consensus mechanism between HEPD and MPnS comes from a bifunctional mutant of HEPD that is able to make both HMP and MPn in a manner consistent with isotope sensitive branching (Chapter 5). Moreover $^{18}\text{O}_2$ KIE and substrate analog studies support key common early intermediates respectively. Taken together, the studies on HEPD and on MPnS described in this thesis led to a mechanistic proposal that can explain these unusual transformations.

1.5: References


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2.1: Introduction

2.1.1: Divergent Hypothetical Mechanisms with Differing Stereochemical Outcomes

Previous work on HEPD included X-ray crystal structures,\textsuperscript{1,2} stable-isotope labeling studies,\textsuperscript{3} substrate-analog incubations,\textsuperscript{4} and computational calculations.\textsuperscript{5-7} The results of these investigations led to the formulation of two possible mechanisms that could explain all experimental observations associated with the reaction between HEPD and its native substrate 2-HEP (Figure 2.1). In either mechanism, 2-HEP would first bind to the Fe(II) site and trigger O\textsubscript{2} binding. One-electron reduction of O\textsubscript{2} by the Fe(II) center would yield Fe(III)-superoxo species Ia, which would then abstract the pro-S C2 hydrogen to form ferric-hydroperoxo IIa. Stereospecific abstraction of the pro-S C2 hydrogen atom was supported by the HEPD•2-HEP cocrystal structure but had not been experimentally validated.\textsuperscript{1,3}

After formation of the C2 radical intermediate IIa, either a hydroperoxylation or hydroxylation route could be operative. In the first mechanism, the hydroperoxo species would directly recombine with the C2 radical (hydroperoxylation mechanism). This hydroperoxylated intermediate IIIa would then undergo a Criegee rearrangement to cleave the carbon-carbon bond, yielding O-formyl-HMP IVa. Though the C2 ester carbon is more electrophilic than the C1 carbon, hydrolysis would have to occur at C1 in an S\textsubscript{N}2 fashion to account for solvent incorporation in the hydroxyl of HMP.\textsuperscript{1} Compelling

\textsuperscript{a} This is an unofficial adaptation of two articles that appeared in ACS publications. ACS has not endorsed the content of this adaptation or the context of its use. The articles can be found online at http://pubs.acs.org/doi/abs/10.1021%2Fja1113326 and http://pubs.acs.org/doi/abs/10.1021%2Fbi200804r.
evidence for this route arose from the finding by Dr. Whitteck that incubating HEPD with 1-hydroxyethylphosphonate (1-HEP) yielded acetyl phosphate (AP) (Figure 2.2A). In analogy to the reaction with 2-HEP, the most facile explanation for the formation of AP from 1-HEP by HEPD also invoked phosphonate binding followed by formation of a ferric superoxo species Ib that would abstract a hydrogen atom from C1 to yield radical IIb (Figure 2.2B). Following C-H activation, hydroperoxylolation would generate intermediate IIIb, a transformation highly reminiscent of IIIa in the catalytic cycle of 2-HEP. In the case of 1-HEP, an ensuing Criegee rearrangement would directly afford AP (IVb). A similar mechanism could thus be operative in the catalytic cycle of 2-HEP.

Figure 2.1: Catalysis with 2-HEP could be explained by one of two possible mechanisms that could possibly be distinguished by their stereochemical outcomes. (E = glutamate; H = histidine; L = unknown ligand.)
Figure 2.2: (A) HEPD oxidizes 1-HEP to acetyl phosphate. (B) The most direct route to AP formation is through a hydroperoxylated intermediate that undergoes a subsequent Criegee rearrangement. Many of the intermediates in the proposed 2-HEP and 1-HEP catalytic cycles are analogous to each other.

Though previous studies on the carbon-carbon bond cleavage mechanism by HEPD made a more compelling case for the hydroperoxylation route, the possibility of direct hydroxylation of C2 by the ferric-hydroperoxo with concomitant scission of the oxygen-oxygen bond could not be excluded. After hydroxylation, the resulting acetal V could collapse in a manner similar to a retro-Claisen reaction, yielding formate with attack on the electrophilic iron(IV)-oxo in a concerted manner to form HMP (hydroxylation route, Figure 2.1). Alternatively, the retro-Claisen reaction might occur in a stepwise fashion in which the methylphosphonate (MPn) carbanion would be a discrete intermediate with possible stabilization by delocalization into the phosphonate group (endowing possible double-bond character to the P-C bond) as well as proximity to the positively charged Fe center. The carbanion would then attack the electrophilic iron(IV)-
oxo species to generate hydroxymethylphosphonate and reset the active site for another round of catalysis.

The hydroperoxylolation and hydroxylation mechanism each make distinct predictions regarding the stereochemical outcome of the reaction with 2-HEP stereospecifically labeled with deuterium at C1. Were hydroperoxylolation active, the Criegee rearrangement would take place with retention due to the orbital overlap alignment in intermediate IIIa followed by net inversion during the subsequent SN2 hydrolysis of IVa, leading to net inversion (Figure 2.1). By contrast, the hydroxylation route would likely take place in a stereospecific manner due to the concerted collapse of the C-C bond and formation of the C-O bond, but it was not possible to exclude the possibility that a stepwise process might result in an alternative stereochemical outcome. To differentiate between these mechanistic hypotheses, the stereochemistry of the HMP product using 2-HEP stereospecifically deuterated at C1 as starting material was therefore determined.

2.1.2: Hydrogen Atom Abstraction by HEPD Is Stereo- and Regiospecific

To determine the stereochemical outcome of the reaction with WT HEPD, 2-[2-\(^2\)H\(_2\)]-HEP was synthesized by Dr. Whitteck and incubated with HEPD. The produced HMP and formate were characterized as previously described\(^1,4\) demonstrating that the formate, but not the HMP, contained deuterium, confirming that the putative hydrogen atom abstraction is regioselective. To test the stereospecificity of this step, (R)- and (S)-2-[2-\(^2\)H\(_1\)]-HEP (84% and 86% ee, respectively; 99% isotopic purity for both compounds) were synthesized\(^8\) by Dr. Whitteck and incubated with HEPD. The formate produced
from \((R)-2-[2^2\text{H}_1]\)-HEP contained deuterium whereas that produced with the \(S\) enantiomer did not. These observations show that the \(\text{pro-S}\) C2 hydrogen is abstracted, which agrees well with the active site geometry and bidentate substrate binding observed in the co-crystal structure of Cd(II)-HEPD with 2-HEP.\(^1\)

2.1.3: Mosher's Ester Analysis of the Stereochemical Outcome at C1

\((R)-\) and \((S)-2-[1^2\text{H}_1]\)-HEP (84\% and 86\% ee, respectively; 99\% isotopic purity for both compounds) were prepared as substrates for HEPD by Ms. Malova in the laboratory of Friedrich Hammerschmidt at the University of Vienna.\(^8\) Moreover, authentic \((R)-\) and \((S)-[2^2\text{H}_1]\)-HMP of 99\% ee were prepared from \((R)-\) and \((S)-\)diisopropyl hydroxy-[\(2^2\text{H}_1]\]-methylphosphonate.\(^9\) As standards for comparison, Ms. Malova also prepared the Mosher's esters of \((S)\) and \((R)\)-dimethyl hydroxy-[\(2^2\text{H}_1]\]-methylphosphonates.

Dr. Whitteck separately incubated synthetic \((R)-\) and \((S)-2-[1^2\text{H}_1]\)-HEP with HEPD that I had prepared, derivatized the produced \([2^2\text{H}_1]\)-HMP to the dimethyl ester using trimethylsilyl diazomethane, and purified the resulting material to afford several milligrams of dimethyl \([2^2\text{H}_1]\)-HMP. The stereochemistry of the \([2^2\text{H}_1]\)-HMP was assessed by conversion to the corresponding Mosher’s esters by Ms. Malova, who also conducted the subsequent \(^1\text{H}\) NMR analysis (Figure 2.3) Unexpectedly, comparison with the Mosher’s esters prepared from authentic \((R)-\) and \((S)-[2^2\text{H}_1]\)-HMP showed that HEPD converted both \((R)-\) and \((S)-2-[1^2\text{H}_1]\)-HEP into near racemic \([2^2\text{H}_1]\)-HMP (Figure 2.3, Figure 2.4A).
Figure 2.3: $^1$H NMR spectra of the PCHD groups of the authentic standards of $(R)$-Mosher esters prepared from dimethyl (A) $(R)$- and (B) $(S)$-$[^{2}H_1]$-HMP. The enzymatically derived $[^{2}H_1]$-HMP produced from (C) $(R)$- or (D) $(S)$-2-$[1^{-2}H_1]$-HEP was derivatized to the $(R)$-Mosher ester in a similar manner and demonstrated that the outcome was racemic. Figure prepared by Ms. Malova.

2.1.4: Analysis of Formate Produced Is Consistent with Racemization

Given the unanticipated outcome found by Mosher's ester analysis, Dr. Whitteck conducted an additional experiment to ensure that the stereochemical information was not lost during conversion of HMP to its dimethyl ester or Mosher’s ester. Previous experiments demonstrated that HMP was also a substrate for HEPD and was slowly converted to phosphate and formate. Dr. Whitteck determined the stereochemistry of this reaction. When $(R)$-$[^{2}H_1]$-HMP was incubated with HEPD, the formate generated contained no deuterium, whereas when $(S)$-$[^{2}H_1]$-HMP was used, the produced formate contained one equivalent of deuterium. This result was expected because it was
consistent with HMP binding in the HEPD active site in the same bidentate fashion as 2-HEP.\(^1\) Having established that oxidation of HMP was stereospecific, Dr. Whitteck incubated \((R)-\) and \((S)-2-[1-^2\text{H}_1]-\text{HEP}\) with HEPD until full conversion of both 2-HEP and the initial HMP product took place, thus resulting in 2 equivalents of formate. If the conversion of 2-HEP to HMP were stereospecific at C1, the formate produced from \((R)-\) and \((S)-2-[1-^2\text{H}_1]-\text{HEP}\) would either contain 50% deuterium or no deuterium (Figure 2.4B and 2.4C). However, experimentally both enantiomers of 2-[1-^2\text{H}_1]-HEP resulted in 25% deuterium incorporation in formate (Figure 2.4D),\(^3,10\) consistent with racemization at C1 during the conversion of 2-HEP to HMP.

\[\text{(A) } \text{HEPD} \quad \text{(B) } \text{HEPD} \quad \text{(C) } \text{Inversion} \quad \text{(D) } \text{Racemization}\]

**Figure 2.4:** Explanation of the stereochemical outcome for the HEPD reaction using stereospecifically monodeuterated substrate. (A) The HMP that was obtained from reacting \((R)-\) or \((S)-2-[1-^2\text{H}_1]-\text{HEP}\) with HEPD was observed to be racemic by Mosher’s ester analysis. (B) Reacting \((R)-2-[1-^2\text{H}_1]-\text{HEP}\) with HEPD all the way to phosphate should produce 0% deuterated formate in the case of retention, (C) 50% deuterated formate in the case of inversion, or (D) 25% deuterated formate in the case of racemization. Similar logic can be applied in the case of \((S)-2-[1-^2\text{H}_1]-\text{HEP}\).

2.2: Results and Discussion

2.2.1: Experiments to Corroborate Racemization

In the previous experiment carried out in section 2.1.4, the ratio of protiated formate to deuterated formate during oxidation of 2-[1-^2\text{H}_1]-HEP to \[^2\text{H}_1]-\text{HMP}\) and then
[\text{\textsuperscript{2}H\textsubscript{1}}]-HMP to phosphate was an indirect assessment of the stereochemical outcome at C1. Therefore, I aimed to quench the reaction after the initial oxidation of 2-[\text{\textsuperscript{1}2\text{H\textsubscript{1}}}]-HEP to [\text{\textsuperscript{2}H\textsubscript{1}}]-HMP and to purify the [\text{\textsuperscript{2}H\textsubscript{1}}]-HMP to analyze it directly. Multiple attempts were made to remove the initial equivalent of formate produced during HMP formation by acidifying the reaction mixture and lyophilizing it to dryness. However, when the residue was dissolved and HEPD was added in order to react the HMP to phosphate and formate, no reaction took place.

I therefore pursued an alternative route to isolate the HMP and characterize it. First, I measured the steady-state kinetics of oxidation of (\text{\textit{R}})- and (\text{\textit{S}})-[\text{\textsuperscript{2}H\textsubscript{1}}]-HMP in comparison with unlabeled HMP using a Clark-type oxygen electrode. As shown in Figure 2.5, this reaction exhibited a substantial primary kinetic isotope effect (KIE) on $k_{\text{cat}}$ of 7.6 ± 0.4 for (\text{\textit{R}})-[\text{\textsuperscript{2}H\textsubscript{1}}]-HMP and a large secondary KIE on $k_{\text{cat}}$ of 1.4 ± 0.1 under phosphonate-saturating conditions. This observed isotope effect provided the opportunity to confirm racemization at C1 by isolating the [\text{\textsuperscript{2}H\textsubscript{1}}]-HMP and using it as a substrate for a kinetic experiment. (\text{\textit{R}})- and (\text{\textit{S}})-2-[\text{\textsuperscript{1}2\text{H\textsubscript{1}}}]-HEP were separately incubated with HEPD until all 2-HEP was consumed as determined by $^{31}\text{P}$ NMR spectroscopy. HEPD was then reacted with the resulting [\text{\textsuperscript{2}H\textsubscript{1}}]-HMP, and the rate of oxidation was measured and compared to the rate of oxidation of unlabeled HMP. The HMP produced with (\text{\textit{S}})-2-[\text{\textsuperscript{1}2\text{H\textsubscript{1}}}]-HEP resulted in an observed KIE of 2.4 ± 0.2 and the HMP produced with (\text{\textit{R}})-2-[\text{\textsuperscript{1}2\text{H\textsubscript{1}}}]-HEP displayed an observed KIE of 2.5 ± 0.3. Importantly, a 1:1 mixture of authentic (\text{\textit{R}})- and (\text{\textit{S}})-[\text{\textsuperscript{2}H\textsubscript{1}}]-HMP displayed an observed KIE of 2.2 ± 0.2 under the same conditions, agreeing well with the expected value of 2.4 for a racemic mixture (see
section 2.4.2). Thus, all experiments come to the same conclusion: the stereochemical integrity at C1 of 2-HEP is lost during the transformation to HMP.

![Figure 2.5](image)

**Figure 2.5:** Steady-state oxidation kinetics by HEPD of HMP (black), (S)-[^2]H_1^-HMP, (blue), or (R)-[^2]H_1^-HMP (red) under air-saturating conditions.

### 2.2.2: Racemization Does Not Occur After the Reaction

A pair of explanations could still allow either the hydroperoxylation or hydroxylation mechanism (Figure 2.1) to be operative even with the loss of stereochemistry.\(^2\) One model consistent with the outcome from either mechanism is shown in Figure 2.6. After HMP formation, the P-C bond in the product could be transiently broken, generating phosphite and formaldehyde in the active site of HEPD. If the formaldehyde is able to rotate along the C=O bond, then phosphite could attack either face of the carbonyl group resulting in loss of stereochemistry; racemization would therefore occur after the reaction rather than during it. To test this possibility, (R)-[^2]H_1^-HMP\(^3,9\) was incubated with HEPD under anaerobic conditions. Subsequently, the solution was sparged with oxygen to effect the slow but stereospecific conversion of HMP to phosphate and formate.\(^3\) If the (R)-[^2]H_1^-HMP had (partially) racemized, deuterium would be incorporated into formate to the same extent as racemization. However, analysis of the formate by LC-MS after derivatization\(^4\) did not show any deuterium incorporation and
hence the experiment provided no evidence for racemization of (R)-[^3]H\textsubscript{1}\textsuperscript{-}HMP (Figure 2.7).^2

![Diagram](image1.png)

**Figure 2.6:** After formation of HMP, racemization could potentially occur via breakdown of the HMP to formaldehyde and phosphite. If the lifetime of the phosphite anion were long enough, formaldehyde could conceivably rotate prior to the P-C bond being reformed.

![Graphs](image2.png)

**Figure 2.7:** (R)-[^2]H\textsubscript{1}\textsuperscript{-}HMP was reacted with HEPD (A) after a preincubation period or (B) without prior preincubation. The produced formate was then derivatized and analyzed by LC-MS. In each panel, the blue trace is the protiated formate adduct and the red trace represents the formate corresponding to either the deuterated formate or the ^13\textsuperscript{C} isotope of the formate nitrophenylhydrazide adduct that was used for detection purposes.

### 2.2.3: A Methylphosphonate Intermediate Does Not Cause Racemization

Another possible mechanism that could account for the production of racemic HMP from (R)- or (S)-2-[^3]H\textsubscript{1}\textsuperscript{-}HEP as well as the generation of acetylphosphate from 1-HEP is the hydroperoxylation route in Figure 2.8. In this model, intermediate III\textsubscript{a}, generated as in Figure 2.1, would undergo C-C bond cleavage to produce performic acid and MPn, an intermediate in which all stereochemical information would be lost. Based on literature precedence with model compounds,^1\textsuperscript{1} the performic acid could oxidize the
iron to the corresponding ferryl species \textbf{VI}, which could abstract a hydrogen atom from MPn. Rebound of the ferric hydroxide and the organic radical would generate the HMP product and reset the enzyme for another turnover. This mechanism could also explain the formation of acetyl phosphate from 1-HEP if the hydroperoxylated intermediate (\textbf{IIIb}, Figure 2.2) generated with this substrate analog were to undergo a Criegee rearrangement instead of formation of performic acid.

\textbf{Figure 2.8}: Either the hydroperoxylation route or the hydroxylation route could involve a methylphosphonate intermediate followed by an iron(IV)-oxo species that abstracts a hydrogen atom, ultimately leading to recombination of the methylphosphonate radical with the ferric-hydroxide. If the orange proton arising from bulk solvent were instead a deuteron, it would likely be retained in the product.

\textit{Prima facie}, the mechanism in Figure 2.8 appears inconsistent with the previously observed retention of both deuterium atoms in HMP after oxidation of 2-[1-\textsuperscript{2}H\textsubscript{2}]HEP by HEPD.\textsuperscript{1} This mechanism would predict incorporation of a proton from solvent in methylphosphonate, and at least partial retention of this proton in the final product. However, abstractions of hydrogen atoms by ferryl species have been reported to result in large deuterium KIEs of 50-60 in TauD\textsuperscript{12} and prolyl-4-hydroxylase.\textsuperscript{13} A similarly large discrimination against deuterium abstraction from [\textsuperscript{2}H\textsubscript{1}]-MPn by the ferryl intermediate
VI in HEPD would account for retention of both deuterium atoms in HMP because the sensitivity of the assay used would not be sufficient to detect <5% monodeuterated HMP. If the mechanism in Figure 2.8 were operational with a large KIE, an experiment performed with unlabeled 2-HEP in D₂O should produce monodeuterated HMP (as the ferryl should leave the deuterium untouched).

HEPD was therefore incubated with 2-HEP in buffer prepared in D₂O (> 99.8% ²H-labeled), and the HMP generated was analyzed by LC-FTMS by Dr. Bradley Evans, a post-doctoral researcher in the Metcalf group at the University of Illinois Urbana-Champaign. Extracted ion chromatograms were obtained using a linear ion-trap Fourier transform hybrid mass spectrometer allowing a 10 ppm mass error with respect to the theoretical masses. This method can distinguish between [²H₄]-HMP and other analytes such as natural abundance ¹³C-HMP. [²H₄]-HMP was not detected (Figure 2.9), indicating that the hydroperoxylation mechanism in Figure 2.8 cannot be correct. This result also excludes an alternative hydroxylation route in which hydroxylation of the C₂ radical with concomitant ferryl formation would be followed by collapse of the acetal and generation of MPn with ferryl VI. As in the hydroperoxylation mechanism, ferryl VI would then abstract a hydrogen atom from the transiently present MPn, ultimately leading to HMP formation; the absence of any solvent wash-in to the HMP product likewise precludes this alternative possibility.
Figure 2.9: Extracted ion chromatograms of reaction mixtures of 2-HEP incubated with wt HEPD in D$_2$O. [H$_2$]-HMP was observed (red, calculated 110.9847 m/z, found 110.9852 m/z), but [H$_1$]-HMP (blue) was not detected.

2.3: Conclusions

Taken together, the evidence presented herein makes a compelling argument that hydroperoxylolation is not operative during the catalytic cycle of 2-HEP and raises the specter that 1-HEP induces alternative chemistry. However, computational calculations have suggested that 1-HEP is oxidized to acetyl phosphate without undergoing a Criegee rearrangement. In the absence of detailed characterization of any intermediates in the catalytic cycle of HEPD with 2-HEP, it is difficult to draw any solid conclusions regarding the mechanism; attempts to characterize reactive species in the HEPD reaction were therefore carried out and are described in Chapter 3.
2.4: Materials and Methods

2.4.1: General Experimental

Substrates were prepared by Dr. John Whitteck (van der Donk lab, University of Illinois) or Petra Malova (Hammerschmidt lab, University of Vienna) as previously detailed elsewhere. Dr. Whitteck was primarily responsible for the milligram-scale preparation of enzymatically produced $^2$H$_1$-HMP, and Ms. Malova was responsible for the Mosher's ester analysis of those products. Dr. Whitteck was also responsible for the determination of racemization from the percentage of deuterated formate that was produced upon complete oxidation of various 2-HEP substrates to P$_i$ and two equivalents of formate.

2.4.2: Steady-State Oxidation Kinetics by HEPD

These experiments were performed on 3/14/10, 4/3/10, 6/3/10, 7/30/10, 8/12/10, and 8/16/10. Steady-state oxidation kinetics were carried out on a Hansatech Oxytherm Electrode Unit with anaerobically reconstituted HEPD (2 µM final concentration, 1 eq. of Fe(II)) in air-saturated buffer (25 mM HEPES pH 7.5) by initiating with substrate (HMP, (R)-$^2$H$_1$-HMP, or (S)-$^2$H$_1$-HMP, varying concentrations) in triplicate. For the indirect examination of the stereochemical outcome in the HEPD reaction with 2-HEP using a kinetic isotope effect, reconstituted HEPD (20 µM) was taken up in buffer (25 mM HEPES pH 7.5, 2.0 mL total volume), and substrate (2.0 mM) was added to initiate the reaction. The reaction was allowed to proceed for 90 min at 23 °C and then protein was removed by centrifuge filtration (Millipore Micron YM-10, 10 kDa nominal molecular...
weight limit). Methylphosphonate (internal standard) and D$_2$O were added to a small aliquot of the flow through, and then the $^{31}$P NMR spectrum was recorded (Varian UI600) to confirm that complete HEP consumption had occurred. Integration of the $^{31}$P NMR spectrum and comparison with the methylphosphonate internal standard afforded the concentration of HMP.

The oxygen electrode was used to determine the rate of O$_2$ consumption. HEPD was freshly reconstituted as before. Substrate (1.0 mM for all substrates, except for ($R$)-$^{2}$-[1-$^2$H$_1$]-HEP which was 0.50 mM) was added to air-saturated buffer (25 mM HEPES, pH 7.5) and the reaction was initiated by the addition of HEPD (20 µM). Samples were run in triplicate and KIEs were determined by comparison to the rate of oxygen consumption of HMP at the same concentration as the substrate in question. HMP was obtained from enzymatically reacted ($R$)-[1-$^2$H$_1$]-HEP, and ($S$)-[1-$^2$H$_1$]-HEP. For comparison, HMP, ($R$)-[$^2$H$_1$]-HMP, ($S$)-[$^2$H$_1$]-HMP, and a 1:1 mixture of chemically synthesized ($R$)-[$^2$H$_1$]-HMP and ($S$)-[$^2$H$_1$]-HMP were also assayed.

For ($S$)-[$^2$H$_1$]-HMP, $k_{cat}$ is 0.042 s$^{-1}$, and for ($R$)-[$^2$H$_1$]-HMP, $k_{cat}$ is 0.0078 s$^{-1}$. Because the $K_m$ values for ($S$)-[$^2$H$_1$]-HMP and ($R$)-[$^2$H$_1$]-HMP are very similar (240 ± 10 and 260 ± 20 µM respectively), any racemic mixture would result in the enzyme being equally likely to act on ($S$)- or ($R$)-[$^2$H$_1$]-HMP, and therefore the $k_{cat}$ for the racemic mixture should be the average of the values for the individual enantiomers. Therefore, the racemic mixture would be predicted to display an apparent $k_{cat}$ of $0.5 \times 0.042$ s$^{-1} + 0.5 \times 0.0078$ s$^{-1} = 0.025$ s$^{-1}$. Since the $k_{cat}$ for protiated HMP is 0.059 s$^{-1}$, the expected apparent KIE for a racemic mixture would be $0.059/0.025 = 2.4$, which is in good agreement with the observed values.
2.4.3: Testing the Possibility of HMP Breakdown After the Reaction

This work was performed on 5/26/10. The possibility that transient breakdown of HMP in the HEPD active site to phosphite and formaldehyde could account for the observed racemization was evaluated. Anaerobically reconstituted WT HEPD (10 µM final concentration) was anaerobically incubated with (R)-[2H1]-HMP (1 mM final) in buffer (25 mM HEPES pH 7.5) and then allowed to react for 1 h or was directly allowed to react aerobically without any anaerobic preincubation. The reaction was quenched, derivatized, and analyzed as described previously.³ Because the formate was analyzed after condensing it with 2-nitrophenylhydrazine and the LC-MS method was insensitive to the presence of a +1 peak due to natural abundance of 13C in the formate adduct or deuterium in the formate, it was necessary to compare the samples with and without preincubation. Both samples had ratios of the parent peak to the +1 peak (10.9:1) that closely matched what would be expected based on the isotopic distribution (11.5:1).

2.4.4: Assaying the Possibility of a Methylphosphonate Intermediate

This assay was carried out on 10/7/10. To evaluate whether D₂O was incorporated into HMP during the reaction of 2-HEP with HEPD, HEPD was exchanged into deuterated buffer (25 mM HEPES pD 7.5) using a NAP-5 desalting column and anaerobically reconstituted. HEPD (10 µM final concentration) was allowed to react with 2-HEP (2 mM) in the presence of O₂ and 25 mM HEPES pD 7.5. The reaction was centrifuge-filtered with an Amicon filter and analyzed by LC-FTMS by Dr. Bradley
Evans (Metcalf lab, University of Illinois) with extracted ion chromatograms used to evaluate the presence of HMP and $[^2\text{H}_1]^{-}\text{HMP}$.

2.5: References


(8) Hammerschmidt, F.; Kählig, H. Biosynthesis of natural products with a P-C bond. 7. Synthesis of $[1,1^{-2}\text{H}_2]$, $[2,2^{-2}\text{H}_2]$, (R)- and (S)-$[1^{-2}\text{H}_1](2$-hydroxyethyl)phosphonic Acid and (R,S)-$[1^{-2}\text{H}_1](1,2$-dihydroxyethyl)phosphonic acid and incorporation studies into fosfomycin in *Streptomyces fradiae*. *J. Org. Chem.* 1991, 56, 2364.


Chapter 3: Characterization of an Unexpected Ferryl Intermediate In HEPD Catalysis

3.1: Introduction

As described in Chapter 2, the stereochemical outcome of HMP produced by HEPD was incompatible with the mechanisms postulated previously. Therefore, an endeavor was undertaken to obtain direct experimental evidence for an intermediate involved in oxidation of 2-HEP, namely the putative initial ferric-superoxo species (intermediate Ia in Figure 2.1).\(^1\) Despite its postulated critical role as the initial reactive oxygen species in catalysis for many non-heme iron enzymes, this intermediate has been characterized as the active C-H bond cleaving intermediate in non-heme iron enzymes only twice: in the diiron enzyme myo-inositol oxygenase (MIOX)\(^2\) and the mononuclear non-heme iron enzyme, 2,3-homoprotocatechuate dioxygenase (2,3-HPCD).\(^3\) In each instance, accumulation of the ferric-superoxide species in quantities necessary for characterization was achieved by utilizing a less activated substrate analog or a deuterium-labeled substrate, either of which could prolong the lifetime of an intermediate. I reasoned that employing 2-[2-\(^2\)H\(_2\)]-HEP might engender a substrate kinetic isotope effect (KIE) that would impose a higher energy barrier to decay of Ia, thus enabling the characterization of this elusive reactive oxygen species.

3.2: Results

3.2.1: Kinetics of Steady-State Oxidation of 2-HEP by WT HEPD

To evaluate whether the wild-type (WT) HEPD reaction with 2-[2-\(^2\)H\(_2\)]-HEP was a suitable candidate for spectroscopic studies, I first determined the Michaelis-Menten
kinetics of 2-[2-2H2]-HEP oxidation by WT HEPD using a steady-state continuous assay with an O2 electrode. WT HEPD oxidizes 2-HEP with catalytic efficiency typical of an enzyme involved in secondary metabolism (Table 3.1 and Figure 3.1). Oxidation of 2-[2-2H2]-HEP by WT HEPD displayed a minimal KIE on $k_{cat}/K_{m,2-HEP}$ and $k_{cat}$, the latter of which suggests that any slower chemical step may be kinetically masked by a rate-limiting physical step such as a conformational change or product release. However, the significant KIE on $k_{cat}/K_{m,O2}$ indicates partial-rate limitation prior to the first irreversible step in the catalytic cycle with respect to O2 activation upon employing dideuterated substrate.

**Figure 3.1:** Michaelis-Menten oxidation kinetics by WT HEPD with (A) constant O2 and varying concentrations of 2-HEP (red) or 2-[2-2H2]-HEP (blue) or (B) constant phosphonate (red: 2-HEP, blue: 2-[2-2H2]-HEP) and varying O2 concentrations.

<table>
<thead>
<tr>
<th>System</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$K_{m,2-HEP}$ (µM)</th>
<th>$K_{cat}/K_{m,2-HEP}$ (M$^{-1}$s$^{-1}$)</th>
<th>$K_{m,O2}$ (µM)</th>
<th>$k_{cat}/K_{m,O2}$ (M$^{-1}$s$^{-1}$)</th>
<th>KIE ($k_{cat}$)</th>
<th>KIE ($k_{cat}/K_{m,2-HEP}$)</th>
<th>KIE ($k_{cat}/K_{m,O2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT HEPD, 2-HEP</td>
<td>0.35 ± 0.01</td>
<td>10 ± 1</td>
<td>(3.6 ± 0.3) x 10$^4$</td>
<td>16 ± 3</td>
<td>(2.2 ± 0.5) x 10$^4$</td>
<td>0.99 ± 0.04</td>
<td>1.1 ± 0.3</td>
<td>4.4 ± 1.4</td>
</tr>
<tr>
<td>WT HEPD, 2-[2-2H2]-HEP</td>
<td>0.36 ± 0.01</td>
<td>14 ± 2</td>
<td>(2.6 ± 0.3) x 10$^4$</td>
<td>62 ± 7</td>
<td>(5.7 ± 0.7) x 10$^4$</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>HEPD-E176A, 2-HEP</td>
<td>0.75 ± 0.02</td>
<td>23 ± 2</td>
<td>(3.3 ± 0.2) x 10$^4$</td>
<td>7 ± 1</td>
<td>(9.9 ± 2) x 10$^4$</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>HEPD-E176A, 2-[2-2H2]-HEP</td>
<td>0.59 ± 0.02</td>
<td>30 ± 3</td>
<td>(1.9 ± 0.2) x 10$^4$</td>
<td>28 ± 3</td>
<td>(2.3 ± 0.3) x 10$^4$</td>
<td>1.3 ± 0.1</td>
<td>1.4 ± 0.3</td>
<td>4.3 ± 1.0</td>
</tr>
</tbody>
</table>

**Table 3.1:** Michaelis-Menten parameters for WT HEPD and HEPD-E176A in H2O to determine if a system was suitable for spectroscopic characterization.
3.2.2: Construction and Characterization of an Active-Site Mutant of HEPD

Though the lack of a KIE on $k_{\text{cat}}$ with WT HEPD oxidation of 2-HEP under steady-state conditions does not preclude trapping a reactive intermediate under pre-steady state conditions, I wondered if a mutant of HEPD might be identified that would exhibit a KIE on $k_{\text{cat}}$ (i.e. even under steady-state conditions). One candidate for mutagenesis was the active site glutamate residue (E176) that ligates the Fe(II). I thought that HEPD-E176A might retain activity with two histidines, as the non-heme iron halogenases SyrB2 and CytC3 are capable of catalysis despite possessing only two endogenous histidine ligands.\(^5\)\(^,\)\(^6\) Additionally, as E176 is cis to the putative O\(_2\) binding site in the HEPD crystal structure,\(^7\) I hypothesized that its removal might disrupt the hydrogen-bonding network responsible for binding H\(_2\)O to the metal at the putative O\(_2\)-binding site in the absence of substrate. A less tightly anchored H\(_2\)O molecule might in turn lead to an active site that was more reactive towards O\(_2\).\(^8\) Additionally, the steady-state WT HEPD oxidation kinetics suggested a physical step such as substrate binding was rate-limiting, and therefore faster dissociation of the H\(_2\)O could conceivably unmask a substrate KIE on $k_{\text{cat}}$ even under steady-state conditions.
Figure 3.2: (A) HEPD-E176A produces HMP (17.1 ppm) from 2-HEP (19.6 ppm) as observed by \(^{31}\)P NMR spectroscopy (B) as well as formate as determined by single ion monitoring (181 m/z) during LC-MS analysis of samples (black = WT HEPD; blue = HEPD-E176A; red = unreacted HEPD-E176A control) that had been derivatized as previously described.\(^9\)

The HEPD-E176A variant was constructed, overexpressed, and purified similar to WT HEPD.\(^7\) As with WT HEPD, HMP and formate were the only products detected after anaerobic reconstitution with Fe(II) and subsequent incubation with 2-HEP and O\(_2\) (Figure 3.2). However, in contrast to WT HEPD, which gained full activity upon reconstitution with 1 equivalent of Fe(II), HEPD-E176A required more than 1 equivalent of Fe(II) to reach maximal activity (Figure 3.3). With saturating concentrations of Fe(II), the Michaelis-Menten kinetics of oxidation of 2-HEP or 2-[\(^{2}\)H\(_2\)]-HEP by HEPD-E176A were determined as before (Figure 3.4). The \(k_{\text{cat}}/K_{m,2\text{-HEP}}\) of HEPD-E176A was very similar to WT HEPD (Table 3.1), though both the \(K_{m,2\text{-HEP}}\) and \(k_{\text{cat}}\) were roughly twofold higher. Consistent with the hypothesis that removal of the glutamate could enhance the reactivity with O\(_2\), the \(k_{\text{cat}}/K_{m,O_2}\) of HEPD-E176A for either 2-HEP or 2-[\(^{2}\)H\(_2\)]-HEP was approximately fivefold higher compared to WT HEPD. A small but reproducible KIE on \(k_{\text{cat}}\) was observed suggesting that chemistry was partially rate-limiting; as with WT HEPD, the KIE on \(k_{\text{cat}}/K_{m,2\text{-HEP}}\) was relatively small and the KIE on \(k_{\text{cat}}/K_{m,O_2}\) was much larger. The steady-state kinetics suggested that both WT HEPD and HEPD-E176A
would be suitable candidates for attempting to trap a reactive intermediate under pre-steady-state conditions.

**Figure 3.3:** WT HEPD (blue) or HEPD-E176A (red) were reconstituted with varying equivalents of Fe(II) followed by assaying the activity of each protein (2 µM final concentration in the reaction) towards 2-HEP using an O₂ electrode. Under these conditions, WT HEPD does not require more than 1 equivalent of Fe(II) to achieve maximal activity, whereas HEPD-E176A does.

**Figure 3.4:** Steady-state oxidation kinetics of HEPD-E176A with (A) constant O₂ and varying concentrations of 2-HEP (red) or 2-[2-²H₂]-HEP (blue) or (B) constant phosphonate (red: 2-HEP, blue: 2-[2-²H₂]-HEP) and varying O₂ concentrations.

### 3.2.3: Pre-Steady State Kinetics Assessed by Stopped-Flow UV-Visible Spectroscopy

To assess whether the ferric-superoxide intermediate accumulated under pre-steady state conditions, a collaboration was established with the Bollinger and Krebs group at the Pennsylvania State University. With the help of Laura Dassama, then a
graduate student in that group, I reconstituted WT HEPD anaerobically with Fe(II) and added an anaerobic solution of 2-HEP or 2-[2-\(^2\)H\(_2\)]-HEP. The reaction was initiated by rapidly mixing the anaerobic protein-phosphonate solution with an O\(_2\)-saturated buffer in a stopped flow UV-Visible experiment. No intermediates that absorbed from 300-700 nm were detected. By contrast, when reconstituted HEPD-E176A was mixed with 2-[2-\(^2\)H\(_2\)]-HEP and then reacted with O\(_2\)-saturated buffer under identical conditions, a species that absorbed maximally at 320 nm was accumulated (Figure 3.5A), suggestive of a possible Fe(IV)=O intermediate\(^{10-12}\) rather than the expected ferric-superoxo species.\(^2\) As described previously (Chapter 1), HEPD does not require an exogenous source of electrons, and therefore I had anticipated that Fe(III)-superoxo I would be responsible for substrate activation.

Because the detection of a putative Fe(IV) species was surprising, this intermediate was further characterized to determine how it was involved in substrate activation. In two separate experiments, Chen Wang, a graduate student in the Bollinger and Krebs group, reacted HEPD-E176A with (R)-2-[2-\(^2\)H\(_1\)]-HEP, and I reacted HEPD-E176A with the (S) enantiomer. The reaction resulted in a similar growth and decay of the intermediate as with 2-[2-\(^2\)H\(_2\)]-HEP for the (S) enantiomer but not the (R) enantiomer, indicating that the intermediate decayed by abstracting the hydrogen atom that originates from the pro-\(S\) C2 position. To determine the most favorable conditions for intermediate accumulation, Ms. Wang reconstituted HEPD-E176A with Fe(II) but did not add 2-[2-\(^2\)H\(_2\)]-HEP. The anaerobic protein solution was then reacted with buffer containing both 2-[2-\(^2\)H\(_2\)]-HEP and O\(_2\). The formation of the species absorbing at 320 nm was substantially diminished in quantity and delayed compared to preloading HEPD-E176A with 2-[2-\(^2\)H\(_2\)]-HEP.
$^{2}H_2$]-HEP (Figure 3.5), demonstrating that pre-forming the phosphonate-enzyme complex leads to greater amounts of intermediate accumulation.

**Figure 3.5:** Stopped-flow experiments with HEPD-E176A under various conditions. (A) HEPD-E176A was reconstituted, preloaded with either 2-HEP (red), 2-[2-$^{2}H_2$]-HEP (blue) or (S)-2-[2-$^{2}H_1$]-HEP (black), and reacted with O$_2$-saturated buffer. The reaction with (R)-2-[2-$^{2}H_1$]-HEP is not shown for clarity. (B) HEPD-E176A was reconstituted and preloaded with 2-HEP and reacted with O$_2$ (black), preloaded with 2-[2-$^{2}H_2$]-HEP and reacted with O$_2$ (blue), or reacted with 2-[2-$^{2}H_2$]-HEP in oxygenated buffer (red, no preloading). Note the significantly delayed maximum in the case of no pre-incubation of enzyme with phosphonate substrate. Panel B was prepared by Ms. Wang.

**3.2.4: Characterization of the Intermediate by Rapid-Freeze Quench Mössbauer Spectroscopy**

To further characterize the species detected in the stopped-flow experiment, Dr. Dassama and I reconstituted HEPD-E176A anaerobically with $^{57}$Fe and then added an anaerobic solution of 2-[2-$^{2}H_2$]-HEP. This mixture was rapidly mixed with O$_2$-saturated buffer before quenching in liquid N$_2$/isopentane at various reaction times. Mössbauer spectra obtained by Professor Carsten Krebs and Dr. Dassama in the presence of a weak external magnetic field (53 mT) revealed the growth and then decay of a peak (Figure 3.6) roughly in accord with the time course of the intermediate observed in the stopped
flow experiment. The quadrupole doublet associated with unreacted Fe(II) was determined by analyzing an unreacted anaerobic control; by subtracting out this Fe(II) signal, the parameters associated with the quadrupole doublet of the new species could be determined. The parameters ($\delta = 0.22 \text{ mm/s}$, $|\Delta E_Q| = 0.69 \text{ mm/s}$) are reminiscent of those associated with high spin Fe(IV)-oxo species observed in other enzymes, such as taurine dioxygenase (TauD), prolyl-4-hydroxylase (P4H), and tyrosine hydroxylase.\textsuperscript{11,13,14} Additionally, the Mössbauer spectrum enabled the quantification of the amount of ferryl isolated, enabling the determination that $\varepsilon_{320} = 1,300 \text{ M}^{-1} \text{ cm}^{-1}$ in conjunction with the stopped-flow experiment, in good agreement with iron(IV)-oxo species trapped previously.\textsuperscript{11,15} When Dr. Dassama and I reacted HEPD-E176A with 2-HEP or reacted WT HEPD with 2-[2-$^2$H$_2$]-HEP in oxygenated buffer, no signal other than a quadrupole doublet characteristic of high spin Fe(II) was detected. However, when WT HEPD was reacted with 2-[2-$^2$H$_2$]-HEP and chlorite dismutase/chlorite was used to generate O$_2$ levels in excess of what can be achieved by employing O$_2$-saturated buffer,\textsuperscript{16} a peak consistent with the ferryl detected in the HEPD-E176A reaction was observed (Figure 3.7). Based on the similarity of the spectroscopic parameters with the parameters from other iron-oxo species, this intermediate was assigned as an iron(IV)-oxo species. After the decay of the putative iron(IV)-oxo species, a second species accumulated with parameters consistent with an Fe(II) species. This species is likely an Fe(II)-product complex similar to those previously observed in TauD and P4H.\textsuperscript{11,15}
Figure 3.6: Mössbauer spectra of $^{57}$Fe-reconstituted HEPD-E176A mixed with 2-[2-^2H_2]-HEP and reacted with O_2-saturated buffer with reaction times indicated (dashed black line). The theoretical spectrum of the ferryl (parameters provided in text) is shown in the solid red line. The spectra were recorded by Professor Krebs and Dr. Dassama. Dr. Dassama assembled the figure.
Figure 3.7: (Top) A time course of Mössbauer spectra of WT HEPD reacted with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP using chlorite dismutase and chlorite to generate a bolus of O\textsubscript{2} followed by quenching at various time points; the solid red line has the parameters that were extracted from the HEPD-E176A Mössbauer experiment and indicate that there is likely a ferryl species in the WT HEPD reaction as well. However, this peak only exists when WT HEPD is reacted with O\textsubscript{2} levels in excess of what is obtainable under O\textsubscript{2}-saturating conditions (bottom). The reactions were performed by me and Dr. Dassama, who also prepared the figure. Spectra were recorded by Dr. Dassama and Professor Krebs. Dr. Dassama made the figure.
3.2.5: Kinetic Fitting of the Spectroscopic Data

I separately fit the stopped-flow UV-Visible data and the Mössbauer time course to an equation describing an intermediate formed from a single reactant and decaying to a single product (A → B → C; A to B is governed by $k_{\text{form}}$ and B to C is governed by $k_{\text{decay}}$). The rate constants extracted from fitting the stopped-flow data yielded $2H_k_{\text{form}}$ as 130 s$^{-1}$ and $2H_k_{\text{decay}}$ as 7.7 s$^{-1}$. These were in good agreement with the rate constants derived from fitting the Mössbauer time course ($2H_k_{\text{form}} = 140$ s$^{-1}$ and $2H_k_{\text{decay}} = 8.7$ s$^{-1}$) (Figure 3.8). With protiated substrate, the ferryl species decayed too fast to obtain an accurate rate, and therefore only a lower bound of 100 s$^{-1}$ can be placed on $1H_k_{\text{decay}}$. The observation of a KIE $\geq 11$ for this step is consistent with the large KIEs associated with the decay of ferryl species observed in TauD and P4H.$^{11,13}$ Such a large KIE suggests that the ferryl decays by abstracting the hydrogen/deuterium atom that originates from the pro-S position at C2 of 2-HEP.

![Figure 3.8: Simulated kinetics of the trapped intermediate. (A) Overlay of the quantity of Fe(IV) as determined by Mössbauer spectroscopy (red dots, left axis) over the stopped-flow trace from reacting HEPD-E176A with [2-$^2$H$_2$]-HEP (blue trace, right axis). The simulation of the stopped-flow data (shown in black) and Mössbauer data gave similar results.](image)
3.2.6: Chemical Quench Experiments Demonstrate Competence of the Intermediate

To ascertain whether the intermediate observed spectroscopically was catalytically relevant, product formation as a function of time was determined by Ms. Wang under similar conditions as in the previous experiments. HEPD-E176A was reconstituted anaerobically and then 2-[2-²H₂]-HEP was added anaerobically. The reaction was initiated by mixing the anaerobic protein-phosphonate solution with O₂-saturated buffer and was quenched at various times. The amount of HMP present at each time point was quantified by LC-MS using synthetic [²H₂]-HMP as an internal standard (Figure 3.9). Fitting the data to an exponential curve yielded a rate of product formation (9.5-10 s⁻¹ from two separate experiments) that closely mirrored the rate at which the ferryl disappeared in both the freeze-quench Mössbauer (8.7 s⁻¹) and stopped-flow UV-Vis experiments (7.7 s⁻¹), consistent with a model in which the Fe(IV) species decays concomitant with product formation. This observation strongly suggested that this species was on-pathway and not an artifact of the assays used to generate it.

Figure 3.9: Rapid chemical quench experiments by Ms. Wang of HEPD-E176A with 2-[2-²H₂]-HEP demonstrate time-dependent HMP formation. Each time point was run in triplicate, and the entire time course was run twice. A representative curve assembled by Ms. Wang is shown.
3.2.7: Reacting HEPD-E176A with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP in Deuterated Buffer Yields Greater Amounts of the Ferryl Species

To fully characterize the reaction of HEPD-E176A with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP, Ms. Wang anaerobically reconstituted the protein with Fe(II) and loaded it with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP in buffer that had been made up in H\textsubscript{2}O or D\textsubscript{2}O. The anaerobic protein-phosphonate solution was then mixed against O\textsubscript{2}-saturated buffer in either H\textsubscript{2}O or D\textsubscript{2}O in a stopped-flow UV-Vis experiment, resulting in greater accumulation of a species that absorbed maximally at 320 nm (Figure 3.10A). To confirm that the species observed by stopped-flow was the same ferryl species detected previously, Ms. Wang also reconstituted HEPD-E176A with \textsuperscript{57}Fe(II) and loaded it with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP in buffer in either H\textsubscript{2}O or D\textsubscript{2}O. Each solution was mixed with O\textsubscript{2}-saturated buffer in H\textsubscript{2}O or D\textsubscript{2}O, respectively, and then quenched by freezing the mixture in liquid N\textsubscript{2}-isopentane after identical reaction times. Mössbauer spectra obtained by Professor Krebs demonstrated that a species with the same parameters as the ferryl from earlier accumulated to greater extent in the reaction run in D\textsubscript{2}O (Figure 3.10B).

![Figure 3.10](image-url)  
**Figure 3.10:** Reactions with HEPD-E176A resulted in increased accumulation of the ferryl intermediate in deuterated buffer compared to protiated buffer. (A) Stopped-flow reactions of with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP and O\textsubscript{2} in protiated buffer (black line) or deuterated buffer (red line) demonstrated that the ferryl formed and decayed at the same rate but has a higher amplitude in deuterated buffer. (B) Mössbauer spectra of HEPD-E176A incubated with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP and O\textsubscript{2} in protiated buffer (left) or deuterated buffer (right). Each reaction was quenched after the same period of time. The solid red line indicates the signal attributed to the ferryl species. Figure compiled by Ms. Wang.
3.2.8: Solvent Wash-in Decreases with Increasing Ferryl Accumulation

In non-heme iron enzymes or catalysts that hydroxylate their substrates, the observation of solvent-derived hydroxyl groups in the product has been rationalized by invoking solvent exchange through an oxo-hydroxo tautomerism at a high-valent iron intermediate (Figure 3.11A) prior to C-H bond cleavage rather than during the short-lived ferric hydroxide substrate radical stage after substrate activation.\(^{17-19}\) I confirmed that when HEPD-E176A was incubated with \(^{18}\)O\(_2\) and 2-HEP, the HMP product displayed retention of roughly 60% \(^{18}\)O, in line with what had previously been observed with WT HEPD under similar conditions.\(^7\) Next, I incubated HEPD-E176A with \(^{18}\)O\(_2\) and 2-[\[^2\]H\(_2\)]-HEP. Though greater ferryl accumulation could enable more solvent exchange to occur, instead 60% \(^{18}\)O retention in the HMP product was again observed.

![Figure 3.11](image)

**Figure 3.11:** (A) The paradigm for solvent exchange in non-heme iron enzymes invokes an oxo-hydroxo tautomerism, in which two positions are equilibrated by a proton transfer step. (B) The data at hand suggests that solvent exchange does not take place during the ferryl intermediate, but rather likely occurs at the ferric hydroxide stage.

Finally, I reasoned that by performing the reaction with HEPD-E176A and \(^{18}\)O\(_2\) with 2-[\[^2\]H\(_2\)]-HEP in D\(_2\)O, thus accumulating the maximal amount of ferryl possible, a greater amount of solvent exchange might take place. In this case, the \(^{18}\)O content in the HMP product increased to roughly 80%, consistent with a decrease in solvent exchange. To further investigate this unexpected observation, the deuterium content of the reaction buffer of the WT HEPD or HEPD-E176A reaction with 2-[\[^2\]H\(_2\)]-HEP was
systematically varied, with the $^{18}$O label originating from $^{18}$O$_2$ or from a combination of H$_2$$^{18}$O/D$_2$$^{18}$O. After correcting for the fraction of isotopic enrichment, the conclusion reached was the same: solvent wash-in is significantly diminished in the presence of deuterated buffer (Figure 3.12), leading to the proposal that solvent exchange occurs not during the ferryl intermediate, but instead at the ferric-hydroxide intermediate (Figure 3.11B and see Discussion). The observed solvent isotope effect on solvent exchange/O$_2$ retention in the product could originate from rebound occurring preferentially with the hydroxide (originally derived from O$_2$) over the water ligand; proton transfer (more energetically challenging in deuterated buffer) would be required to equilibrate these two positions and effect solvent wash-in.

Figure 3.12: The percent of label retention from the gas source increases as the deuterium content of the reaction increases. The results from using the label in the gas ($^{18}$O$_2$, blue) agree well with using labeled water (red, H$_2$$^{18}$O and/or D$_2$$^{18}$O as appropriate).

3.2.9: Kinetics of Steady-State Oxidation of 2-HEP by WT HEPD and HEPD-E176A in Deuterated Buffer

Given these changes in the pre-steady state reactivity and the solvent exchange in reactions run in D$_2$O, I decided to examine whether there was a solvent kinetic isotope effect under steady-state conditions. With either WT HEPD or HEPD-E176A, steady-state kinetics in deuterated buffer completely abolished the small substrate KIE on $k_{cat}$
while introducing a large solvent KIE on $k_{cat}$ (Figure 3.13 and Table 3.2). Proton inventories of WT HEPD or HEPD-E176A with either 2-HEP or 2-[2-$^2$H$_2$]-HEP appeared linear in all cases (Figure 3.14), consistent with a single solvent-exchangeable proton moving in the isotopically-sensitive rate-determining step. The kinetic data is consistent with proton transfer during product release as the slow step under steady-state conditions.

**Figure 3.13:** Michaelis-Menten kinetics in D$_2$O with WT HEPD (A) with varying 2-HEP and constant O$_2$ and (B) with varying O$_2$ and constant 2-HEP. Michaelis-Menten kinetics of HEPD-E176A were similarly determined in D$_2$O (C) with varying 2-HEP and constant O$_2$ and (D) with varying O$_2$ and constant 2-HEP. In each graph, the protiated 2-HEP curve is in red, and the 2-[2-$^2$H$_2$]-HEP curve is in blue.

**Table 3.2:** Steady-state kinetic parameters of 2-HEP or 2-[2-$^2$H$_2$]-HEP oxidation by WT or HEPD-E176A in D$_2$O.
Figure 3.14: Proton inventories of (A) WT HEPD with 2-HEP (red) or 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP (blue) demonstrate linear relationships, consistent with a single proton moving in the isotopically sensitive step. The same phenomenon is observed with (B) HEPD-E176A with 2-HEP (red) or 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP (blue). Crossing at (or near) \( n = 1.0 \) indicates that the \( k_{\text{cat}} \) for either substrate in D\textsubscript{2}O is roughly the same, consistent with the Michaelis-Menten kinetics observed in Figure 3.11.

3.3: Discussion

A combination of isotope labeling studies, use of substrate analogs, and DFT calculations were previously used to formulate a pair of mechanistic hypotheses for HEPD catalysis (Figure 2.1). Each of these mechanisms postulated that a Fe(III)-superoxo species (I\textsubscript{a} in Figure 2.1) cleaves the C-H bond of 2-HEP. Attempts to trap this species in this study instead resulted in identification of an intermediate with UV-Vis and Mössbauer spectral characteristics strongly resembling Fe(IV)-oxo species trapped in other enzymes such as TauD, P4H, and tyrosine hydroxylase.\textsuperscript{11,14,15} Each of these enzymes requires two electrons from the Fe(II) center and two electrons from a cosubstrate (either \( \alpha \)-ketoglutarate for TauD and P4H or tetrahydropterin for tyrosine hydroxylase) to completely cleave the O\textsubscript{2} and to form the Fe(IV)=O species. By contrast, HEPD requires no cosubstrate other than O\textsubscript{2} for catalysis, and therefore formation of the iron-oxo intermediate can only occur after two electrons have been extracted from the substrate rather than prior to substrate activation. Conceivably two tyrosines in the active
site could each donate a single electron to cleave O₂ prior to substrate activation, but mutation of these residues to phenylalanine residues yields constructs that remain active. A second compelling reason that the Fe(IV)=O reported herein cannot be responsible for the initial C-H cleavage is that reacting HEPD-E176A with 2-[2-²H₂]-HEP in deuterated buffer increases the amount of ferryl detected by stopped-flow UV-Vis and freeze-quench Mössbauer spectroscopy (Figure 3.10). Neither of the C2 protons is solvent-exchangeable prior to substrate activation, and thus the observation of a solvent kinetic isotope effect indicates that the ferryl probably decays by abstracting a hydrogen atom from a rapidly exchangeable position that is likely a heteroatom.

Given these results, a reformulated mechanism is proposed in Figure 3.15. For the reasons discussed above, a ferric-superoxo species I (rather than the characterized ferryl) likely cleaves the C-H bond to activate the substrate. From ferric-hydroperoxo II, at least two possible pathways could explain the formation of the trapped Fe(IV). Based on DFT calculations on HEPD²⁰ as well as studies with substrate analogs with hydroxypropylphosphonate epoxidase, which demonstrated rapid electron transfer from a ketyl radical to the Fe center,²¹,²² one scenario involves electron transfer from the ketyl radical to the metal, reducing the Fe(III) to Fe(II) ("electron transfer" route). The ferrous-hydroperoxo III could then undergo heterolytic scission with nucleophilic attack on the nearby aldehyde to form the ferryl species. In an alternative route, homolytic bond cleavage of the peroxy species would directly yield the acetal and iron(IV) oxo species IV ("hydroxylation" route).
In either of these routes, several intermediates can undergo exchange of the original pro-$S$ hydrogen at C2 of 2-HEP (blue in Figure 3.15) for a deuterium derived from bulk solvent. Such exchange could explain all of the observations with labeled substrates or solvent. First, the observation that the ferryl did not accumulate with 2-HEP but was observed with 2-$[2^{-2}\text{H}_2]$-HEP in protiated buffer is consistent with incomplete wash-out of the deuterium from the substrate on the time-scale of the intermediates leading to the ferryl. Indeed, as predicted, when the reaction was performed in buffered D$_2$O, a significantly greater amount of ferryl was observed, consistent with the abstraction of a solvent-exchangeable hydrogen atom. The model can also explain several other observations. First, the reaction in D$_2$O resulted in increased build-up of the ferryl with 2-$[2^{-2}\text{H}_2]$-HEP but use of D$_2$O did not lead to observation of the intermediate with unlabeled HEP. The inability to observe any intermediate in the reaction with unlabeled HEP in H$_2$O suggests that the conversion of intermediate IV to gem-diol V is fast. If so, it may be too fast for solvent exchange, explaining why conducting the experiment with unlabeled HEP in D$_2$O did not result in observation of the ferryl species. On the other
hand, when the substrate was deuterium labeled, the hydroxyl group in IV (blue in Figure 3.15) would be deuterated. Apparently the KIE on the conversion of IV to V is sufficiently large to allow detection of IV. This prolonged lifetime may in turn allow solvent exchange, resulting in more build-up of the intermediate when the reaction was conducted with 2-[2-^2H_2]-HEP in D_2O than in H_2O because in D_2O intermediate IV would be fully deuterium labeled, whereas in H_2O, some of the deuterium originating from 2-[2-^2H_2]-HEP would exchange with protium from solvent. The inability to detect the iron(IV)-oxo intermediate in the reaction of unlabeled HEP in D_2O demands that the exchange must take place at intermediate IV and not earlier (e.g. intermediates II or III).

The identical rates of formation of the ferryl when the experiment was conducted with 2-[2-^2H_2]-HEP in H_2O and D_2O are readily explained since all steps up to the formation of the ferryl do not involve a chemical step cleaving/forming a bond to a hydrogen/deuterium that is in an exchangeable position. The identical rates of decay in H_2O and D_2O are at first glance more puzzling. Once again, it is important to note that the intermediate is not observed with unlabeled 2-HEP suggesting IV decays too fast to be detected by stopped-flow UV-Vis if the hydroxyl group of the acetal carries a protium. Therefore, all of the species observed in the stopped-flow experiments presumably contain a deuterium on the hydroxyl group, and the decay rate of this species will be identical in H_2O and D_2O.

One facet of this mechanism that needs discussion is that the bond dissociation energy for an O-H bond is at least 7-10 kcal/mol higher than the C-H bonds typically targeted for cleavage by iron(IV)-oxo species. Though high spin Fe(IV)=O intermediates typically require the targeted H atom to align collinearly with the Fe-O bond, recent work
has shown that these intermediates can activate their substrates even when collinear arrangements are impossible by using intermediates with π character. Due to the constraints of the active site, the latter route is likely operative in the case of HEPD; indeed, two separate theoretical studies postulated that such a π attack by a ferryl in HEPD could cleave the O-H bond depicted in Figure 6. Hirao and Morokuma concluded that ferryl IV was not smoothly connected to the intermediates that came before it and that it would have to traverse energy barriers higher than their preferred alternative mechanism. By contrast, Du et al. argued that in the preferred pathway, a ferryl IV performs water-assisted abstraction of the hydrogen in the O-H bond of the acetal to cause β-scission in a concerted fashion. The detection of a chemically and kinetically competent ferryl intermediate provides experimental support for its intermediacy during HEPD catalysis, and the observed substrate and solvent kinetic isotope effects provides support for its abstraction of a hydrogen atom from an H-O bond.

After hydrogen abstraction by the ferryl IV, the proposed catalytic mechanism is unchanged from previous proposals. Cleavage of the O-H bond of IV yields a gem-diol radical V that could undergo beta scission to generate a methylphosphonate radical and ferric hydroxide VI. The P-C bond in the methylphosphonate radical has been calculated to have a low energy barrier to rotation. Rotation before recombination would explain the racemization in the HMP product when 2-HEP stereospecifically deuterated at C1 was employed as substrate. Subsequent product release is likely the rate-determining step under steady-state conditions, as evidenced by the small substrate KIEs on $k_{cat}$ under steady-state conditions with wt HEPD under steady-state conditions and the
accumulation of an Fe(II)-product complex in the Mössbauer experiment even in the presence of excess substrate.

The combination of pre-steady-state kinetics and steady-state kinetics has enabled the placement of this ferryl intermediate in the context of the catalytic cycle for HEPD. It is unclear why it is possible to trap the Fe(IV) species in HEPD-E176A but not WT HEPD. One possibility is that HEPD-E176A exhibits enhanced reactivity with O₂ compared to WT HEPD. This hypothesis is consistent with the greater $k_{cat}/K_{m,O₂}$ for HEPD-E176A compared to WT HEPD as well as the detection of the Fe(IV) species in WT HEPD when it was reacted with higher O₂ concentrations accessible only by using chlorite dismutase/chlorite to generate a bolus of O₂. Yet another possibility is that deletion of the glutamate residue compromises reactivity of reactive intermediates up to and including the ferryl, and (through means not fully understood) delays the formation and decay of the ferryl to a time regime that is compatible with the spectroscopic techniques employed herein. It is tempting to speculate that this mechanistic hypothesis for HEPD could also explain the catalytic cycle for a related non-heme iron dioxygenase, methylphosphonate synthase, as discussed in Chapters 4 and 5.
3.4: Materials and Methods.

3.4.1: Fe(II) Titrations for WT HEPD and HEPD-E176A

These assays were performed on 2/1/10 and 12/8/11. Steady-state oxidation kinetics for WT HEPD and HEPD-E176A were determined using a Clark-type Oxytherm Electrode Unit (Hansatech, Inc). First, the affinity for Fe(II) was established. WT HEPD (40 µM final concentration) or HEPD-E176A (70 µM final concentration) was anaerobically reconstituted in 25 mM HEPES pH 7.5 by adding varying equivalents of Fe(II) (0-3 eqs. for WT HEPD, 0-5 eqs. for HEPD-E176A), and the protein solution was allowed to incubate on ice for 10 min. Activity was then assayed by monitoring O₂ consumption in air-saturated buffer (25 mM HEPES pH 7.5, 280 µM O₂) at 20 °C in the presence of WT HEPD or HEPD-E176A (2 µM) after addition of 2-HEP (250 µM for WT HEPD or 400 µM for HEPD-E176A). Activity at each concentration of Fe(II) was normalized to the maximum observed; assays were run in triplicate.

3.4.2: Steady-State Kinetic Assays with WT HEPD and HEPD-E176A

These experiments were carried out on 10/29/13, 3/20/14, 3/21/14, 3/23/14, 3/26/14, 3/28/14, 4/4/14, 4/7/14, 4/8/14, and 4/10/14. WT HEPD or HEPD-E176A were purified as previously specified with an additional size exclusion purification step as detailed elsewhere to isolate the active dimer fraction.⁴,⁷ In a typical reconstitution, WT HEPD or HEPD-E176A was first anaerobically reconstituted on ice with Fe(II) (1 eq. for WT HEPD, 3 eq. for HEPD-E176A) to a final concentration of 40 µM in 50 mM HEPES pH 7.5. After at least 10 min of incubation, the protein was removed from the anaerobic chamber. For Michaelis-Menten kinetics with varying phosphonate and a constant O₂
concentration, 1 µM of protein was diluted into air-saturated 50 mM HEPES pH 7.5 (~280 µM O₂ at 20 °C); then the phosphonate substrate (5-100 µM for WT HEPD, 10-250 µM for HEPD-E176A) was added and the rate of O₂ consumption was monitored. For varying the concentration of O₂, the buffer (50 mM HEPES pH 7.5) was sparged with N₂ from a house source for various amounts of time to generate solutions with different concentrations of O₂ (10-250 µM). The protein (1 µM final concentration) and then phosphonate were added (250 µM for either WT HEPD or HEPD-E176A), and the rate of O₂ consumption was monitored in triplicate. For the Michaelis-Menten kinetics determined in D₂O, the assays were run as above, except the buffer of the protein solution was first exchanged to buffer made in D₂O (50 mM HEPES pD 7.5) by washing the protein in a 0.5 mL Amicon centrifuge filter five times such that the theoretical residual amount of protiated buffer was <1%. Additionally, substrate stocks were diluted into D₂O such that in any given reaction, the deuterium content was >98%. For the proton inventory assay, WT HEPD or HEPD-E176A were reconstituted as before; either protein (1 µM final concentration) was then assayed for activity at a saturating concentration of phosphonate (250 µM for either enzyme with 2-HEP or 2-[2-²H₂]-HEP) in buffer with varying D₂O content (adjusted by varying the concentration of 50 mM HEPES pH 7.5 and 50 mM HEPES pD 7.5).

3.4.3: Confirming the Products of HEPD-E176A Catalysis

These experiments were done on 4/1/10 and 5/26/10. The products of incubating 2-HEP with HEPD-E176A were confirmed to be HMP by ³¹P NMR spectroscopy and formate by an LC-MS analysis following derivatization. To confirm HMP formation,
HEPD-E176A was reconstituted as before and then incubated (10 µM final concentration) with O$_2$-saturated buffer (25 mM HEPES pH 7.5) in the presence of 2-HEP (2 mM). After 2 h incubation, EDTA (25 mM final concentration), dithionite (10 mM final concentration), and D$_2$O (20% final v/v) were added, and the $^{31}$P NMR spectrum was recorded. Signals were confirmed by spiking with the authentic samples.

To confirm formate production, reconstituted HEPD-E176A (2 µM final concentration) was incubated with 2-[2-13C]-HEP (2 mM, to circumvent spurious formate contamination) in O$_2$-saturated buffer (25 mM HEPES pH 7.5). After 5 min of reaction, the solution was quenched, derivatized, and analyzed as previously described in detail. A positive control run with WT HEPD under identical conditions confirmed that HEPD-E176A produced formate under these conditions.

### 3.4.4: Stopped-Flow UV-Visible Experiments

This experiment was carried out on 9/5/11. I assayed WT HEPD and HEPD-E176A by stopped-flow for a possible intermediate under nearly-identical conditions. Either construct was reconstituted (4.0 mM stock, 1.0 mM final concentration) anaerobically with Fe(II) (1 eq. for WT, 3 eqs. for HEPD-E176A) in 50 mM HEPES pH 7.5 at 4 °C for >10 min. An anaerobic solution of 2-HEP, 2-[2-2H$_2$]-HEP, or (S)-2-[2-2H$_1$]-HEP was added (2.0 mM final concentration), and the protein-substrate solution was allowed to incubate at 4 °C for a further >10 min. This solution was mixed (1:1 v/v) with buffer (50 mM HEPES pH 7.5) that had been O$_2$-saturated at 0 °C by repeated pump-refill cycles, and the UV-Vis spectrum was monitored as a function of time at ~4 °C. Additional experiments without preloading the protein with the phosphonate substrate,
reacting HEPD-E176A with (R)-2-[2-²H₁]-HEP, and comparing the accumulation of the ferryl intermediate in a reaction run in H₂O vs. D₂O were performed by Ms. Wang.

3.4.5: Mössbauer Time Course of HEPD-E176A and 2-[2-²H₂]-HEP

This assay was carried out on 9/6/11. Aided by Dr. Dassama, I constructed a Mössbauer time course of HEPD-E176A reacting with 2-[2-²H₂]-HEP by first reconstituting HEPD-E176A (4.0 mM stock, 3.4 mM final concentration) with 1 eq. ⁵⁷Fe in 50 mM HEPES pH 7.5 at 4 °C for 10 min. An anaerobic solution of 2-[2-²H₂]-HEP was added (6.8 mM final concentration) at 4 °C, and the solution was allowed to incubate for 10 min. An aliquot of the protein-phosphonate solution was removed as an unreacted anaerobic control to serve as a baseline for comparison; the remainder was transferred into a 1 mL glass-barrel syringe. Buffer (50 mM HEPES pH 7.5) that had been O₂-saturated at 0 °C by repeated pump-refill cycles was loaded from a tonometer into a 2 mL glass-barrel syringe. The solutions were mixed together (1:2 v/VI mix of protein-phosphonate:O₂-saturated buffer) at 4 °C followed by quenching the reaction by rapidly freezing it in a solution of liquid N₂/isopentane maintained at -155 °C (final reaction concentrations: 1.1 mM ⁵⁷Fe-HEPD-E176A, 2.3 mM 2-[2-²H₂]-HEP, 1.3 mM O₂ in 50 mM HEPES pH 7.5) after varying reaction times (10-500 ms). Samples were packed into Mössbauer cups and analyzed by Mössbauer spectroscopy in the presence of a weak external magnetic field (53 mT) by Professor Krebs and Dr. Dassama.
3.4.6: Generating the Ferryl Intermediate in WT HEPD

These assays were performed on 7/29/11 and 7/31/11. Dr. Dassama also helped me in an attempt to generate the same ferryl by reacting WT HEPD with 2-[2-²H₂]-HEP. To do this, WT HEPD (3.4 mM final concentration prior to mixing) was reconstituted with ⁵⁷Fe (1 eq.) in 50 mM HEPES pH 7.5, and then 2-[2-²H₂]-HEP was added anaerobically (2 eqs., 6.8 mM pre-mix concentration). An aliquot was removed as an unreacted control sample, and the remainder of the anaerobic protein-phosphonate solution was transferred to a 2 mL glass-barrel syringe. Chlorite (96 mM premix) and chlorite dismutase (125 µM premix) were separately prepared in 50 mM HEPES pH 7.5 and loaded into two 0.5 mL glass-barrel syringes. The reaction was initiated at 4 °C by mixing the protein-phosphonate solution with the chlorite dismutase and then rapidly mixing the resulting solution with the chlorite solution (final reaction conditions: 2.3 mM ⁵⁷Fe-WT HEPD, 4.6 mM 2-[2-²H₂]-HEP, 20 µM chlorite dismutase, 16 mM chlorite in 50 mM HEPES pH 7.5) followed by rapid quenching after 70-80 ms in a liquid N₂/isopentane cold bath. Samples were also generated at 10 and 25 ms using identical conditions but with lower concentrations of WT HEPD (900 µM) and 2-[2-²H₂]-HEP (1.8 mM). Samples were packed into Mössbauer cups and analyzed by Mössbauer spectroscopy in the presence of a weak external magnetic field (53 mT) by Professor Krebs and Dr. Dassama.

3.4.7: Quantifying Solvent-Exchange in HEPD-E176A

These reactions were performed on 10/31/13, 1/28/14, 2/3/14, and 4/4/14. To quantify solvent wash-in or retention of oxygen from O₂ in the HMP product, ¹⁸O₂ (97%
$^{18}$O) was obtained from Sigma Aldrich while $^{2}H^{18}$O (97% $^{18}$O) and $D_{2}^{18}$O (97.6% $D$, 97% $^{18}$O) were obtained from Cambridge Isotope Labs. In a typical assay using labeled $^{18}$O$_2$, a stock solution of HEPD-E176A was made anaerobic by using a NAP-5 column equilibrated with anaerobic buffer (50 mM HEPES pH 7.5, degassed on a Schlenk line by repeated pump-refill cycles). The degassed HEPD-E176A solution (40 µM final concentration) was anaerobically reconstituted with Fe(II) (3 eqs.) in 50 mM HEPES pH 7.5 and 2-$[2^{2}H_{2}]$-HEP was added (1 mM final concentration). For the reaction in D$_2$O, the same conditions were used as in the protiated case except the HEPD-E176A bulk solution was exchanged into buffer made in D$_2$O (50 mM HEPES pH 7.5) using a 0.5 mL 30 kDa MWCO Amicon centrifuge filter. This process ultimately allowed a deuterium content of ~99% to be reached in the final reaction. For the ~50% deuterated buffer conditions, equal parts of the anaerobic protein-phosphonate solution in H$_2$O and in D$_2$O were mixed. Each reaction (500 µL total) was transferred to a 15 mL round-bottom flask equipped with a stir-bar and sealed tightly with a septum and Parafilm. A canister of $^{18}$O$_2$ was fitted with the appropriate regulator and septum, and the regulator headspace was purged with $^{18}$O$_2$ several times. A 10 mL sample lock syringe was used to remove $^{18}$O$_2$ from the canister and to charge each of the three reaction flasks (~10 mL of $^{18}$O$_2$, pressure unknown). The reactions were allowed to stir in an anaerobic chamber at room temperature for 2 h. The reactions were quenched by removing the protein in each with a 0.5 mL 30 kDa centrifuge filter that had been rinsed with degassed water to remove any traces of contaminating $^{16}$O$_2$.

The filtrates (1 µL) were separated by liquid chromatography coupled to multi-reaction mode, which monitors the transition of a given $m/z$ value fragmenting to another
The components of the filtrate were separated by using LC (Thermo Scientific Hypercarb 100 x 4.6 mm, 5 µm mesh) with a gradient of 25 mM ammonium acetate in H₂O (buffer A) and acetonitrile (buffer B). The column was equilibrated with a 98:2 mixture of buffer A:buffer B. Following injection, this ratio was maintained for 2 min, after which the mobile phase was gradually changed to 50:50 mixture of buffer A:buffer B over the course of 8 min. The mobile phase was returned to a 98:2 mixture of A:B over 1 min and held at that concentration for a further 6 min to reequilibrate the column. At all stages, the flow rate was maintained at 0.3 mL/min. After chromatographic separation, the samples were analyzed by in-line detection with a 5500 QTrap (AB Sciex) by monitoring the 113->62.9 m/z (¹⁸O-HMP) and 111->62.9 m/z (¹⁶O-HMP) transitions in negative mode (curtain gas: 32 psi; voltage: 4,500 V; temperature: 450 °C; N₂ gas one: 50 psi; N₂ gas two: 65 psi). Percent retention of the label was corrected for based on the isotopic composition of the reaction.

For the labeling reactions using H₂¹⁸O and/or D₂¹⁸O, WT or HEPD-E176A (final concentration of 40 µM) was reconstituted with Fe(II) (1 eq. for WT HEPD, 3 eq. for HEPD-E176A) directly in H₂O, or first exchanged into buffer made in D₂O and then reconstituted depending on the desired final D₂O content of the reaction. The buffer used in all cases was 25 mM HEPES pH/D 7.5. The protein solution (5 µM final concentration in the reaction) was removed from the anaerobic chamber and additional H₂¹⁸O, H₂O, D₂O, and/or D₂¹⁸O were added as needed to adjust the total deuterium content of the solution to the desired concentration (0-98%) while maintaining the ¹⁸O content of the reaction at 48.5%. Substrate (2-HEP or 2-[²H₂]-HEP) was added (1 mM final concentration), and the solution (150 µL) was allowed to react under atmospheric
conditions for 2 h at room temperature. The reaction was quenched by filtering with a 0.5 mL 30 kDa MWCO centrifuge filter and analyzed by LC-MRM as before.

3.5: References


(13) Price, J. C.; Barr, E. W.; Glass, T. E.; Krebs, C.; Bollinger, J. M. Evidence for Hydrogen Abstraction from C1 of Taurine by the High-Spin Fe(IV) Intermediate


Chapter 4: Stereochemical Outcome of the MPnS Reaction

4.1: Introduction

As outlined in Chapter 1, methylphosphonate synthase (MPnS) is a non-heme iron enzyme with low sequence similarity to 2-hydroxyethylphosphonate dioxygenase (HEPD) and 2-hydroxypropylphosphonate epoxidase (HppE). Despite low sequence similarity, many of the residues known to be important either for binding the phosphonate substrate or Fe(II) in HEPD or HppE are conserved in the MPnS sequence (Figure 1.8). MPnS was shown to accept 2-HEP in an O$_2$-dependent manner to produce MPn$^1$ and, as with HEPD, the enzyme does not require external electrons for catalysis. However, HEPD transfers the oxygen atoms from O$_2$ to both C1 and C2 of 2-HEP whereas MPnS completely oxidizes C2 to CO$_2$ or bicarbonate while reducing C1. This unprecedented reaction between MPnS and 2-HEP requires the removal of both hydrogen atoms at C2. Understanding the chemistry associated with MPnS catalysis not only is important for understanding phosphonate metabolism but also has environmental implications as the enzyme may represent a key step in methane production in the oceans.$^1$

To investigate the mechanism of catalysis by MPnS with 2-HEP, a post-doctoral researcher in the van der Donk group, Dr. Heather Cooke, first determined the ratio of O$_2$ consumed to MPn produced during the reaction. An assay mixture containing 1 µM MPnS, which was anaerobically reconstituted with one equivalent of Fe(II), was allowed

$^a$ This is an unofficial adaptation of an article that appeared in an ACS publication. ACS has not endorsed the content of this adaptation or the context of its use. The article can be found online at http://pubs.acs.org/doi/abs/10.1021%2Fja306777w.
to equilibrate in the chamber of a Clark-type oxygen electrode. Catalysis was initiated by the addition of 2-HEP, and the consumption of O₂ was monitored. An aliquot of the assay mixture was removed when about 25 µM O₂ had been consumed. The sample was quenched, and the mixture was analyzed by LC-MS to determine the amount of MPn produced using a standard curve. The ratio of MPn produced to O₂ consumed was 1.05 ± 0.07 based on three independent experiments.² Consistent with this unity stoichiometry of O₂ consumed to product oxidized (both transformations are four-electron processes), I was unable to detect H₂O₂ formation (a two-electron reduction of O₂) during 2-HEP turnover by MPnS.²

4.2: Results

4.2.1: Steady-State Oxidation Kinetics of 2-HEP Isotopologues

With the stoichiometry of O₂ consumption established, the kinetic parameters of the oxidation of 2-HEP by MPnS were determined by both Dr. Cooke and me. The dependence of the rate of O₂ consumption on the concentration of O₂ was established by a steady-state continuous assay using the oxygen electrode, establishing a $K_{m,O₂} < 5$ µM. The dependence of O₂ consumption on the concentration of 2-HEP, determined by initial rate measurements, also exhibited Michaelis-Menten kinetics with a $K_m$ of 4.5 µM for 2-HEP and a $k_{cat}$ of 0.18 ± 0.01 s⁻¹ at an oxygen concentration of 280 µM (Table 4.1, Figure 4.1).² To provide information on the timing of the removal of the two hydrogen atoms at C2 and to investigate whether either hydrogen transfer occurred in a (partially) rate-limiting step, the kinetics of three previously synthesized isotopologues,³⁴ (R)- and (S)-2-[2⁻²H₁]-HEP, and 2-[2⁻²H₂]-HEP were determined (Figure 4.1). At saturating 2-HEP
concentrations, the isotopologues displayed rates that were not significantly different from that of unlabeled 2-HEP at close to identical O₂ concentrations (280 µM), indicating that hydrogen transfers do not contribute substantially to the overall rate equation for \( k_{\text{cat}} \) under these conditions. Although the errors on \( K_m \) are relatively large, making strong conclusions difficult to draw, the values of \( k_{\text{cat}}/K_m \) suggest that a small kinetic isotope effect (KIE) on the bimolecular rate constant for both enantiomers of 2-[2-\(^2\text{H}_1\)]-HEP may exist (Table 4.1). One possible explanation for these observations is that the rate of \( k_{\text{cat}} \) may be limited by a physical step such as substrate binding or product release whereas the steps involved in removal of each of the hydrogen atoms at C2 contribute to \( k_{\text{cat}}/K_m \).

### Table 4.1: Kinetic parameters of phosphonate oxidation by MPnS.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( k_{\text{cat}} ) (µM)</th>
<th>( K_m ) (µM)</th>
<th>( k_{\text{cat}}/K_m ) (M⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-HEP</td>
<td>0.18 ± 0.01</td>
<td>4.5 ± 1.4</td>
<td>(4.0 ± 1.3) x 10⁴</td>
</tr>
<tr>
<td>(R)-2-[2-(^2\text{H}_1)]-HEP</td>
<td>0.17 ± 0.01</td>
<td>7.1 ± 2.3</td>
<td>(2.4 ± 0.8) x 10⁴</td>
</tr>
<tr>
<td>(S)-2-[2-(^2\text{H}_1)]-HEP</td>
<td>0.17 ± 0.01</td>
<td>6.6 ± 2.0</td>
<td>(2.5 ± 0.7) x 10⁴</td>
</tr>
<tr>
<td>2-[2-(^2\text{H}_2)]-HEP</td>
<td>0.17 ± 0.01</td>
<td>7.2 ± 1.2</td>
<td>(2.4 ± 0.4) x 10⁴</td>
</tr>
</tbody>
</table>

**Figure 4.1:** Steady-state oxidation kinetics by MPnS of (A) 2-HEP, (B) (R)-2-[2-\(^2\text{H}_1\)]-HEP, (C) (S)-2-[2-\(^2\text{H}_1\)]-HEP, or (D) 2-[2-\(^2\text{H}_2\)]-HEP under air-saturating conditions.
4.2.2: Stereochemistry of Initial Hydrogen Atom Abstraction from 2-HEP

Based on previous observations when the enantiomers of 2-hydroxypropylphosphonate ((2R)- and (2S)-HPP) were reacted with HEPD, only the (2R)-HPP substrate was expected to bind in the MPnS active site in a conformation that would present a hydrogen atom for abstraction. Reactivity with one substrate but not the other would therefore indicate which hydrogen atom of 2-HEP is removed by MPnS in the initial activation of the substrate. Dr. Cooke observed negligible oxygen consumption with (2S)-HPP, suggesting that this enantiomer is not a substrate for MPnS. In contrast, when Dr. Cooke reacted (2R)-HPP with MPnS, oxygen consumption was observed but the activity ceased well before complete consumption of the substrate. Furthermore, the recovered enzyme was inactive towards 2-HEP. These findings suggest a time-dependent inactivation of MPnS by (2R)-HPP. Next, Dr. Cooke and I separately carried out an assay containing 1.5 mM (2R)-HPP and 0.5 mM MPnS and analyzed the reaction by $^{31}$P NMR spectroscopy, revealing three new resonances (Figure 4.2). Nearly all of the substrate was converted to 2-oxopropylphosphonate (OPP; 10.8 ppm), with small amounts of MPn (23.1 ppm) and inorganic phosphate (P$_i$, 3 ppm) also formed. Very little substrate remained (20.3 ppm). The identity of all products was confirmed by spiking with authentic synthetic materials.$^{3,5}$ Oxidation of an alcohol group to a ketone in a substrate analog was also observed with HEPD$^5$ and HppE.$^{6,7}$ That only the $R$ enantiomer of 2-HPP was a substrate for MPnS suggested that the pro-$S$ hydrogen on C2 of 2-HEP was removed initially, consistent with a similar first step of HEPD with 2-HEP. Furthermore, the stereochemical removal of the pro-$S$ hydrogen of 2-HEP is also congruent with the X-ray structure of HEPD$^3$ and the sequence homology of HEPD with MPnS (Figure 1.8).
(2S)-HPP is likely not a substrate because the C2 hydrogen is not positioned correctly in the active site for abstraction.

**Figure 4.2:** $^{31}$P NMR spectrum demonstrating that MPnS abstracts the pro-$S$ hydrogen atom from (2$R$)-HPP and subsequently converts it to a variety of products. Figure assembled by Dr. Cooke.

The inactivation of MPnS by (2$R$)-HPP must be a complex process because several phosphorus-containing products are observed: methylphosphonate, phosphate, and 2-oxopropylphosphonate. The mechanism in Figure 4.3 tentatively explains the formation of the detected products as well as the observed enzyme inactivation. Assuming that the initial steps for MPnS catalysis with (2$R$)-HPP are the same as those for 2-HEP (vide infra), superoxo intermediate Ia would abstract the C2 hydrogen atom to form hydroperoxo species IIa. Following reduction of the Fe center to the ferrous state, intermediate IIIa could partition to generate either 2-oxopropylphosphonate and release hydrogen peroxide (which can oxidize the enzyme to an inactive ferric state) or, by analogy to the HEPD catalytic cycle with 2-HEP, hydrogen atom abstraction by species IVa would yield a gem diol radical that would then induce $\beta$-scission and form a MPn radical (Figure 4.3, red arrow). In the absence of formate (see below), this MPn radical
may abstract a hydrogen atom from the methyl group of acetate bound to the metal. Alternatively, hydrolysis of intermediate IIIa would yield phosphate and an enolate anion; protonation of this species would form acetone.

Figure 4.3: A possible mechanism by which both 2-OPP and MPn could be produced from incubating MPnS with (2R)-HPP. The rate of hydroxylation of ketone IIIa would govern how much 2-OPP is made compared to how much MPn is formed. Phosphate could be formed if the 2-OPP substrate were attacked in a manner reminiscent of how many phosphonate hydrolases operate.

Formation of MPn via the mechanism outlined in Figure 4.3 would also yield an equivalent of glycolate if recombination with the hydroxide took place. I therefore further examined the byproducts of the reaction between MPnS and (2R)-HPP. Indeed, glycolate was observed after derivatization of the organic acids and subsequent analysis by LC-MS (Figure 4.4). Control reactions in which MPnS was not reconstituted but the reaction mixture was derivatized did not display any derivatized glycolate. Similarly, a control in which the MPnS reaction was run normally but 2-nitrophenol hydrazine (2-NPH) was omitted during the derivatization procedure displayed no derivatized glycolate. Hence,
the glycolate appears to be enzyme-generated. Our proposed mechanism was further supported indirectly by showing, via O\textsubscript{2} consumption with 2-HEP as substrate, that MPnS was reactivated upon addition of fresh Fe(II) (100 \mu M) or ascorbic acid (1 mM) signaling that inactivation appears to be a consequence of oxidation of the iron, possibly as a consequence of H\textsubscript{2}O\textsubscript{2} release.

**Figure 4.4:** Extracted ion chromatograms (210 m/z, negative mode, corresponding to derivatized glycolate) of various experiments after derivatization. The glycolate standard (black) eluted with the same retention time as an unknown organic acid found in the reaction between MPnS and (2R)-HPP (red). No glycolate was detected in the unreconstituted MPnS control reaction (blue) or when 2-NPH was omitted from the derivatization reaction (green).

### 4.2.3: Origin of the Incipient Hydrogen in MPn

The conversion of 2-HEP to MPn requires the acquisition of a proton or hydrogen atom at C1 during or following C-C bond cleavage. This hydrogen can come from either bulk solvent (or enzyme) or from the substrate itself. Dr. Cooke and I independently examined both possibilities by assaying MPnS in 99.9% D\textsubscript{2}O with 2-HEP, and in H\textsubscript{2}O with (S)- or (R)-2-[2-\textsuperscript{2}H\textsubscript{1}]-HEP. The resulting MPn products were analyzed by LC-FTMS by Dr. Bradley Evans to determine whether \[^2\text{H}_1\]-MPn (m/z\textsubscript{calc} = 95.99663) or unlabeled MPn m/z\textsubscript{calc} = 94.99036) was produced (Figure 4.5). The assays containing D\textsubscript{2}O and (S)-2-[2-\textsuperscript{2}H\textsubscript{1}]-HEP produced unlabeled MPn, signifying that the new hydrogen on the carbon
of MPn does not originate from solvent (or solvent exchangeable residues on the enzyme) or from the pro-\textit{S} hydrogen on C2 of 2-HEP (Figure 4.5A and 4.5B). In the assay containing (\textit{R})-2-[2\textsuperscript{2}H\textsubscript{1}]-HEP, however, only deuterium labeled MPn was observed by FT-MS (Figure 4.5C), indicating that the pro-\textit{R} hydrogen of 2-HEP is transferred to MPn during catalysis. A \textsuperscript{1}H-coupled \textsuperscript{3}P NMR spectrum enabled observation of the coupling of the protons to the \textsuperscript{3}P nucleus, and confirmed the transfer of the pro-\textit{R} hydrogen on C2 of 2-HEP to the methyl group of the MPn product (Figure 4.6).

**Figure 4.5:** MPnS labeling results by MS. FT-MS analysis (negative mode) after incubation of MPnS with (\textbf{A}) 2-HEP in D\textsubscript{2}O, (\textbf{B}) \((\textit{S})\)-2-[2\textsuperscript{2}H\textsubscript{1}]-HEP in H\textsubscript{2}O or (\textbf{C}) \((\textit{R})\)-2-[2\textsuperscript{2}H\textsubscript{1}]-HEP in H\textsubscript{2}O to determine the origin of the third hydrogen on MPn. The ion at m/z 95.02508 in panel C is not unlabeled MPn but an unknown impurity outside the 10 ppm error range calculated for MPn. The MS data was corroborated by \textsuperscript{3}P NMR analyses of the products (Figure 4.6). Figure made by Dr. Evans.

**Figure 4.6:** Confirming incorporation of the pro-\textit{R} hydrogen in the MPn product. \textsuperscript{1}H-coupled \textsuperscript{3}P NMR analysis of with (\textbf{A}) 2-HEP in D\textsubscript{2}O, (\textbf{B}) \((\textit{S})\)-2-[2\textsuperscript{2}H\textsubscript{1}]-HEP in H\textsubscript{2}O or (\textbf{C}) \((\textit{R})\)-2-[2\textsuperscript{2}H\textsubscript{1}]-HEP in H\textsubscript{2}O demonstrated the presence of a -CH\textsubscript{3} group in the MPn product in the first two cases and a CH\textsubscript{2}D moiety (hence the triplet) in the last case.
4.3: Discussion

Taken together, the data presented herein suggest that the initial abstraction step in MPnS catalysis mirrors that in HEPD catalysis and that the final intermediate is also a MPn radical (Figure 4.7).\textsuperscript{2,4} Therefore, as in HEPD catalysis, after abstraction of the pro-S hydrogen atom from C2 of 2-HEP by the ferric superoxo species \textbf{Ib}, the ketyl radical \textbf{IIb} might transfer an electron to the Fe(III), generating phosphonoacetaldehyde ligated to the iron (Figure 4.7).\textsuperscript{8} These two steps could also occur in a concerted process with electron transfer commencing during hydrogen atom abstraction from C2. As with HEPD, attack of ferrous-hydroperoxo \textbf{IIIB} with cleavage of the $O-O$ bond could be one possible route to generate ferryl \textbf{IVB} (Figure 4.7); direct hydroxylation would also afford intermediate \textbf{IVB}. A second hydrogen atom abstraction event by the ferryl would generate a gem-diol radical,\textsuperscript{8} ultimately resulting in C-C bond cleavage, forming a MPn radical and formate bound to the ferric-iron center (\textbf{Vb}). In MPnS, however, the MPn radical is proposed to abstract the hydrogen atom on formate, resulting in MPn and a CO\textsubscript{2} radical anion. The latter is a very strong reductant\textsuperscript{9} that can reduce the metal center back to its ferrous state, preparing the enzyme for another round of catalysis following release of CO\textsubscript{2} and MPn. Alternatively, hydrogen atom abstraction from formate and electron transfer to the Fe(III) could occur simultaneously.
**Figure 4.7:** A possible mechanism to explain the incorporation in MPn of the hydrogen atom originating from the pro-\(R\) position at C2 (shown in orange) of 2-HEP.

In HEPD catalysis, MPn radical \(\text{Vb}\) likely has a low barrier to rotation along the P-C bond axis, resulting in scrambling of the stereochemistry at C1 and racemic HMP.\(^4\) The MPnS active site likely traps formate in the active site and positions it to facilitate abstraction of the hydrogen atom (originally derived from the pro-\(R\) position at C2). Though there is no direct evidence for equivalent intermediates in HEPD and MPnS catalysis (i.e. a consensus mechanism), the likelihood that the active site architectures are similar (Figure 4.8) coupled with the hypothesis that the first and last intermediates are similar constrains the energetic landscape and mechanistic trajectory. As described in Chapter 5, the characterization of a mutant of HEPD that produces both MPn and HMP with isotope-sensitive branching is consistent with a late-stage intermediate that determines product identity.
Figure 4.8: Overlay of the active site of MPnS (light blue sticks) from a homology model generated by Phyre\textsuperscript{10} with the HEPD•Cd(II)•2-HEP cocrystal structure (green sticks).\textsuperscript{3}

In summary, this study provides the first mechanistic insights into catalysis by MPnS. The data suggest that following removal of the pro-\textit{S} hydrogen, the pro-\textit{R} hydrogen is transferred from C2 of 2-HEP to the methyl group of MPn. Neither step is rate-limiting under saturating substrate concentrations. Further elucidation of the details of the early steps requires spectroscopic characterization of the intermediates\textsuperscript{11} or possibly a combination of \textsuperscript{2}H and \textsuperscript{18}O-kinetic isotope effects.\textsuperscript{12,13} Preliminary studies using the latter are described in Chapter 5. MPnS is another addition to the impressive array of transformations catalyzed by non-heme iron dependent enzymes.\textsuperscript{14,15}

4.4: Materials and Methods

4.4.1: Kinetic Assays

These experiments were performed on 9/2/12, 9/6/12, and 3/25/14. MPnS was purified and reconstituted as previously reported.\textsuperscript{1,2} For steady-state kinetic assays of MPnS, MPnS was anaerobically reconstituted with 1 eq. of Fe(II) in 50 mM HEPES pH
7.5. Kinetic assays were performed by holding one substrate (O₂ or phosphonate) at a saturating concentration and varying the concentration of the other substrate with MPnS (1 µM final concentration) in 50 mM HEPES pH 7.5 at 20˚C. Dr. Heather Cooke (van der Donk group, University of Illinois) independently assayed MPnS steady-state kinetics and obtained very similar numbers.

**4.4.2: Stereochemistry of Hydrogen Atom Abstraction**

These experiments were performed on 8/19/12, 8/23/12, and 9/6/12. Dr. Cooke also established the activity of MPnS towards (2R)-HPP and not (2S)-HPP. I confirmed that glycolic acid was formed during the incubation of MPnS (first anaerobically reconstituted with 1 eq. Fe(II), 500 µM final concentration) with 2R-HPP (1.5 mM) in 25 mM HEPES pH 7.5 by subsequently derivatizing the reaction with 2-nitrophenyl hydrazine and the coupling reagent EDC as specified elsewhere. I also confirmed that after incubation with (2R)-HPP (1.5 mM), MPnS (10 µM) activity towards 2-HEP was partially rescued by adding ascorbic acid (1 mM) or Fe(II) (100 µM).

**4.4.3: Determining the Origin of the Incipient Hydrogen Atom in MPn**

This assay was performed on 9/2/12. Samples to determine the origin of the incipient proton in MPn were generated by both Dr. Cooke and myself; LC-FTMS analysis was carried out by Dr. Bradley Evans (Metcalf group, University of Illinois), and ³¹P NMR analysis was carried out by me. Briefly, anaerobically reconstituted WT MPnS in deuterated buffer (25 mM HEPES pD 7.5, total ²H content > 98%) or in protiated buffer (25 mM HEPES pH 7.5) was added (20 µM final protein concentration) to
substrate (2-HEP in D$_2$O, (S)-2-[2-$^2$H$_1$]-HEP in H$_2$O or (R)-2-[2-$^2$H$_1$]-HEP in H$_2$O, 1 mM final concentration) and the reaction mixture was allowed to incubate at room temperature. After 2 h, the reaction mixtures were filtered through an Amicon centrifuge filter (30 kDa MWCO); 20% (v/v) D$_2$O was added to all reaction samples, and the $^{31}$P NMR spectrum was recorded with the $^1$H decoupler turned off. An aliquot was also reserved for LC-FTMS analysis.

4.5: References


Chapter 5: Evidence of a Consensus Mechanism for HEPD and MPnS

5.1: Introduction

The sequence homology between HEPD and MPnS combined with the observation that MPnS transfers the pro-R hydrogen atom from C2 of 2-HEP to the methyl group in MPn led to the hypothesis that these proteins operate by a similar mechanism in the early stages of catalysis but then diverge at a late intermediate. Based on the accumulated evidence for HEPD (Chapters 2 and 3) and MPnS (Chapter 4), this intermediate is likely a carbon-centered radical that can either recombine with the ferric hydroxide to afford HMP or abstract a hydrogen atom from formate to generate MPn (Figure 5.1). That neither enzyme requires an exogenous source of electrons implies that putative ferric-superoxo species I is responsible for the initial substrate activation in each enzyme. In each case, cleaving the pro-S hydrogen atom at C2 of 2-HEP would yield ketyl radical II (Figure 5.1). As discussed in Chapter 3, this intermediate is then hypothesized to convert to a ferryl that was observed for HEPD, but these early steps of catalysis in each protein remained uncharacterized experimentally.

Figure 5.1: Hypothesized early and late intermediates in the catalytic cycle of HEPD and MPnS. Studies on active site mutants of HEPD and the HEPD/MPnS homologs described in this chapter were designed to find a system that could facilitate accumulation of intermediate I. The substrate analogs were designed to report on the existence of ketyl radical II. $^{18}$O KIE studies could in theory report on any intermediate as long as it occurs before the first irreversible step (here shown as the hydrogen atom abstraction step).
Several different approaches were pursued to characterize these hypothesized early intermediates in catalysis. Given the success at trapping an intermediate with the HEPD-E176A construct (Chapter 3), I hypothesized that it might be possible to trap ferric-superoxof I by perturbing the active site. To do so, the iron-binding glutamate residue in WT HEPD was mutated to a histidine to endow the active-site Fe(II) in HEPD with a 3-His coordination sphere and perturb the kinetics or stability of the ferric-superoxof species, possibly enabling its accumulation. In a second approach to identify the early pathway intermediates, I characterized a series of HEPD and MPnS homologs identified in bacterial genomes with the hope that different active site architectures in one of these proteins might sufficiently stabilize the ferric-superoxof species such that it could be trapped. Cleavage of the C-H bond would generate ketyl radical II, which could be indirectly detected by using a moiety that reports on the presence of an α radical such as a cyclopropane group, which can undergo ring-opening in the presence of a nearby radical or cation,1,3 or halogenated substrate analogs, which can undergo halide ion elimination in the presence of a nearby radical.4,5 Because it was unknown which of these substrate analogs would be accepted for turnover by HEPD and MPnS, a series of analogs were synthesized and tested. Finally, competitive 18O KIE studies were performed with WT HEPD and WT MPnS and 2-HEP and 2-[2-2H2]-HEP as substrates in collaboration with the Klinman laboratory at U.C. Berkeley. This measurement reflects the reactive intermediates up to and including those in the first irreversible step in the catalytic cycle6 and therefore could report on putative intermediates in the mechanism including ferric-superoxof I and ferric-hydroperoxof II.
5.2 Results and Discussion

5.2.1: A Bifunctional Mutant of HEPD

HEPD-E176H was constructed via site-directed mutagenesis, overexpressed, and purified using the same procedure as has been detailed elsewhere for WT HEPD. The protein was reconstituted anaerobically with varying equivalents of Fe(II), and the activity of the mutant was determined with a continuous, steady-state assay using a Clark O₂ electrode as reported previously. In contrast to WT HEPD, which required only 1 equivalent of Fe(II) to reconstitute full activity, HEPD-E176H needed more than 1 equivalent of Fe(II) to attain maximal activity, similar to the observations with the E176A construct (Chapter 3). Use of the O₂ electrode also enabled determination of Michaelis-Menten parameters for both 2-HEP and 2-[2-²H₂]-HEP as well as those of O₂ (Table 5.1, Figure 5.3). HEPD-E176H appeared to have a minimal $k_{cat}$ enhancement compared to that observed when the glutamate was mutated to alanine and overall exhibited similar kinetic parameters to WT HEPD and HEPD-E176A (Table 3.1). The high affinity for O₂ however precluded any determination if there was a KIE on $k_{cat}/K_{m,O₂}$.

Figure 5.2: Dependence of the activity of HEPD-E176H on the equivalents of Fe(II) demonstrating that more than 1 equivalent of Fe(II) was required to saturate the active site. In the assay used as a proxy for Fe(II) binding, the HEPD-E176H concentration was 2 μM, and maximal activity was achieved with roughly 6 μM Fe(II) in the reaction.
Figure 5.3: Steady-state kinetics with HEPD-E176H were determined with 2-HEP (red) or 2-[2-^2^H_2]-HEP (blue).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_{\text{cat}}$ (s(^{-1}))</th>
<th>$K_{m,2\text{-HEP}}$ (µM)</th>
<th>$K_{m,O_2}$ (µM)</th>
<th>KIE, $k_{\text{cat}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-HEP</td>
<td>0.38 ± 0.01</td>
<td>23 ± 3</td>
<td>&lt; 5</td>
<td>1.50 ± 0.05</td>
</tr>
<tr>
<td>2-[2-^2^H_2]-HEP</td>
<td>0.26 ± 0.01</td>
<td>25 ± 2</td>
<td>&lt; 5</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.1: Steady-state Michaelis-Menten kinetic parameters with HEPD-E176H.

To confirm HMP formation, the products generated by incubation of HEPD-E176H and 2-HEP were analyzed by \(^{31}\)P NMR spectroscopy, which showed complete consumption of starting material and the appearance of three species (Figure 5.4A). The signals at 17 and 3 ppm were identified as HMP and P\(_i\), respectively, as determined by spiking with the authentic compounds; P\(_i\) is likely the result of HEPD-like oxidation of HMP. However, the additional resonance at 23 ppm remained unidentified. Because of the homology between MPnS and HEPD, the additional resonance was likely MPn. This hypothesis was confirmed by spiking the sample with authentic MPn, indicating that the E176H mutation had endowed this protein with the ability to bifurcate activity between native HEPD activity and novel MPnS functionality.

As discussed in Chapter 4, the incipient hydrogen atom in MPn generated by reacting 2-HEP with MPnS is derived from the pro-\(R\) hydrogen at C2 rather than from
bulk solvent or solvent-exchangeable protons from the protein itself.\(^9\) This result enabled the formulation of a mechanism that could explain this observation (Figure 4.7). To investigate whether an identical labeling pattern occurred in MPn produced by HEPD-E176H, 2-HEP was incubated with enzyme in buffer made up in D\(_2\)O, and (\(R\))-2-[2-\(^2\)H\(_1\)]-HEP or (\(S\))-2-[2-\(^2\)H\(_1\)]-HEP were separately incubated with the enzyme in buffer made up in H\(_2\)O. After Chelex treatment to remove Fe(II), removal of enzyme, and addition of D\(_2\)O, the \(^1\)H-decoupled \(^{31}\)P NMR spectra were recorded (Figure 5.4). While the experiment with 2-HEP in D\(_2\)O and the reaction of (\(S\))-2-[2-\(^2\)H\(_1\)]-HEP in H\(_2\)O had similar MPn to HMP and P\(_i\) ratios, the reaction with (\(R\))-2-[2-\(^2\)H\(_1\)]-HEP displayed a greatly reduced amount of MPn compared to HMP and P\(_i\). In addition, the \(^1\)H-coupled \(^{31}\)P NMR spectra demonstrated that the additional proton in MPn did not derive from either bulk solvent (Figure 5.4A inset) or the pro-\(S\) C2 hydrogen (Figure 5.4B inset) as observed by a quartet splitting pattern for the phosphorus in MPn in each case. In the case of (\(R\))-2-[2-\(^2\)H\(_1\)]-HEP, the MPn signal appeared to be a triplet (Figure 5.4C inset). However, a triplet would be consistent with transfer of the pro-\(R\) hydrogen at C2 into the methyl group of MPn, which is the labeling pattern observed in MPn produced by MPnS.\(^9\) The approximately 1:1 ratio of HMP and phosphate to MPn when 2-HEP was the substrate indicated that the energy barriers for hydroxyl radical recombination (Figure 5.5, blue arrow, "HEPD") or hydrogen atom abstraction from formate (green arrow, "MPnS") were roughly equal in height. The dramatic shift in product distribution to 11:1 in favor of HEPD activity with a deuterium atom installed at the C2 pro-\(R\) position implied a large kinetic isotope of approximately 10 in the step that determined whether MPn or HMP was formed.
Figure 5.4: $^1$H-decoupled $^{31}$P NMR spectra of the reaction of HEPD-E176H with (A) 2-HEP in buffered D$_2$O, (B) (S)-2-[2-$^2$H]$_1$-HEP in buffered H$_2$O or (C) (R)-2-[2-$^2$H]$_1$-HEP in buffered H$_2$O. Inset: the MPn signal from the $^1$H-coupled $^{31}$P NMR spectrum of each reaction; the MPn derived from 2-HEP in D$_2$O or (S)-2-[2-$^2$H]$_1$-HEP in H$_2$O split into quartets whereas that from (R)-2-[2-$^2$H]$_1$-HEP in H$_2$O split into a triplet.

Figure 5.5: Energy landscape of the partitioning of HEPD-E176H. His176 could form a hydrogen bond to the formate and orient it for attack by the MPn radical.
Because of the limited quantity of (R)-2-[2-\textsuperscript{2}H\textsubscript{1}]-HEP available and the small amount of MPn produced from this substrate, I sought to corroborate transfer of the pro-R C2 hydrogen by using 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP as a substrate in a relatively large-scale reaction with HEPD-E176H. After treating the reaction with Chelex to remove Fe(II), removing the enzyme, and lyophilizing the reaction mixture, the residue was redissolved in D\textsubscript{2}O. Separately, protiated and monodeuterated MPn were generated enzymatically as standards by reacting MPnS with either 2-HEP or 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP. A \textsuperscript{1}H-decoupled \textsuperscript{31}P NMR spectrum of the HEPD-E176H reaction with 2-[2-\textsuperscript{2}H\textsubscript{2}]-HEP displayed two resolved peaks at \textasciitilde23 ppm (Figure 5.6). The identities of the two peaks were confirmed as protiated and monodeuterated isotopologues of MPn by spiking with the authentic standards. The upfield peak corresponded to protiated MPn, whereas the downfield peak corresponded to monodeuterated MPn. The observation of protiated MPn is a consequence of incomplete deuterium-labeling in the substrate. Though the lack of baseline resolution between the isotopologues of MPn in the \textsuperscript{31}P NMR spectrum precludes unambiguous quantitation, the product distribution agrees relatively well with the outcome observed in the reactions of HEPD-E176H with the monodeuterated enantiomers (Figure 5.4).
Figure 5.6: To corroborate the transfer of the pro-R hydrogen at C2 into MPn, (A) a large scale reaction (5 mL) was carried out between HEPD-E176H and 2-[2-^2H]_-HEP resulting in the formation of two signals corresponding to different MPn isotopologues. (B) The upfield peak was confirmed to be protiated MPn by spiking with enzymatically generated MPn, and (C) the downfield peak was determined to be monodeuterated MPn by spiking with enzymatically generated [^2H]_-MPn using MPnS and 2-[2-^2H]_-HEP. The integration numbers are such that the total product (including HMP and P_i, not shown) is 100.

5.2.2: Pre-Steady-State Reactivity of the HEPD-E176H Mutant

As mentioned previously, I hypothesized that a 3-His coordination sphere might perturb the kinetics and/or stability of the reactive intermediates leading to the ferryl intermediate that was trapped in HEPD-E176A (Chapter 3) and permit the accumulation of the still-elusive Fe(III)-superoxo complex. Therefore, HEPD-E176H was anaerobically reconstituted and either 2-HEP or 2-[2-^2H]_-HEP was added. The anaerobic protein-phosphonate solution was rapidly mixed with O_2-saturated buffer, and the reaction was
monitored in a stopped-flow UV-Visible spectroscopic experiment (Figure 5.7). Whereas no intermediates absorbing from 300-700 nm accumulated when the reaction was carried out with 2-HEP, an intermediate with maximal absorbance at 320 nm formed with 2-[2-\(^2\)H\(_2\)]-HEP and decayed at faster rates (\(2^Hk_{\text{form}} = 170 \pm 10 \text{ s}^{-1}\) and \(2^Hk_{\text{decay}} = 19 \pm 1 \text{ s}^{-1}\)) than the ferryl observed in the case of HEPD-E176A (\(2^Hk_{\text{form}} = 140 \text{ s}^{-1}\) and \(2^Hk_{\text{decay}} = 8.7 \text{ s}^{-1}\)) (Chapter 3). The origin of the rate enhancement for formation and decay of the ferryl species in HEPD-E176H is unclear, but the observation of the ferryl suggested that it is operative in catalysis by both HEPD-E176H and HEPD-E176A. Unfortunately, this result also indicated that HEPD-E176H and 2-[2-\(^2\)H\(_2\)]-HEP would not enable trapping the putative Fe(III)-superoxo species.

**Figure 5.7:** Stopped-flow experiments with HEPD-E176H under various conditions. HEPD-E176H was reconstituted, premixed with either 2-HEP (red) or 2-[2-\(^2\)H\(_2\)]-HEP (blue) and reacted with O\(_2\)-saturated buffer.

**5.2.3: Tuning the Product Distribution of the HEPD-E176H Mutant**

To determine if the active site architecture of HEPD-E176H might provide an explanation for its unanticipated bifunctional activity, HEPD-E176H was cocrystallized with Cd(II) by Jon Chekan, a graduate student in the Nair group at the University of Illinois Urbana-Champaign. Rather surprisingly, the Cd(II) structure exhibited ill-defined
electron density for His176 (Figure 5.8), in contrast to the well-defined electron densities for the native histidines, which were conformationally fixed by virtue of binding to the active site Cd(II). The multiple conformers observed for His176 suggested that it did not bind the active site metal, and that E176H exists as a 2-His-ligated enzyme rather than a 3-His enzyme. The flexibility also explained why more than one equivalent of Fe(II) was necessary to reconstitute full activity of the construct. Moreover, compared to the Cd(II)•WT HEPD crystal structure, the Cd(II) was displaced farther from the residue at position 176 (Figure 5.8); this cavity might enable the His176 residue to form a hydrogen bond with formate in the active site of HEPD-E176H and anchor it so as to facilitate abstraction of the hydrogen atom from formate to generate MPn rather than HMP.

**Figure 5.8:** Crystal structure of Cd(II)•HEPD-E176H (green sticks) superimposed on the Cd(II)•WT HEPD•2-HEP cocrystal structure (blue sticks). (B) A rotated view of panel A. The Cd(II) was displaced and might have caused a cavity to form that formate could occupy. Note the multiple conformations adopted by His176, indicated by the arrow.

To explore the hypothesis that formate lingering in the active site of HEPD-E176H enabled formation of MPn (Figure 5.5), HEPD-E176H was reacted with 2-HEP with or without exogenous formate and the product distribution was determined by $^{31}$P NMR spectroscopy. If the HEPD-E176H active site were solvent-exposed during
catalysis, formate could dissociate from the protein; additional formate in the reaction buffer might then saturate the active site and skew the product ratio towards MPn and away from HMP. However, the reaction supplemented with formate had a nearly identical ratio of MPn to HMP as the reaction without formate (Figure 5.9).

Figure 5.9: $^{31}$P NMR spectra of the reaction of (A) HEPD-E176H (5 µM) with 2-HEP (1 mM) or with (B) additional formate (1 mM) added prior to the start of the reaction. The ratio of MPn (21.5 ppm) to HMP (16.5 ppm) was approximately the same in either case. The signal at 18.7 ppm corresponded to residual 2-HEP.

In the crystal structures of WT HEPD and HEPD-E176H, the hydroxyl group of Ser196 was observed to be 4.1 Å from the Cd(II). Mutating Ser196 to a larger residue that projects farther into the active site and has the capacity to donate a hydrogen bond to the formate might lock the formate into a conformation better suited for attack by the methylphosphonate radical and skew product distribution towards MPn. Therefore, the
HEPD-E176H/S196D and E176H/S196N double mutants were constructed by site-directed mutagenesis, expressed, and purified. After anaerobic reconstitution with Fe(II), the constructs were incubated with 2-HEP and the reaction mixtures were analyzed by $^{31}$P NMR spectroscopy. HMP was the only product observed in the samples (Figure 5.10). The increased size of the Asp and Asn residues might have caused a steric clash that precluded retention of the formate in the active site, or they may have reoriented the formate such that hydrogen atom abstraction by the MPn radical was no longer possible.

![Figure 5.10: $^{31}$P NMR spectrum of the reaction of 2-HEP (20.5 ppm) with (A) HEPD-E176H/S196D or (B) HEPD-E176H/S196N indicated that HMP (17.6 ppm) was the sole product of catalysis. MPn (~23 ppm) was not detected.](image-url)
5.2.4: Enzymes with Homology to HEPD and MPnS

In an effort to discover novel phosphonate biosynthetic gene clusters and possible bioactive compounds, Dr. Kou-San Ju, a post-doctoral scholar in the Metcalf group at the University of Illinois Urbana-Champaign, led a team in screening several thousand strains from the Agricultural Research Service/Northern Regional Research Laboratory (NRRL) for phosphonate biosynthetic gene clusters. To do so, the team screened the genomic DNA of these species with degenerate primers for the gene encoding phosphoenolpyruvate mutase (PepM), which catalyzes the initial step in most known phosphonate biosynthetic pathways. From this initial screen, several hundred strains encoding PepM were identified; the genomes of these strains were subsequently sequenced to obtain information about their phosphonate biosynthetic gene clusters, many of which were found to harbor HEPD/MPnS analogs. With the help of Dr. James Doroghazi, a post-doctoral associate in the Metcalf group at the University of Illinois, I constructed a phylogenetic tree based on the amino acid alignment of the various homologs revealed that putative HEPD and MPnS proteins separated into two distinct clades (Figure 5.11). Notably, the gene cluster responsible for argolaphos biosynthesis in Streptomyces monomycinic contained an HEPD/MPnS-like protein that clustered with MPnS even though HEPD-like activity would enable a more direct route to the isolated phosphonotripeptide product (Figure 5.12). Therefore, I sought to clarify this discrepancy between the hypothesized reactivity and the phylogeny of these enzymes. Additionally, it was hoped that homologous enzymes might be more amenable either to crystallization conditions (to which MPnS has proved recalcitrant) or to spectroscopic techniques to trap the putative ferric-superoxo species I.
Figure 5.11: A phylogenetic tree based on an amino acid alignment of the proteins with homology to MPnS or HEPD. The originally characterized HEPD from *S. viridochromogenes* is indicated in red; MPnS from *N. maritimus* is in blue. 2-Hydroxypropylphosphonate epoxidase from *S. wedmorensis* was used as an outgroup. Figure assembled with help from Dr. Doroghazi.
Figure 5.12: (A) The phosphonate gene cluster from *S. monomycini* contains a number of proteins widely found in other phosphonate gene clusters. (B) A retrosynthetic analysis of the phosphonotripeptide natural product isolated from *S. monomycini*. After the successive action of PepM, phosphonopyruvate decarboxylase (Ppd) and a group III alcohol dehydrogenase (AD) to furnish 2-HEP, a number of other proteins in the cluster could lead to the final product. The implicated ligases are outside the depicted window of the gene cluster. The cluster lacks an appropriate hydroxylase, further supporting the notion that the HEPD/MPnS homolog is functionally an HEPD.

The homolog from *S. monomycini* was cloned as an N-terminal His₆-fusion, overexpressed, and purified. The protein was anaerobically reconstituted with one equivalent of Fe(II) and incubated with 2-HEP. The O₂ concentration during the reaction was monitored using an O₂ electrode (Figure 5.13A), which demonstrated that O₂ consumption occurred in an enzyme-dependent fashion. $^{31}$P NMR spectroscopy confirmed that HMP was the sole product of 2-HEP oxidation by the *S. monomycini* HEPD homolog (Figure 5.13B). Interestingly, this protein retains many residues observed in the HEPD$^7$ or HppE$^{10}$ crystal structures that are important for substrate or metal binding (Figure 5.14) but the *S. monomycini* protein lacks a metal-binding residue at the positions where HEPD and HppE harbor carboxylic acids. The absence of a conserved carboxylic acid ligand is more reminiscent of MPnS from *Nitrosopumilis maritimus* rather than HEPD from *Streptomyces viridochromogenes*. 
Figure 5.13: Activity of the homolog from *S. monomycini* towards 2-HEP. (A) The protein was observed to consume O$_2$ in the presence of 2-HEP; additional aliquots of enzyme were added at 100 s and 200 s to confirm that this consumption of O$_2$ was enzyme-dependent. (B) The sole product that was observed by $^{31}$P NMR spectroscopy was HMP.

**Table 5.13**: Activity of the homolog from *S. monomycini* towards 2-HEP.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Time (s)</th>
<th>Activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. viridochromogenes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HppE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. maritimus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. albus NRR_12346</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N. maritimus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. griseofuscus NRR_12346</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 5.14: A Clustal alignment of the characterized HEPD/MPnS homologs. Residues thought to be important for substrate binding are highlighted in red; those responsible for binding Fe(II) are highlighted in blue. As with MPnS, the four homologs that cluster with MPnS but are functional HEPDs lack a carboxylic acid at either position where HEPD or HppE have a glutamate residue (indicated by a blue arrow).
Curious about how many other functional HEPD homologs might be clustered based on sequence with MPnS in the phylogenetic analysis, the HEPD/MPnS homologs from *Streptomyces albus*, *Streptomyces griseus*, and *Streptomyces griseofuscus* were cloned as N-terminal His$_6$-fusions, overexpressed, and purified to homogeneity. Though all these homologs are clustered in the same clade as the *S. monomycini* protein, these three additional proteins were picked because Dr. Kou-san Ju in the Metcalf group had already isolated genomic DNA from those three organisms. After anaerobic reconstitution with Fe(II), each protein was found to consume O$_2$ in the presence of 2-HEP and oxidized 2-HEP to HMP as determined by $^{31}$P NMR spectroscopy (Figure 5.15). To understand the structural features that induce MPnS activity over HEPD activity, an attempt was made to obtain crystal structures in collaboration with a post-doctoral researcher in the Drennan group at the Massachusetts Institute of Technology, Dr. Jennifer Rabb. Unfortunately, this effort has not yet yielded tractable crystals, nor have the proteins been evaluated for their potential to trap the putative ferric-superoxo species.
Figure 5.15: $^{31}$P NMR spectroscopy analyzing the reactions of 2-HEP with the HEPD/MPnS homologs from (A) *S. albus*, (B) *S. griseus*, or (C) *S. griseofuscus*. In each case, HMP was the sole product detected.

5.2.5: Incubation of HEPD and MPnS with Cyclopropyl Substrate Analogs

Because HEPD-E176H did not enable trapping of intermediates early in the catalytic cycle, indirect methods that might allow their characterization were pursued. Abstraction of the C2 pro-S hydrogen of 2-HEP by the ferric-superoxo species I would result in ketyl radical II (Figure 5.1). Indirect evidence for its intermediacy could conceivably be obtained by incubating either HEPD or MPnS with a series of substrate analogs with intramolecular traps that could undergo ring-opening of the adjacent strained cyclopropane ring (Figure 5.16, compounds 5.4 and 5.6). I reasoned that these substrate analogs could provide us with insight about the lifetime of the ketyl radical II.
and perhaps allow us to distinguish between a divergent electron transfer route and direct hydroxylation route (Figure 3.15 and Figure 4.7) by possibly undergoing ring-opening (Figure 5.16C). Additionally, substrate analogs with cyclopropane rings have been used to establish the presence and lifetime of the C2 ketyl radical in the case of 2-hydroxypropylphosphonate epoxidase (HppE),\textsuperscript{11,12} an enzyme with distant homology to HEPD and MPnS.

![Figure 5.16](image)

**Figure 5.16:** The synthetic scheme to accessing (A) 2-cyc-2-HEP and (B) 1-cyc-1-HEP. (C) A general scheme for protein inactivation via cyclopropane ring-opening upon incubation with compound 5.4 (incubation with 5.6 would be expected to provide similar results). Damage to the protein active site by the primary radical or by interruption of the catalytic cycle (possibly leaving the Fe in a ferric state) would result in protein inactivation.

Compound 5.4 was synthesized following a previously published literature procedure (Figure 5.16A).\textsuperscript{11} Bromination of cyclopropyl methyl ketone followed by an Arbuzov reaction between the brominated derivative 5.1 and triethyl phosphite afforded phosphonate 5.2. Reduction of the ketone moiety with NaBH₄ in MeOH yielded alcohol 5.3; deprotection of the phosphonate ethyl ester groups with trimethylsilyl bromide
(TMSBr) produced the desired substrate analog 2-cyc-2-HEP (5.4). Although 5.4 was synthesized as a racemic mixture, it was anticipated based on the Cd(II)•WT HEPD•2-HEP cocrystal structure that the (R) enantiomer would present a hydrogen atom for abstraction and that binding of the (S) enantiomer would occur in a fashion that rendered hydrogen atom abstraction impossible.

WT HEPD and WT MPnS (400 μM of each) were anaerobically reconstituted and then separately incubated with 2 molar equivalents of 5.4 (800 μM, only 1 equivalent with respect to the (R) enantiomer). The reactions were treated with Chelex, filtered through a centrifuge filter to remove protein, added to D₂O, and analyzed by ³¹P NMR spectroscopy (Figure 5.17). Only a single peak corresponding to starting material was observed, suggesting that either no reaction had taken place or that the (R) enantiomer had been partially converted in a one-turnover inactivation process to products with concentrations too low to observe by ³¹P NMR spectroscopy. To distinguish between these possibilities, WT HEPD and WT MPnS were incubated with 5.4, and the O₂ concentration of each reaction was monitored using a Clark-type O₂ electrode. No O₂ consumption was detected in the reaction with this substrate analog, but robust O₂ consumption was observed when 2-HEP was added to either reaction (Figure 5.18), indicating that the enzymes did not accept 5.4 as a substrate analog.
Figure 5.17: $^{31}$P NMR spectroscopy of (A) 2-cyc-2-HEP in buffer or (B) reacted with WT MPnS or (C) reacted with WT HEPD. Only starting material was detected in the samples.

Figure 5.18: WT HEPD (blue) and WT MPnS (red) were incubated with 2-cyc-2-HEP, but no $O_2$ consumption was observed. After ~100 s, 2-HEP was added, resulting in rapid $O_2$ consumption and demonstrating that the lack of activity towards 2-cyc-2-HEP was not due to inactive enzyme.
Examination of the active site of WT HEPD suggested that the steric bulk of Y184 might preclude binding of \((R)-5.4\) by clashing with the cyclopropane moiety (Figure 5.19); the phenyl ring of Y184 was only 3.8 Å from C2 of 2-HEP in the WT HEPD•2-HEP cocrystal structure. To potentially relieve this interference, the HEPD-Y184A and -Y184L mutants were constructed, expressed, and purified. The HEPD-Y184A variant did not express well, so only the HEPD-Y184L mutant was tested for activity. HEPD-Y184L only weakly consumed \(\text{O}_2\) in the presence of 2-HEP (Figure 5.20); no activity towards compound 5.4 was observed by \(\text{O}_2\) electrode or \(^{31}\text{P}\) NMR spectroscopy with this construct. Enlarging the active site of HEPD may perturb it in an unforeseen fashion that prevents binding of 5.4 and disrupts binding of phosphonates in general.

\[ \text{Figure 5.19: The phenyl ring of Y184 is directly in-line of the projected cyclopropane position of } (R)-5.4, \text{ likely causing a steric clash.} \]
Figure 5.20: HEPD-Y184L weakly consumed O\textsubscript{2} in the presence of 2-HEP (red) but not in the presence of 2-cyc-2-HEP. In each case, substrate was added after roughly 10 s.

I therefore turned our attention to a different cyclopropane analog, 1-cyc-1-HEP (5.6). With the help of an undergraduate summer rotation student, Victor Garcia-Lopez, I condensed dimethyl phosphite with cyclopropanecarbaldehyde to afford phosphonate alcohol 5.5 (Figure 5.16B); deprotection of the phosphonate methyl groups with TMSBr yielded the substrate analog 5.6. As with compound 5.4, no attempt was made to initially synthesize this analog 5.6 in a stereospecific fashion; were activity observed, then a stereospecific synthesis might be pursued. WT HEPD and WT MPnS were reconstituted and incubated with multiple equivalents of compound 5.6 while the O\textsubscript{2} concentration was monitored. However, no O\textsubscript{2} consumption was detected with either protein during the incubation with this substrate analog (Figure 5.21). When 2-HEP was added to the reactions as a positive control, the rate of O\textsubscript{2} consumption was roughly equal to the rate of O\textsubscript{2} consumption in the absence of compound 5.6. As with compound 5.4, this observation suggests that substrate 5.6 was too bulky to bind in the active site of WT HEPD or WT MPnS, but no single residue in the active site stood out as the reason for the failure of 5.6 to bind in the active site.
Figure 5.21: WT HEPD (blue) and WT MPnS (red) were incubated with substrate analog 5.6 while the O₂ was monitored. After not observing any decrease in the O₂ levels for ~60 s, 2-HEP was added, and O₂ was rapidly consumed, demonstrating that the proteins were still active.

5.2.6: Halogenated Substrate Analog Studies with WT HEPD

The steric bulk of the cyclopropane moiety at either the C1 or C2 position did not appear to be well tolerated by HEPD or MPnS, and therefore a smaller functionality that might report on the presence of a C2 radical was sought. One possibility was to employ halogenated substrate analogs in a manner that has been useful for elucidating the mechanism of ribonucleotide reductase.⁴,¹³⁻¹⁵ When the halogen is alpha to a carbon-centered radical, it has been observed to undergo elimination as the halide anion or halogen atom and often results in the inactivation of the enzyme.¹⁶ This expulsion is responsible for one mode of action of the potent drug gemcitabine.¹⁷ By reducing the commercially available phosphonates 5.7 and 5.8 with BH₃-THF to yield alcohols 5.9 and 5.10 (Figure 5.22A) followed by removal of the phosphonate ethyl ester groups in refluxing HCl, I was able to access the racemic phosphonic acids 5.12 and 5.13.
**Figure 5.22:** (A) Synthetic scheme for various halogenated 2-HEP analogs. (B) Possible explanations for the enzymatic inactivation of HEPD and MPnS that account for the formation of phosphate. Either an elimination of the halogen (blue arrow) followed by hydrolysis or direct hydrolysis (red arrow) could account for the formation of phosphate in a manner reminiscent of the hydrolysis of PnAA by phosphonatase. Halogen atom elimination is unlikely in the case of the reaction of 1-F-2-HEP, as fluoride radical formation would be unfavorable. (C) A possible scheme for the nonenzymatic release of halide anions from halohydroxymethylphosphonate. The resulting phosphonoformaldehyde could be hydrolyzed or inactivate HEPD or MPnS. (D) Synthetic scheme for the synthesis of chloromethylphosphonate.

WT HEPD was incubated with 1-Cl-2-HEP under multiple turnover conditions, and O₂ consumption was continuously monitored via a Clark-type oxygen electrode. Rapid inactivation of WT HEPD occurred, though the partial restoration of activity after addition of reductant (ascorbic acid) or Fe(II) suggested that inactivation was not damaging the overall scaffold of the protein (Figure 5.23). When 1-F-2-HEP was incubated with WT HEPD under identical conditions, a very similar result was observed:
inactivation of WT HEPD after approximately 1 equivalent of O₂ was consumed, with activity mostly restored via addition of either reductant or fresh Fe(II) (Figure 5.24). When either 1-Cl-2-HEP or 1-F-2-HEP was incubated with HEPD under single turnover conditions, approximately 1 equivalent of O₂ was consumed (Figure 5.23C and 5.24D). This result indicated that even though 1-F-2-HEP and 1-Cl-2-HEP were racemic mixtures, either enantiomer was accepted for turnover by WT HEPD. Additionally, when WT HEPD (100 µM) was incubated with 1-F-2-HEP (1 mM) and the fluoride concentration was monitored with a fluoride electrode, fluoride was released over time (Figure 5.25). A control experiment with a solution of (NH₄)₂Fe(II)(SO₄)₂ (100 µM) showed no corresponding increase. Because of the high response time of the fluoride electrode at low concentrations of fluoride and the non-linear response below ~10 µM fluoride, the data in Figure 5.25 should be treated as qualitative rather than quantitative. After 24 h, the reaction of WT HEPD with 1-F-2-HEP had resulted in release of 150 µM free fluoride, suggesting that 1-F-2-HEP does not quantitatively inactivate WT HEPD.

In the single turnover reactions between WT HEPD and compound 5.12 (Figure 5.23C) or compound 5.13 (Figure 5.24D), O₂ consumption occurred with biphasic kinetics, consistent with a model in which one enantiomer was consumed preferentially over the other. However, in each case, the amplitude associated with the fast phase was roughly four-fold larger than the amplitude associated with the slow phase; if each rate corresponded to consumption of a separate enantiomer, the amplitudes of each would be equal. The reason for this kinetic complexity is unclear at this time, and further investigations will be required to elucidate whether one enantiomer is preferentially turned over with WT HEPD.
Figure 5.23: Reaction of WT HEPD with 1-Cl-2-HEP. (A) WT HEPD underwent inactivation when incubated with 1-Cl-2-HEP as demonstrated by the lack of significant $O_2$ consumption when incubated with 2-HEP. However, activity could partially be restored by addition of ascorbic acid or (B) a fresh aliquot of Fe(II). (C) Approximately 1 equivalent of $O_2$ was consumed when WT HEPD (200 µM) was incubated with 1-Cl-2-HEP (200 µM), suggesting that both enantiomers were accepted for turnover.
Figure 5.24: Reactions of WT HEPD with 1-F-2-HEP. (A) WT HEPD (10 µM) was mostly inactivated under multiple turnover conditions (1 mM substrate) after turning over slightly more than 1 equivalent of 1-F-2-HEP (dashed line indicates 1 equivalent of O₂). (B) Activity could be partially restored by the addition of ascorbic acid or (C) a fresh aliquot of Fe(II). (D) In a single turnover experiment in which WT HEPD (200 µM) was incubated with 1-F-2-HEP (200 µM), roughly 1 equivalent of O₂ was consumed (indicated by the dashed line), consistent with either enantiomer being accepted for catalysis.

Figure 5.25: Fluoride release occurred in a time-dependent manner when 1-F-2-HEP (1 mM) was incubated with WT HEPD (100 µM, blue) or WT MPnS (100 µM, red) but not when it was incubated with Fe(II)-only (100 µM, black). After 24 h, 1.5 and 0.3 equivalents of fluoride were released with in the WT HEPD and WT MPnS reactions respectively; the Fe(II)-only reaction had free fluoride concentrations < 1 µM.
An attempt was made to determine what products resulted from 1-X-2-HEP turnover with WT HEPD and whether these could help corroborate the existence of radical II. I therefore incubated WT HEPD with either 1-Cl-2-HEP or 1-F-2-HEP and then analyzed the reaction mixture by $^{31}$P NMR spectroscopy (Figures 5.26 and 5.27). In each case, residual starting material and phosphate were identified by spiking experiments, suggesting that inactivation might proceed through one of two pathways depicted in Figure 5.22B. In the elimination pathway (blue), the halogen would be eliminated as the halogen atom. Protonation of C1 could be followed by hydrolysis of the halo-phosphonoacetaldehyde (PnAA) molecule in a manner reminiscent of the hydrolysis of phosphonates by various phosphonate hydrolases. However, an alternative possibility is that halide anions were released nonenzymatically from halohydroxymethylphosphonate (the product of normal turnover of 1-X-2-HEP without inactivation), and that HEPD was instead inhibited by the resulting phosphonoformaldehyde (Figure 5.22C). Given that fluorine atom release is highly unlikely, the mechanism shown in Figure 5.22C is the most likely explanation of the data. In an attempt to determine the carbon-containing products and distinguish between these possibilities, the reaction mixture and the headspace were analyzed by GC-MS. Despite repeated attempts to identify what should presumably be volatile one- or two-carbon products, no products were detected, possibly due to their low concentrations.
Figure 5.26: (A) $^{31}$P NMR spectrum of the reaction of WT HEPD (100 µM) with 1-Cl-2-HEP (2 mM) in the presence of ascorbic acid (2 mM). (B) The peak at 2 ppm spiked with authentic phosphate. (C) The singlet at 12 ppm was confirmed to be starting material by spiking the reaction with 1-Cl-2-HEP.
Figure 5.27: (A) $^{31}$P NMR spectrum of the reaction of WT HEPD (750 µM) with 1-F-2-HEP (1 mM). (B) The doublet at 9 ppm was confirmed to be starting material by spiking the reaction with 1-F-2-HEP, resulting in an increase in intensity relative to the peak at 2 ppm. Because of the low signal-to-noise ratio of the spectrum, discerning whether the signal at 8 ppm is real was challenging. The peak at 2 ppm is likely to be phosphate, in analogy to the WT HEPD reaction with 1-Cl-2-HEP.

5.2.7: Halogenated Substrate Analog Studies with WT MPnS

In contrast to WT HEPD, if WT MPnS were to undergo normal turnover with 1-X-2-HEP without expulsion of the halogen atom during the catalytic cycle, the presumed product would be halomethylphosphonate. This product would be unable to undergo spontaneous non-enzymatic elimination of the halogen, and therefore WT MPnS might be less readily inactivated by incubation with these substrate analogs. Indeed, WT MPnS consumed multiple equivalents of O$_2$ prior to inactivation by 1-Cl-2-HEP (Figure 5.28A). As with WT HEPD, addition of fresh Fe(II) or ascorbic acid could partially restore...
oxygen consumption, and both enantiomers of 1-Cl-2-HEP were accepted for turnover (Figure 5.28C). As with WT HEPD, the consumption of O_2 by WT MPnS in the presence of 1-Cl-2-HEP occurred with biphasic kinetics, consistent with a model in which one enantiomer is preferentially accepted by WT MPnS. However, also as before, the amplitude of the fast phase was roughly 2.4 times larger than the amplitude of the slow phase. The reason for this discrepancy is at present unclear.

Upon incubating WT MPnS (100 µM) with 1-F-2-HEP (1 mM), fluoride release was detected by using a fluoride electrode assay, though the release was much slower than in the case of WT HEPD (Figure 5.25). Analysis of the reaction after 24 h indicated that approximately 27 µM fluoride had been released, whereas a control experiment with a solution of (NH_4)_2Fe(II)(SO_4)_2 resulted in fluoride concentrations below the detection limit. Moreover, inactivation of MPnS by 1-F-2-HEP was much slower than inactivation by 1-Cl-2-HEP, and was sufficiently slow that steady-state Michaelis-Menten parameters of 1-F-2-HEP oxidation by MPnS could be obtained with an oxygen electrode (Figure 5.29). The $K_{m,1-F-2-HEP}$ (23 ± 3 µM) was higher than that of the native substrate 2-HEP (∼5 µM), but quite surprisingly, $k_{cat}$ was also several-fold larger than that of the native substrate (0.99 ± 0.04 s\(^{-1}\) vs ∼0.2 s\(^{-1}\)). This observation suggests that the rate-limiting step in MPnS catalysis with 2-HEP could be deprotonation of the hydroxyl at C2 to spur subsequent H_2O dissociation and subsequent O_2 activation. Similar to how the fluorine in 2-fluoroethanol depresses the hydroxyl group pK_a (by 1.8 units) compared to ethanol, the C1 fluorine of 1-F-2-HEP may be able to inductively stabilize the incipient negative charge on the deprotonated hydroxyl at C2. Deprotonation has been posited to be necessary for subsequent C-H cleavage. To test this hypothesis, dideuterated substrate
(1-F-2-[2-²H₂]-HEP, 5.14) was synthesized in an analogous manner and assayed. No significant change in $K_m$ (20 ± 3 µM) was observed, but a significant decrease in $k_{cat}$ (0.37 ± 0.01) was noted, resulting in a moderate KIE on $k_{cat}$ (2.7 ± 0.1). This KIE suggests that it might be possible to use 1-F-2-[2-²H₂]-HEP and MPnS to trap the initial putative ferric-superoxo species, although the KIE is lower than that observed with MIOX or other reactions involving ferric-superoxo species.²²,²³

**Figure 5.28:** Incubations of WT MPnS (10 µM) with 1-Cl-2-HEP (1 mM). (A) After inactivation with 1-Cl-2-HEP, WT MPnS activity could be partially restored by addition of ascorbic acid (1 mM) or (B) a fresh aliquot of Fe(II) (100 µM). (C) A single-turnover experiment between WT MPnS and 1-Cl-2-HEP (200 µM each) demonstrated that approximately 200 µM O₂ was consumed, suggesting that both enantiomers of 1-Cl-2-HEP are accepted for turnover.
Figure 5.29: (A) WT MPnS (10 µM) catalyzes multiple turnovers with 1-F-2-HEP (1 mM) (the dashed line is where 1 turnover would have been). (B) Inactivation was sufficiently slow that steady-state Michaelis-Menten kinetics with WT MPnS and 1-F-2-HEP (red) or 1-F-2-[2-2H₂]-HEP (blue) could be determined.

To identify the products formed upon incubation of MPnS with 1-F-2-HEP and 1-Cl-2-HEP, I analyzed the reaction mixtures by ³¹P NMR spectroscopy. In the reaction with 1-Cl-2-HEP, three signals were observed. The signals at 12 ppm and 2 ppm were identified by spiking with starting material and phosphate, respectively, leaving the singlet at 13 ppm unidentified (Figure 5.30). In the case of 1-F-2-HEP, both phosphate at 0 ppm and an unidentified doublet at 12.7 ppm were observed (Figure 5.31). Based on NMR spectroscopic evidence, it was hypothesized that the unidentified peak in each spectrum was likely the corresponding halomethylphosphonate, i.e. the products of normal catalysis. To confirm this supposition, chloromethylphosphonate was synthesized²⁴,²⁵ (Figure 5.22D) and spiked into the assay of WT MPnS and 1-Cl-2-HEP, confirming the production of this compound. By analogy, the product of the reaction of WT MPnS with 1-F-2-HEP likely was fluoromethylphosphonate, consistent with the spectral data obtained from a variety of NMR techniques (Figure 5.31). The observation of halomethylphosphonates as products strongly suggests that partitioning occurs
between release of the halide during catalysis, and catalysis proceeding as normal without release of the halide. However, either pathway depicted in Figure 5.22B could lead to formation of phosphate, so the observation of phosphate does not allow any conclusions to be drawn about the inactivation pathway.

Figure 5.30: MPnS reaction with 1-Cl-2-HEP analyzed by $^{31}$P NMR spectroscopy. (A) The MPnS reaction with 1-Cl-2-HEP. (B) Spiking with 1-Cl-2-HEP confirmed that the resonance at 12 ppm is residual starting material and (C) spiking with synthetically prepared chloromethylphosphonate confirmed that the resonance at 13 ppm is chloromethylphosphonate. Separately, the broad resonance at 2 ppm was confirmed to be phosphate by spiking.
Figure 5.31: (A) $^1$H-decoupled $^{31}$P NMR spectrum of the reaction of WT MPnS with 1-F-2-HEP. Inset: The $^1$H-coupled $^{31}$P NMR signal of the doublet at 12.7 ppm, which splits into a doublet of triplets due to a pair of $\alpha$ protons. (B) Spiking the sample with 1-F-2-HEP creates a new doublet. (C) The peak at 0 ppm was determined to be phosphate by spiking with a standard of phosphate. (D) A two dimensional [$^1$H-$^{31}$P] HMBC spectrum demonstrated that the doublet at 12.7 ppm in the $^{31}$P dimension (vertical axis) was coupled to a doublet of doublets at 4.47 ppm in the $^1$H dimension (horizontal axis). The spectral characteristics of this peak are consistent with fluoromethylphosphonate.
5.2.8: $^{18}$O KIE Studies

As described in the preceding sections, neither the attempts to obtain direct spectroscopic evidence nor the use of substrate analogs has been productive in characterizing the putative initial ferric-superoxo species. I therefore pursued $^{18}$O KIE studies in collaboration with the Klinman group at U.C. Berkeley to examine the early intermediates in the catalytic cycle of WT HEPD and WT MPnS. Due to the very small nature of heavy atom oxygen KIEs (< 4%), these experiments are run with natural abundance $^{16}$O$_2$ and $^{18}$O-$^{16}$O to improve the precision of the measurement. As a consequence of the competition between these isotopologues, the KIE reflects the early steps of catalysis up to and including the first irreversible step in the mechanism (i.e. the KIE is measured on $k_{cat}/K_m$ rather than $k_{cat}$).$^6$ This technique has been useful to glean insight into the intermediates in the catalytic cycles of non-heme iron enzymes such as 1-aminocyclopropane-1-carboxylic acid oxidase (ACCO)$^{26}$ and taurine dioxygenase$^{27}$ that would otherwise have been difficult to characterize.

Although the actual magnitude of the KIE is of somewhat limited value, the relative values with protiated and deuterated substrate can give an indication as to the timing of the abstraction of the hydrogen atom with respect to steps involving O$_2$ activation. If the $^{18}$O KIE were to increase with deuterated substrate compared to protiated substrate, abstraction of the hydrogen atom must be at least partially reversible because events that occur after the first irreversible step are not measured by this technique. If, by contrast, the hydrogen atom abstraction occurs after the first irreversible step with respect to O$_2$ (thought to be oxygen-oxygen bond cleavage), the $^{18}$O KIE would
be insensitive to whether a deuterium or protium were abstracted from substrate, and therefore the $^{18}\text{O}$ KIEs would be identical for both substrates. In other words, if the hydrogen abstraction is performed by a ferric superoxide (i.e. before the first irreversible step with respect to oxygen), a difference may be observed between the $^{18}\text{O}$ KIE with protiated and deuterated substrates, whereas if the hydrogen abstraction from substrate is performed by the ferryl (i.e. after the first irreversible step), the $^{18}\text{O}$ KIE should be unaffected by labeling of 2-HEP. Therefore, $^{18}\text{O}$ KIE measurements could provide support for or against our favored mechanism.

I therefore prepared both protein (WT HEPD and WT MPnS) and substrates (2-HEP and 2-[2-$^2\text{H}_2$]-HEP) in quantities necessary for applying this technique. Two post-doctoral researchers in the Klinman group at U.C. Berkeley, Dr. Florence Bonnot and later Dr. Hui Zhu, then reacted WT HEPD and WT MPnS with 2-HEP or 2-[2-$^2\text{H}_2$]-HEP to varying stages of completion. The reactions were quenched by acidifying the mixture, and the residual $^{16}\text{O}_2/^{18}\text{O}-^{16}\text{O}$ atmosphere was combusted in the presence of excess graphite to CO$_2$. Isotope ratio mass spectrometry was then used to evaluate the $^{18}\text{O}$ content of the CO$_2$. The $^{18}\text{O}$ KIEs that were extracted for WT HEPD were $1.015 \pm 0.001$ with 2-HEP and $1.020 \pm 0.001$ with 2-[2-$^2\text{H}_2$]-HEP; with WT MPnS the KIEs were measured as $1.016 \pm 0.001$ with 2-HEP and $1.022 \pm 0.003$ with 2-[2-$^2\text{H}_2$]-HEP. The observation of an altered $^{18}\text{O}$ KIE upon deuterium-labeling of the substrate indicates that the hydrogen atom abstraction step must occur before the first irreversible step with respect to oxygen chemistry. This observation then necessitates that hydrogen atom abstraction occurs prior to ferryl formation, consistent with the mechanisms previously discussed in Chapters 3 (Figure 3.15) and 4 (Figure 4.7).
5.3: Conclusions

The experiments herein detail the attempts to detect and characterize early intermediates in the catalytic cycle of HEPD and MPnS. I hoped that in doing so, evidence that the early intermediates in the catalytic cycle of HEPD and MPnS were identical would be acquired. $^{18}$O KIE studies in collaboration with the Klinman lab at U.C. Berkeley strongly suggest that each protein uses a ferric-superoxo species I to effect the initial hydrogen atom abstraction and yield ketyl radical II (Figure 5.1). Efforts to indirectly detect this carbon-centered radical with cyclopropane substrate analogs failed, and attempts with halogenated substrate analogs yielded mixed results. WT HEPD was potently inactivated by either 1-Cl-2-HEP or 1-F-2-HEP and seemingly formed phosphate as the sole product detectable by $^{31}$P NMR spectroscopy. However, it was unclear if inhibition was due to halogen atom expulsion during or after catalysis. Any halohydroxymethylphosphonate products formed by normal turnover could eliminate halide anions to yield phosphonoformaldehyde. Although this product was not observed by $^{31}$P NMR spectroscopy, its formation could account for enzyme inactivation. By contrast, MPnS catalysis with these halogenated substrate analogs yielded halomethylphosphonates, which would be unable to non-enzymatically eliminate the halide anion. Inactivation of WT MPnS with these substrates might then reflect the existence of ketyl radical II. Indeed, MPnS was only inactivated very slowly and catalyzed multiple turnovers. Hence, whereas the substrate analogs were designed to promote non-native chemistry, the enzymes appeared to mostly convert the substrate analogs using the same chemistry as with their native substrates.
Though the investigation into active site mutants of HEPD and homologs of HEPD/MPnS began as an attempt to find a system that would be amenable to trapping of the putative ferric-superoxo intermediate I, these experiments ultimately yielded compelling evidence that a consensus mechanism is operative in HEPD and MPnS. The isotope-sensitive branching in HEPD-E176H is particularly compelling evidence that the early intermediates are identical in HEPD and MPnS catalysis. Thus far, conditions that transform HEPD completely into MPnS have not been found. However, the existence of several homologs that cluster with the authentic MPnS but carry out HEPD activity suggests that the shared mechanism between these proteins may extend to their evolutionary history as well.

5.4: Materials and Methods

5.4.1: HEPD-E176H Construction and Kinetic Characterization

These experiments were performed on 4/28/11 and 5/5/11. HEPD-E176H was constructed by using the QuikChange kit. Primers were as follows with the mutated codon in red and bolded:

Forward primer:
```
gcgactctacgtgcataccctctctactgc
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Reverse complement primer:
```
gcgagtacgagggactgagtaggcgc
```

Following sequencing to confirm the mutation of the desired codon, the protein was expressed and purified by immobilized metal affinity chromatography followed by
size exclusion chromatography as previously described for WT HEPD.\textsuperscript{28} HEPD-E176H was anaerobically reconstituted with about 0.50 to 5.0 equivalents of (NH\textsubscript{4})\textsubscript{2}Fe(II)(SO\textsubscript{4})\textsubscript{2} (actual numbers were 0.51 to 5.1 equivalents, once corrected for small variation in protein concentration as determined by the UV-Vis absorbance at 280 nm) in 25 mM HEPES pH 7.5 and the protein solution was allowed to incubate on ice for at least 10 min. The activity was assayed by monitoring the rate of O\textsubscript{2} consumption in air-saturated buffer (280 \mu M O\textsubscript{2}, 25 mM HEPES pH 7.5) at 20 °C in the presence of HEPD-E176H (2 \mu M) and 2-HEP (500 \mu M).

Following the determination that approximately three equivalents of Fe(II) were required to reach maximal activity with HEPD-E176H, the steady-state Michaelis-Menten parameters of 2-HEP oxidation were determined by incubating HEPD-E176H (2 \mu M) with 2-HEP or 2-[2\textsuperscript{-2}H\textsubscript{2}]-HEP (10-250 \mu M) at a constant O\textsubscript{2} concentration (280 \mu M) in buffer (25 mM HEPES pH 7.5) at 20 °C. The $K_m$ for O\textsubscript{2} was determined by incubating HEPD-E176H (2 \mu M) with saturating concentrations of each phosphonate substrate (2-HEP and 2-[2\textsuperscript{-2}H\textsubscript{2}]-HEP, 250 \mu M) and varying O\textsubscript{2} concentration (adjusted by sparging the reaction buffer with N\textsubscript{2} from a house source) in buffer (25 mM HEPES pH 7.5).

5.4.2: Identification of the Products Formed by HEPD-E176H

These assays were carried out on 11/4/11, 9/15/12, 9/17/12, 9/25/12, 3/28/14, 3/30/14, and 4/6/14. HEPD-E176H was anaerobically reconstituted with one equivalent of Fe(II) in either buffer made up in H\textsubscript{2}O (25 mM HEPES pH 7.5) or in buffer made up in D\textsubscript{2}O (25 mM HEPES pD 7.5). The HEPD-E176H (20 \mu M final concentration) was then incubated with substrate ((R)-, (S)-2-[2\textsuperscript{-2}H\textsubscript{1}]-HEP or 2-HEP, 1 mM) in either buffer made
up in H₂O for the monodeuterated enantiomers (25 mM HEPES pH 7.5) or buffer made up in D₂O in the case of 2-HEP (25 mM HEPES pD 7.5). The reaction volume was 500 µL. After incubation for 2 h, the enzyme was removed with a centrifuge filter (30 kDa MWCO), treated with Chelex, and then D₂O was added (20% final v/v). The samples were analyzed by ³¹P NMR spectroscopy with the proton decoupler on and off.

To corroborate the incorporation of the hydrogen atom originating from the pro-R face at C2 of 2-HEP into MPn produced by HEPD-E176H, reconstituted HEPD-E176H (80 µM) was incubated with 2-[2-²H₂]-HEP (1 mM) in 50 mM HEPES pH 7.5 on a larger scale (5 mL). The reaction was allowed to react for 8 h, frozen in liquid N₂, and lyophilized to afford a white residue. The residue was redissolved in D₂O, treated with Chelex, and filtered by centrifugation (30 kDa MWCO). The sample was analyzed by ¹H-coupled ³¹P NMR spectroscopy as well as ³¹P NMR spectroscopy with the ¹H decoupler enabled. To confirm the identity of the two singlets observed in the ³¹P NMR spectrum, the sample was spiked with enzymatically-generated MPn and [²H₁]-MPn, which had been obtained by reacting reconstituted WT MPnS (10 µM) with either 2-HEP or 2-[2-²H₂]-HEP (2 mM) in 50 mM HEPES pH 7.5.

5.4.3: Analysis of HEPD-E176H by Stopped-Flow UV-Vis Experiments

This assay was performed on 8/4/11. Similar to the protocol detailed for the stopped-flow experiment with HEPD-E176A (Section 3.4.4), HEPD-E176H (0.6 mM) was anaerobically reconstituted with Fe(II) (3 equivalents, 1.8 mM) in 50 mM HEPES pH 7.5 at 4 °C for 10 min. The protein was mixed with either 2-HEP or 2-[2-²H₂]-HEP (2 equivalents, 1.2 mM) and allowed to incubate for a further 10 min at 4 °C. This solution
was mixed (1:1 v/v) with buffer (50 mM HEPES pH 7.5) that had been O$_2$-saturated at 0 
°C by repeated pump-refill cycles, and the UV-Vis spectrum was monitored as a function of time at −4 °C.

5.4.4: HEPD-E176H/S196D and -E176H/S196N Double Mutant Construction and Characterization

These experiments were carried out on 1/30/14, 2/4/14, and 2/13/14. The double mutant constructs were generated by using the QuikChange site-directed mutagenesis kit (Agilent) using HEPD-E176H as the template. Primers were as follows, with the mutated codon in bold and red:

HEPD-E176H/S196D forward:
ccgcctccgattcgcgtgactacaccgcagtcgaac

HEPD-E176H/S196D reverse complement:
gtgacgtcccgccgccgcgcgtgcgcgacgcgag

HEPD-E176H/S196N forward:
ccgcctccgattcgtcgaactacaccgcagtcgaac

HEPD-E176H/S196N reverse complement:
gttgacgtcccgccgccgctgcgcgcgcgcgagcgag

The double mutants were purified as previously described by immobilized metal affinity chromatography. The mutants (80 µM) were anaerobically reconstituted with one equivalent of Fe(II) in 50 mM HEPES pH 7.5 on ice for 10 min. The double mutants (10 µM final concentration) were reacted with 2-HEP (1 mM) in buffer (50 mM HEPES pH 7.5, 500 µL total reaction volume) for 4 h at room temperature, treated with Chelex,
and filtered with an Amicon centrifuge filter (30 kDa MWCO). D₂O was added (20% final v/v), and the reaction mixtures were analyzed by ³¹P NMR spectroscopy.

5.4.5: Attempting to Skew the HEPD-E176H Product Distribution by Addition of Exogenous Formate

This reaction was carried out on 1/21/14. HEPD-E176H (40 µM) was anaerobically reconstituted with Fe(II) (120 µM) in buffer (50 mM HEPES pH 7.5) on ice for 10 min. The protein (5 µM) was incubated with 2-HEP (1 mM) in the absence or presence of formate (1 mM) in buffer (50 mM HEPES pH 7.5, 500 µL total reaction volume) for 2 h at room temperature. The reaction mixture was treated with Chelex and filtered with an Amicon centrifuge filter (30 kDa MWCO). D₂O was added (20% final v/v) and the reaction mixtures were analyzed by ³¹P NMR spectroscopy.

5.4.6: Cloning and Characterization of HEPD/MPnS Homologs

These experiments were carried out on 7/13/13, 7/16/13, 7/18/13, 7/19/13, 7/23/13, 7/29/13, 8/5/13, 8/6/13, 12/13/13, and 1/3/14. Genomic DNA from S. monomycini, S. albus, S. griseus, and S. griseofuscus were obtained from Dr. Kou-san Ju. The S. monomycini genome was not closed in the N-terminal region of the homolog, and therefore this gap was first amplified and sequenced, revealing that the missing coding sequence was: ATGTCCGTACCGGACCCTGCCTTCGACCGGCGG. With help from a post-doctoral scholar in the van der Donk group, Dr. Despina Bougioukou, the homolog from S. monomycini was amplified in Failsafe Buffer I (Epicentre) using the instructions with the kit with primers designed for ligation into pET15b using Gibson assembly; the
other homologs were similarly amplified except Failsafe Buffer G was used (all primers are listed below). The amplicon was purified by using a QIAQuick PCR Purification kit (Qiagen) or Promega Wizard SVI Gel and PCR Clean-Up kit (Promega) per the instruction manual. Amplification was checked with a 1% agarose gel made and run in 0.5X TAE buffer (20 mM tris(hydroxymethyl)aminomethane, 10 mM acetic acid, 0.5 mM EDTA). After initial amplification, the amplicon was ligated into a pET15b vector using a Gibson Assembly kit (New England Biolabs) and E. coli electrocompetent cells were transformed with the plasmid (either homemade DH5α or NEB 5α competent from New England Biolabs). Following recovery, selection was achieved by plating on a 1.5% agar LB plate supplemented with 100 µg/mL of ampicillin (LB-Amp100) and growing at 37 °C overnight. Individual colonies were picked and grown in 5 mL of LB-Amp100, and then the plasmids were isolated using a QIAprep Spin Miniprep kit (Qiagen). Sequencing (ACGT, Inc) was used to confirm that the correct sequence had been amplified. Proteins were expressed by transforming the plasmid into Rosetta 2 and expressing and purifying as has been described elsewhere for HEPD and MPnS.7

Primers used for amplification are shown below. In each case, the underlined portion has homology to the pET15b vector that was used for expression, and the capitalized portion is for the homolog of interest. The forward primers were designed with an NdeI restriction site; the reverse primers contain an XhoI restriction site.

*S. monomycini* forward primer:

```
ccagctgtgtgcgcgggcgcgATGTCCGTACCGGACCCTGCCTTCGACCG
```

*S. monomycini* reverse complement primer:
In a typical reconstitution, each homolog (40 \( \mu \)M) was anaerobically reconstituted with one equivalent of Fe(II) in buffer (25 mM HEPES pH 7.5). The protein (2 \( \mu \)M) was incubated with 2-HEP (500 \( \mu \)M) in buffer (25 mM HEPES pH 7.5) while the \( O_2 \) concentration was monitored using a Clark-type \( O_2 \) electrode. The reaction mixture was supplemented with additional aliquots of protein to confirm that the observed \( O_2 \) consumption occurred in an enzyme-dependent fashion. For characterization of the
reaction product by NMR spectroscopy, a similar assay was carried out, except higher concentrations of protein (10 µM) and 2-HEP (1 mM) were used. Following incubation overnight at room temperature, the reactions were treated with Chelex and filtered through a centrifuge filter (30 kDa MWCO). D$_2$O (20% final v/v) was added, and the $^{31}$P NMR spectrum was recorded.

5.4.7: Syntheses of Cyclopropyl Substrate Analogs

These experiments were performed on 7/1/10, 7/4/10, 7/5/10, 7/6/10, 7/7/10, 5/23/13, 5/24/13, 5/26/13, 5/27/13, 6/4/13, 6/5/13, 6/12/13, 6/13/13, and 6/17/13. Compound 5.1 (bromomethylcyclopropane ketone) was synthesized following a known literature procedure.$^{29}$ The spectral data matched the reported literature values. Compounds 5.2, 5.3, and 5.4 were synthesized as described elsewhere in greater detail.$^{11}$ The spectral data for the isolated compounds matched the reported literature values.

Synthesis of Compound 5.5 (Dimethyl 1-cyclopropane-1-hydroxymethyl-phosphonate). To a scintillation vial on ice was added CH$_2$Cl$_2$ (1 mL), dimethyl phosphite (260 µL, 2.8 mmol) and cyclopropane carboxaldehyde (160 µL, 2.8 mmol) followed by KF/Al$_2$O$_3$ (1:1 wt/wt, 130 mg total). The white suspension was stirred and allowed to warm to room temperature. After 4 h, the slurry was filtered, and the cake was washed with CH$_2$Cl$_2$. The filtrates were concentrated under a stream of N$_2$. Compound 5.5 was purified by column chromatography on silica gel (gradient of 0% to 5% MeOH/CH$_2$Cl$_2$ v/v). The fractions containing the product were combined and concentrated under reduced pressure to afford a colorless oil. Yield: 228 mg, 44%. $^1$H NMR (500 MHz,
CDCl$_3$): $\delta$ 3.14 (dd, 1H, $J = 8.9, 6.3$ Hz), 1.07 (m, 1H), 0.50 (m, 2H), 0.33 (m, 2H) ppm.

$^1$H-coupled $^{13}$C NMR (126 MHz, CDCl$_3$): $\delta$ 71.6 (dd, $J = 164, 6.5$ Hz), 53.4 (m), 12.3 (d, $J = 6.7$ Hz), 3.6 (d, $J = 13$ Hz), 3.0 (s) ppm.

**Synthesis of compound 5.6 (1-cyclopropane-1-hydroxymethylphosphonate).** To a solution of starting material (228 mg, 1.26 mmol) dissolved in dry CH$_2$Cl$_2$ (3 mL) under a N$_2$ atmosphere was added trimethylbromosilane (TMSBr, 0.5 mL, 3 equivalents). The mixture was refluxed under N$_2$ for 3 h. The reaction was allowed to cool to room temperature, and the solvent was removed under reduced pressure. EtOH/H$_2$O (3 mL, 1:1 v/v) was added, and the mixture was stirred for 5 min. $^{31}$P NMR spectroscopy indicated that the reaction had not proceeded to completion, so the material was concentrated under reduced pressure and an additional 3 equivalents of TMSBr were added in dry CH$_2$Cl$_2$ (3 mL), and the mixture was refluxed for an additional 3 h. Solvent was removed, H$_2$O (3 mL) was added, and the cloudy suspension was stirred for 10 min. Solvent was removed on the high vacuum line to afford an orange oil. Yield: 192 mg, 92%. $^1$H NMR (500 MHz, D$_2$O): $\delta$ 2.47 (dd, 1H, $J = 9.5, 7.5$ Hz), 0.41 (br, 1H), -0.020 (m, 2H), -0.027 (m, 2H) ppm. $^{31}$P NMR (202 MHz, CDCl$_3$): $\delta$ 24.2 (s) ppm. HRMS (ESI)$^+$: calculated 153.0371, found 153.0321.

**5.4.8: Enzymatic Assays with the Cyclopropyl Substrate Analogs**

These assays were performed on 6/19/13, 6/20/13, 9/19/13, 9/24/13, 10/8/13, 10/9/13, and 7/26/14. WT HEPD and WT MPnS (700 µM of each) were anaerobically reconstituted with one equivalent of Fe(II) in 25 mM HEPES pH 7.5. Each protein (400
µM final concentration) was removed from the anaerobic chamber and incubated with potential substrate analog 5.4 (2-cyc-2-HEP, 800 µM) in buffer (25 mM HEPES pH 7.5) for 30 min at room temperature. The samples were treated with Chelex and filtered through a centrifuge filter (30 kDa MWCO) to remove the enzyme. D$_2$O (20% v/v) was added, and the $^{31}$P NMR spectrum was recorded. To assay activity with 5.4 using an O$_2$ electrode, WT HEPD and WT MPnS were reconstituted as before but to a final concentration of 200 µM. The enzyme (50 µM) was incubated with 5.4 (1 mM) in air-saturated buffer (25 mM HEPES pH 7.5) at 20 °C while O$_2$ consumption was monitored. After ~100 s of no O$_2$ being consumed, 2-HEP (250 µM) was added to the reaction mixture as a positive control to ensure that the proteins were still active.

HEPD-Y184A and HEPD-Y184L were constructed using the standard site-directed mutagenesis protocol for the QuikChange kit (Agilent) with pET15b-WT HEPD as a template. Primers are provided below, with the mutated codon in bold and red. Proteins were expressed and purified as previously described,$^7$$^8$ but HEPD-Y184A expressed poorly (~1 mg/L) and may have been poorly folded. Therefore, only HEPD-Y184L was further characterized. The mutant (100 µM final concentration) was anaerobically reconstituted with 1 equivalent of Fe(II) in buffer (25 mM HEPES pH 7.5). A positive control with HEPD-Y184L (2 µM) and 2-HEP (250 µM) in buffer (25 mM HEPES pH 7.5) indicated that the protein was active with the native substrate. HEPD-Y184L (40 µM) was incubated with compound 5.4 (1 mM) in buffer (25 mM HEPES pH 7.5) while the O$_2$ concentration was monitored, but no O$_2$ consumption was detected.

HEPD-Y184A forward:
Assays to evaluate the activity of WT HEPD and WT MPnS towards compound 5.6 (1-cyc-1-HEP) were conducted by anaerobically reconstituting the protein (100 µM) with one equivalent of Fe(II) in buffer (50 mM HEPES pH 7.5). The protein was then incubated with 5.6 (1 mM) while the O₂ consumption was monitored with an O₂ electrode. After approximately one minute, no O₂ consumption was observed, so 2-HEP (250 µM) was spiked into the reaction mixtures to confirm that the enzymes had not been inactivated.

5.4.9: Syntheses of Halogenated Substrate Analogs

These experiments were carried out on 12/12/11, 1/19/12, 1/25/12, 1/29/12, 7/12/12, 7/13/12, 7/18/12, 7/19/12, and 7/24/12.

Synthesis of compound 5.9 (Diethyl 1-F-2-HEP). The starting material 5.7 (118 mg, 0.49 mmol) was cooled on ice, and then a solution of 1.0 M BH₃-THF (1.5 mL, 3 equivalents) was added dropwise under N₂. The reaction was allowed to warm to room
temperature and then stirred for 20 h. The reaction was cooled to 0 °C and then EtOH (1 mL) was slowly added to quench the reaction. The reaction mixture was concentrated under reduced pressure and then purified by column chromatography on silica gel (gradient of 0% to 6% v/v MeOH in CH₂Cl₂) to afford a yellow oil as product. Yield: 88 mg, 44%. ¹H NMR (500 MHz, CDCl₃): δ 4.88 (m, 1H, PCHF), 4.24 (m, 4H, POCH₂), 4.05 (m, 2H, CH₂OH), 2.77 (br, 1H, CH₂OH), 1.38 (dt, Jₜ-H = 7.2 Hz, Jₚ-H = 1.5 Hz, 6H, CH₂CH₃)¹³C NMR (126 MHz, CDCl₃): δ 89.2 (dd, PCF, J = 184, 166 Hz) 63.65 (d, POCH₂, J = 6.7 Hz), 63.11 (d, POCH₂, J = 6.7 Hz), 61.42 (dd, CH₂OH, J = 21, 4.0 Hz), 16.4 (d, CH₂CH₃, J = 5.7 Hz). ¹⁹F NMR (470 MHZ, CDCl₃): δ -2.35 (m). ³¹P NMR (202 MHz, CDCl₃): δ 16.60 (d, J = 74.5 Hz, P-CF) ppm. HRMS (ESI⁺) calculated (C₆H₁₄O₄FNaP)⁺ 223.0511, found 223.0511.

**Synthesis of compound 5.10 (Diethyl 1-Cl-2-HEP).** The starting material (508 mg, 2.0 mmol) was cooled on ice under N₂, and then a solution of 1.0 M BH₃-THF was added dropwise. The reaction was allowed to come to room temperature and stirred for 24 h. After concentration under reduced pressure, column chromatography on silica gel (gradient of 0% to 4% v/v MeOH in CH₂Cl₂) afforded a yellow oil. Yield: 250 mg, 59%. ¹H NMR (400 MHz, CDCl₃): δ 4.25 (m, 4H, OCH₂CH₃), 4.01 (m, 3H, PCH₂CH₂OH), 3.18 (t, 1H, CH₂OH, J = 6.3 Hz), 1.38 (t, 6H, OCH₂CH₃, J = 7.1 Hz) ppm. ¹³C NMR (126 MHz, CDCl₃): δ 64.21, (d, POCH₂, J = 6.9 Hz), 63.66 (d, CH₂OH, J = 6.8 Hz) 63.01 (s, POCH₂), 52.8 (d, PCH₂, J = 154 Hz), 16.41 (d, CH₂CH₃, J = 5.6 Hz), 16.39 (d, CH₂CH₃, J = 5.6 Hz) ppm. ³¹P NMR (202 MHz, CDCl₃): δ 19.2 ppm. HRMS (ESI⁺) calculated (C₆H₁₅O₄ClP): 217.0397, found 217.0392.
Synthesis of compound 5.11 (Diethyl 1-F-2-[2-^2H_2]-HEP).

Starting material 5.7 (292 mg, 1.2 mmol) was added and then the atmosphere was purged with N\textsubscript{2}. The flask was cooled to 0 °C, and then a 1.0 M BD\textsubscript{3}-THF solution (2.0 mL, 1.6 equivalents) was added under N\textsubscript{2}. The mixture was stirred at 0 °C for 1 h and then at room temperature until it was quenched on ice after 5 days by slow addition of 1 mL EtOH and then 1 mL H\textsubscript{2}O. The solution was then dried under reduced pressure. The crude residue was purified by column chromatography on silica gel (loaded in CH\textsubscript{2}Cl\textsubscript{2} and eluted with a very gradual gradient of 0-5\% MeOH in CH\textsubscript{2}Cl\textsubscript{2} (v/v)) to afford . \Delta R\text{f} of the starting material and product was very low in the elution solvent (~0.03). Yield: 59 mg, 24\%. \textsuperscript{1}H NMR (500 MHz, CDCl\textsubscript{3}): δ 4.80 (dd, 1H, PCF\textsubscript{H}, J = 48, 5.2 Hz), 4.19 (m, 4H, PO\textsubscript{C}H\textsubscript{2}), 3.52 (br, 1H, -OH), 1.34 (td, 6H, CH\textsubscript{2}CH\textsubscript{3}, J = 7.1, 2.1 Hz) ppm. \textsuperscript{13}C NMR (126 MHz, CDCl\textsubscript{3}): δ 89.4 (dd, PCF, J = 183, 165 Hz), 63.6 (d, POCH\textsubscript{2}, J = 6.7 Hz), 63.1 (d, POCH\textsubscript{2}, J = 6.6 Hz), 60.7 (m, CD\textsubscript{2}OH), 16.4 (d, CH\textsubscript{2}CH\textsubscript{3}, J = 5.7 Hz) ppm. \textsuperscript{19}F NMR (470 MHz, CDCl\textsubscript{3}): δ -2.6 (dd, J = 75, 45 Hz) ppm. \textsuperscript{31}P NMR (242 MHz, CDCl\textsubscript{3}): δ 16.7 (d, J = 74 Hz) ppm. HRMS (ESI\textsuperscript{+}) calculated (C\textsubscript{6}H\textsubscript{13}\textsuperscript{2}H\textsubscript{2}O\textsubscript{4}FP)\textsuperscript{+} 203.0818, found 203.0820.

Synthesis of compound 5.12 (1-F-2-HEP). Diethyl 1-F-2-HEP (43 mg, 0.2 mmol) was dissolved in 6 M HCl (1 mL) and then refluxed for 16 h with stirring. The reaction was cooled and then concentrated under reduced pressure. Deionized water (5 mL) was added and the solution was again concentrated under reduced pressure to afford a yellow oil. Yield: 28 mg, 90\%. \textsuperscript{1}H NMR (500 MHz, D\textsubscript{2}O): 4.50 (m), 3.73 (m) ppm. \textsuperscript{13}C NMR (126
MHz, D<sub>2</sub>O): 91.7 (dd, J = 175, 156 Hz), 61.1 (dd, J = 19, 8.2 Hz). <sup>19</sup>F NMR (470 MHz, DMSO-D<sub>6</sub>): δ 0.17 (m) ppm. <sup>31</sup>P NMR (202 MHz, DMSO-D<sub>6</sub>): 17.4 (d, J = 73 Hz) ppm. HRMS (ESI<sup>+</sup>): calculated (C<sub>2</sub>H<sub>7</sub>FO<sub>4</sub>P)<sup>+</sup> 145.0066, found 145.0064.

**Synthesis of 5.13 (1-Cl-2-HEP).** Diethyl 1-Cl-2-HEP (108 mg, 0.50 mmol) was dissolved in 6 M HCl (3 mL) and refluxed for 24 h with stirring. The solution was allowed to cool and then concentrated under reduced pressure. Yield: 71 mg, 88%. <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): 3.75 (m, 2H, C<sub>2</sub>H<sub>2</sub>OH), 3.47 (m, 1H, PCCl<sub>2</sub>OH). <sup>13</sup>C NMR (126 MHz, DMSO-D<sub>6</sub>): δ 62.9 (s, CH<sub>2</sub>OH), 57.4 (d, J = 145 Hz, PCHCl). <sup>31</sup>P NMR (162 MHz, D<sub>2</sub>O): δ 15.3. HRMS (ESI<sup>+</sup>) calculated (C<sub>2</sub>H<sub>7</sub>O<sub>4</sub>ClP)<sup>+</sup> 160.9770, found 160.9771.

**Synthesis of compound 5.14 (1-F-2-[2-<sup>2</sup>H<sub>2</sub>]-HEP).** Compound 5.11 (59 mg, 0.29 mmol) was added to 6 M HCl (2 mL), and the mixture was refluxed for 16 h. The mixture was cooled and then concentrated under reduced pressure. Deionized water (5 mL) was added and the solution was again concentrated under reduced pressure to afford a yellow oil. Yield: 39 mg, 92%. <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): 4.60 (m). <sup>13</sup>C NMR (152 MHz, D<sub>2</sub>O): 91.0 (m), 60.2 (m). <sup>19</sup>F NMR (470 MHz, D<sub>2</sub>O): δ 0.4 (m) ppm. <sup>31</sup>P NMR (202 MHz, D<sub>2</sub>O): 12.6 (d, J = 173 Hz) ppm. ppm HRMS (ESI<sup>+</sup>): calculated (C<sub>2</sub>H<sub>5</sub>2HFO<sub>4</sub>P)<sup>+</sup>: 147.0192, found 147.0198.

**Synthesis of 5.15 (Chloromethylphosphonate) (5/25/12, 6/5/12, 6/8/12).** Compound 5.15 was synthesized by following a previously published literature procedure as described in greater detail elsewhere.24,25
5.4.10: Assays of HEPD and MPnS with Halogenated Substrate Analogs

These assays were performed on 1/20/12, 1/25/12, 2/1/12, 2/10/12, 2/16/12, 2/18/12, 2/20/12, 2/22/12, 3/4/12, 3/19/12, 8/12/12, and 9/11/12. In a typical experiment, WT HEPD and WT MPnS (500 µM) were anaerobically reconstituted with Fe(II) (1 equivalent) in 25 mM HEPES pH 7.5. The protein (10 µM) was then incubated with 1-F-2-HEP or 1-Cl-2-HEP (1 mM) in 25 mM HEPES pH 7.5, and the O$_2$ concentration was monitored over time. Once O$_2$ consumption ceased, 2-HEP (500 µM) was added to confirm that the majority of the protein had been inactivated. A fresh aliquot of Fe(II) (100 µM, previously dissolved in anaerobic H$_2$O in a glovebag) or ascorbic acid (1 mM) was added and confirmed that partial restoration of activity could occur. For single turnover experiments, the protein (200 µM) was incubated with 1-F-2-HEP or 1-Cl-2-HEP (200 µM) in 25 mM HEPES pH 7.5 and the O$_2$ concentration was monitored as a function of time. 2-HEP (500 µM) was added after apparent O$_2$ consumption had ceased to check for residual protein activity. Consumption of O$_2$ was fit to the standard equation governing biphasic kinetics i.e. $C(t) = C_0 + A_1 e^{-k_1 t} + A_2 e^{-k_2 t}$, where $C$ is the concentration of O$_2$ as a function of time (t), $C_0$ is the concentration of O$_2$ after a very long time, $A_1$ and $A_2$ are the respective amplitudes of the two phases, and $k_1$ and $k_2$ are the respective rates of the two phases. By visual inspection, it was apparent that the consumption of O$_2$ was not well fit to a single exponential curve. To establish the Michaelis-Menten parameters of WT MPnS oxidation of 1-F-2-HEP, WT MPnS (40 µM) was anaerobically reconstituted with 1 equivalent of Fe(II) in buffer (25 mM HEPES pH
7.5). The protein (2 µM final concentration) was reacted with varying concentrations (10-300 µM) of compounds 5.12 and 5.14 in air-saturated buffer (25 mM HEPES pH 7.5).

Fluoride electrode assays were run in a 15 mL plastic conical tube with the electrode inserted in the tube and a small stirbar at the bottom. Calibration with standards of known concentrations of fluoride in 25 mM HEPES pH 7.5 allowed the voltage reading to be converted to fluoride concentrations. Reactions were run on a 1.5-1.7 mL scale, which was sufficient to cover the bottom of the electrode. After reconstituting protein as before, Fe(II), WT HEPD, or WT MPnS was removed from the glovebag and diluted with oxygenated buffer (25 mM HEPES pH 7.5) to a final concentration of 100 µM, and the electrode was allowed to equilibrate. After no more than 30 s (prevent loss of dissolved O₃), 1-F-2-HEP was added (1 mM final concentration) and the voltage was manually recorded every 5 seconds. The reactions were then allowed to proceed unmonitored for 24 h at room temperature, and the final voltage reading was recorded once more.

WT HEPD (1.5 mM) was reconstituted with 1 equivalent of Fe(II) in anaerobic buffer (25 mM HEPES pH 7.5), and then the protein (750 µM) was incubated with 1-F-2-HEP (1 mM) in O₂-saturated buffer (25 mM HEPES pH 7.5) at room temperature for 20 min. Dithionite (several grains), EDTA (25 mM) and D₂O (20% v/v) were added, and the ⁳¹P NMR spectrum was recorded. The sample was spiked with residual starting material and Pᵢ. To analyze the reaction of WT HEPD with 1-Cl-2-HEP, WT HEPD (500 µM) was anaerobically reconstituted with 1 equivalent of Fe(II) (500 µM) in 25 mM HEPES pH 7.5. The protein (100 µM final concentration) was then incubated with 1-Cl-2-HEP (2 mM) and ascorbic acid (2 mM) in O₂-saturated buffer (25 mM HEPES pH 7.5) at room
temperature for 2 h. The solution was filtered by centrifugation (30 kDa MWCO), snap frozen, and lyophilized. The lyophilized powder was taken up in D$_2$O and the $^{31}$P NMR spectrum was recorded.

WT MPnS (500 µM final concentration) was exchanged into a volatile buffer (100 mM NH$_4$OAc pH 7) by using a centrifuge filter, and anaerobically reconstituted with 5 equivalents of Fe(II). 1-F-2-HEP and 1-Cl-2-HEP (1 mM) were added to the reconstituted WT MPnS (30 µM for the reaction with 1-F-2-HEP and 250 µM for the reaction with 1-Cl-2-HEP) in O$_2$-saturated 100 mM NH$_4$OAc, and then the reactions were allowed to proceed at room temperature for 12 h. Chelex was added to the reactions to remove the free Fe, and the protein was removed by centrifuging through filters (30 kDa MWCO) that had been washed repeatedly (5 x 500 µL ddH$_2$O). The filtrates were combined, snap frozen, and lyophilized to dryness. The residue was redissolved in ddH$_2$O (2 mL), snap frozen, and lyophilized. The resulting residue was dissolved in H$_2$SO$_4$ (100 mM, 1 mL). An aliquot (100 µL) was transferred to a separate tube, and the solutions were snap frozen and lyophilized. The smaller aliquot was dissolved in 500 µL D$_2$O, analyzed by $^{31}$P NMR spectroscopy, and spiked with residual starting material and P$_i$ to confirm the identities of the peaks. The reaction of WT MPnS with 1-Cl-2-HEP was also spiked with chloromethylphosphonate. The larger aliquots were analyzed by $^1$H, $^1$H-coupled $^{31}$P, and [$^1$H-$^{31}$P] gHMBC NMR spectroscopies.

### 5.4.11: $^{18}$O KIE Studies

These assays were performed on 3/11/14. WT HEPD and WT MPnS were prepared and shipped to the Klinman lab at U.C. Berkeley. 2-HEP and 2-[2-$^2$H$_2$]-HEP
were likewise prepared and shipped to the Klinman lab. Dr. Florence Bonnot and, later, Dr. Hui Zhu, two post-doctoral researchers in the Klinman lab, performed all the $^{18}$O KIE studies.

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Chapter 6: Enzymatic Transformations Acting on Phosphonoacetaldehyde

6.1: Introduction

6.1.1: Background on PhnY

Herein, detailed biochemical and crystallographic characterization of the phosphonoacetaldehyde (PnAA) dehydrogenase enzyme, PhnY, from the soil-dwelling bacterium *Sinorhizobium meliloti* 1021 are presented. As outlined in Chapter 1, this enzyme plays a critical role in enabling certain bacteria to grow on the product of PnAA oxidation, phosphonoacetate (PnA), via phosphonoacetate hydrolase (Figure 1.3). PhnY is the only enzyme known to oxidize to PnAA to PnA, as the enzyme hypothetically responsible for this transformation in the fosfazinomycin biosynthetic pathway has not yet been identified. To better understand the mechanism of PhnY, its structure was solved by X-ray crystallography both in the apo state as well as in the presence of various phosphorus containing molecules including the native substrate and product. Characterization of the site-directed mutants described in this chapter that were generated on the basis of the crystal structure demonstrated the importance of the active site residues in vitro. Finally, PhnY was assayed for activity with a pair of substrate analogs, 3-oxopropylphosphonate (3-OPP) and glyceraldehyde-3-phosphate (G3P). Its activity towards these substrate analogs leads to the conclusion that PhnY does not discriminate between the phosphonate and phosphate moiety, but rather that the physical dimensions of the active site modulates substrate preference.

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6.1.2: Overall Structure of PhnY

The structure of PhnY was determined in the apo state to 2.1 Å resolution by Dr. Vinayak Agarwal (Nair group, University of Illinois Urbana-Champaign). The crystallographic asymmetric unit consists of eight protein chains comprising four sets of biologically relevant homodimers (Figure 6.1A). The arrangement of protein chains as sets of homodimers is consistent with the behavior of PhnY as a dimer in solution, as judged by size exclusion chromatography performed by Dr. Agarwal. In the structure of the unliganded PhnY, electron density corresponding to a phosphate ion (presumably from the crystallization medium) was observed at the interface of the cofactor binding and catalytic domains. The phosphate oxygen atoms were coordinated by hydrogen bonds with the side chains of His159, Arg290, Thr292, and Arg447 (Figure 6.1B). The Arg108 side chain, which is in the vicinity of the phosphate-binding site, showed alternative conformations throughout the different molecules in the asymmetric unit. In one conformation, Arg108 pointed towards the phosphate binding-pocket with the guanidino group within interaction distance of the phosphate. In the alternate conformation, this residue pointed away from the active site and was stabilized by interactions with the side chain of Asp111. This basic, phosphate-binding pocket is strictly conserved among ALDHs that catalyze oxidation of phosphorylated substrates, but is missing in ALDHs that function on non-phosphorylated substrates (Figure 6.2).
Figure 6.1: Three-dimensional crystal structure of apo PhnY. (A) Structure of the PhnY homodimer showing the relative positions of the three domains with one monomer colored grey, and the second monomer colored with the cofactor binding and catalytic domains in blue, and the oligomerization domain in pink. Cofactor NAD$^+$ is shown in ball-and-stick representation. (B) Stereo view showing the active site features of PhnY in complex with inorganic phosphate. Superimposed is a difference Fourier electron density map (contoured at 2.7σ over background in blue, and 6.0σ in red) calculated with coefficients $|F_{\text{obs}}| - |F_{\text{calc}}|$ and phases from the final refined model with the coordinates of the inorganic phosphate deleted prior to one round of refinement. Figure made by Dr. Agarwal and reproduced with permission from ref 3.
Figure 6.2: Primary sequence alignment, constructed by Dr. Agarwal, between characterized ALDHs. Sm_PhnY: S. meliloti 1021 PhnY, Rn_PhnY: R. nubinhibens PhnY, Sm_GAPDH: S. mutans GAPDH, Ec_SsALDH: E. coli succinic semialdehyde dehydrogenase, Pa_BetALDH: P. aeruginosa betaine ALDH. The absolutely conserved catalytic nucleophilic cysteine, catalytic base glutamate and the oxyanion stabilizing asparagine residue are highlighted in pink and marked by (^) under the sequences. The phosphate/phosphonate binding pocket forming arginine and tyrosine/histidine residues are highlighted in blue and boxed. These residues are not conserved in Ec_SsALDH and Pa_BetALDH. All residues of interest are highlighted by (*) above the sequences.
6.1.3: Cocrystal Structure with the Physiological Substrate PnAA

The 2.1 Å resolution cocrystal structure of PhnY in complex with PnAA was generated and solved by Dr. Agarwal, and showed unambiguous density for the ligand in the active site of all eight copies in the crystallographic asymmetric unit (Figure 6.3A). PnAA was bound at the interface of the cofactor-binding and catalytic domains. The side chain thiol of Cys291 underwent a 120° rotation relative to the apo structure, which positioned the Cys291 side chain suitably for interactions with the substrate. The Cys291-Sγ atom in this conformation was 3.0 Å and 3.3 Å away from the backbone amide nitrogen atoms of Cys291 and Thr292, respectively. Similar interactions have been implicated in modulating the nucleophilicity of the Cys side chain for nucleophilic attack in related members of the ALDH superfamily.⁴

Strong and continuous electron density was observed between the Cys291-Sγ and the PnAA-Cβ atom (at a distance of 1.8 Å) indicative of a covalent bond between these two atoms. Unbiased difference Fourier electron density maps were indicative of tetrahedral geometry at the PnAA-Cβ atom that was covalently bound to the Cys291 sulfur atom (Figure 6.3A). This tetrahedral geometry is presumably generated by nucleophilic attack of the Cys291-Sγ thiolate at the Re face of planar PnAA to generate a tetrahedral thiohemiacetal intermediate with an (R) stereochemical configuration. Consequently, the phosphonate in the PhnY-PnAA binary complex was modeled as a thiohemiacetal instead of an aldehyde, the first such instance of the observation of the thiohemiacetal intermediate in a member of the ALDH superfamily of enzymes.
Figure 6.3: PhnY active site in complex with substrate PnAA and cofactor NAD$^+$. All atoms are shown in ball-and-stick representation with PhnY side chain carbon atoms colored yellow, and PnAA and NAD$^+$ carbon atoms colored green. (A) Stereo view showing the active site features of PhnY in the binary complex with the substrate PnAA. Superimposed is a difference Fourier electron density map (contoured at 2.5σ). Stabilizing interactions of PnAA with PhnY are shown as black dashes. (B) Stereo view of the PhnY active site in the ternary complex with substrate PnAA and cofactor NAD$^+$. Superimposed is the difference electron density map contoured at 2.5σ. Note that the nicotinamide and ribose rings for the cofactor have not been modeled. Figure made by Dr. Agarwal and reproduced with permission from ref 3.

The oxygen atom of the thiohemiacetal intermediate was positioned 3.1 Å away from the amide side chain of Asn158, and 3.2 Å away from the main chain amide nitrogen atom of Cys291 (Figure 6.3A). By analogy to the serine/cysteine protease reaction mechanism, these two interactions constitute the ‘oxyanion hole’ that stabilizes the tetrahedral intermediate. Binding of PnAA ordered the side chain of Arg108 in a solitary conformation pointing towards the active site, such that the guanidine moiety also interacted with the phosphonate oxygen atoms.
6.1.4: Cocrystal Structure of the Ternary Complex with PnAA and NAD$^+$

In the 2.0 Å resolution PhnY-NAD$^+$-PnAA ternary complex (Figure 6.3A), solved by Dr. Agarwal, unambiguous electron density could be observed for all atoms of PnAA. However, electron density was absent for the nicotinamide ring and the adjoining ribose of the cofactor. Only the adenine diphosphate (ADP) moiety of the cofactor was modeled in the final structure (Figure 6.3B). The PnAA substrate in the PhnY-NAD$^+$-PnAA ternary complex was positioned analogously to the thiohemiacetal intermediate described previously, with all the interactions with the side chains of Asn158, His159, Arg108, Arg290, Thr292 and Arg447 conserved. However, the $F_o - F_c$ difference electron density map was indicative of planar geometry around the PnAA-$C\beta$ atom, which presumably arose due to the transfer of the hydride from the PnAA-$C\beta$ to the cofactor, and the concomitant conversion of the tetrahedral thiohemiacetal intermediate to an $sp^2$ hybridized planar thioester. The bond angles around the PnAA-$C\beta$ were thus refined to a planar geometry. Variability was observed in the positioning of the Glu254 side chain in the vicinity of the thioester intermediate. Based on sequence homology (Figure 6.2), this residue is the catalytic base that primes a water molecule for nucleophilic attack on the thioester intermediate generated in the catalytic cycle for PhnY \textit{(vide infra)}. Overall, I hoped to confirm the importance of the residues that were observed in the various crystal structures by using an in vitro steady-state kinetics assay with site-directed mutants as well as assess the scope of substrate tolerance for PhnY.
6.1.5: Reduction of PnAA in Phosphonate Biosynthetic Pathways

As highlighted in the previous sections, PnAA is a biosynthetic intermediate common to catabolic pathways and biosynthetic routes such as the pathways leading to fosfomycin, dehydrophos, and phosphinothricin (Chapter 1). The biosynthetic gene clusters of all three compounds share a gene encoding a group III metal dependent alcohol dehydrogenase (AD). FomC (fosfomycin), DhpG (dehydrophos), and PhpC (phosphinothricin) use NAD(P)H to catalyze the reduction of PnAA to 2-hydroxyethylphosphonate (2-HEP). However, the exact stereochemistry of the 2-HEP product had never been determined. Thus, it was postulated that by employing a deuterium-labeled cofactor, the face selectivity of substrate reduction of a group III metal-dependent AD could be determined for the first time. It was hoped that this result would also provide additional insights into the fosfomycin biosynthetic pathway in *Streptomyces fradiae* (Figure 6.4). The overall pathway in this organism is still incomplete as a consequence of the inability to definitively reconstitute the activity of a presumed methyltransferase, Fom3, as well as uncertainty over where in the biosynthetic pathway to place the reductase FomC (Figure 6.4). At the time of the work presented in this chapter, Fom3 had only been hypothesized to catalyze formation of 2-hydroxypropylphosphonate (HPP) from 2-HEP in vitro.

Previous feeding studies have shown that 2-HEP is converted to fosfomycin by *S. fradiae*. Although the mechanism for this conversion is currently unknown, two explanations have been offered. In the first hypothesis, FomC acts on 2-HEP to generate phosphonoacetaldehyde (PnAA), which is subsequently methylated by Fom3 (Figure 6.4, path A). Alternatively, genetic studies combined with feeding studies suggested FomC
reduces PnAA to 2-HEP, which is then methylated by Fom3 (Figure 6.4, path B). Studies by Hammerschmidt and coworkers demonstrated that feeding (R)- or (S)-2-[2-2H]2-HEP to S. fradiae resulted in 32% incorporation of the deuterium label in fosfomycin from the S isomer and no deuterium incorporation from the R isomer. This observation places a restriction on the stereochemistry of hydride transfer from 2-HEP by FomC for path A. Only if FomC were to remove the pro-R hydrogen from C2 of 2-HEP could pathway A explain the experimental results. Thus, experimental determination of the stereochemistry of hydride transfer could support or refute pathway A. Though PhnY and the group III ADs operate on an identical substrate, the former oxidizes it for the catabolic reasons while the latter proteins reduce it for secondary metabolism. The results in this chapter will therefore be discussed separately, and the investigation into the group III ADs will be considered first.

**Figure 6.4:** Proposed biosynthetic routes of fosfomycin in Streptomyces species.

### 6.2: Results and Discussion for the Investigation of ADs that Act on PnAA

#### 6.2.1: Homology of ADs Involved in Phosphonate Biosynthesis

Of the three homologous enzymes discussed in section 6.1.5, DhpG was used in this study as it proved more stable under the conditions used and expressed better in *Escherichia coli* compared to FomC and PhpC. DhpG has 33% sequence identity with
FomC with conservation of the predicted active site residues identified through a multiple sequence alignment with other group III alcohol dehydrogenases (Figure 6.5).\textsuperscript{7,13} The cofactor binding motif (Figure 6.5, orange box) and metal binding residues (Figure 6.5, blue boxes) are conserved in the three ADs involved in phosphonate biosynthesis. The residues responsible for binding of the aldehyde/alcohol substrate are not conserved in this class of proteins, but they were identified for the three proteins of interest via homology models constructed based on the structures of 1,3-propanediol dehydrogenase from \textit{Thermotoga maritima} (PDB code 1O2D) and lactaldehyde reductase from \textit{E. coli} (PDB code 2BL4).\textsuperscript{7} Two residues that are conserved only in the 2-HEP dehydrogenases, a Ser and a Tyr residue, boxed in red in Figure 6.5, engage in hydrogen bonding to the phosphonate moiety in the homology model.\textsuperscript{7} The conservation of these residues across FomC, DhpG, and PhpC along with the overall sequence similarity indicates that hydride transfer from NAD(P)H to the aldehyde very likely occurs with the same facial selectivity for each protein.
Figure 6.5: Sequence alignment of select group III alcohol dehydrogenases. The boxed residues are responsible for NAD(P)H binding (orange), metal cofactor binding (blue), or, in the case of FomC, DhpG, and PhpC, phosphonate binding (red), and were identified through homology modeling.\textsuperscript{7}

6.2.2: An Enzymatic Cascade to Analyze the Stereochemistry of Reduction

In vitro assays with DhpG did not produce sufficient quantities of product to determine the absolute configuration of 2-[2-\textsuperscript{2}H\textsubscript{1}]-HEP formed upon reduction of PnAA with NADP\textsuperscript{2}H by NMR spectroscopy.\textsuperscript{14} Instead, I took advantage of the known stereochemistry of oxidation of 2-HEP to HMP and formate by 2-HEPD (Figure 6.6).

Previous studies have shown that oxidation of (R)-2-[2-\textsuperscript{2}H\textsubscript{1}]-HEP with HEPD produced deuterium labeled formate, whereas oxidation of the (S) enantiomer resulted in unlabeled formate.\textsuperscript{14} To eliminate the possibility of spurious formate interfering with analysis of the enzymatically produced formate,\textsuperscript{15} a \textsuperscript{13}C label was introduced at the C2 position of PnAA.
such that either $^{13}$C,$^1$H- or $^{13}$C,$^2$H-formate would be generated, both of which can be readily distinguished from spurious formate by mass spectrometry. [2-$^{13}$C$_1$]-PnAA was prepared enzymatically from commercial [2-$^{13}$C$_1$]-phosphoenolpyruvate (PEP), using a recombinant N-terminally His$_6$-tagged PEP mutase (RhiH) and a C-terminally His$_6$-tagged PnPy decarboxylase (ppd) from Bacteroides fragilis via a modification to a previously reported procedure. Conversion of [2-$^{13}$C$_1$]-PEP of [2-$^{13}$C$_1$]-PnAA was confirmed by $^{31}$P NMR spectroscopy (Figure 6.7A).

**Figure 6.6:** Outline of the strategy used to determine the stereochemistry of the DhpG-catalyzed reduction of PnAA.
Figure 6.7: The progress of the enzymatic cascade outlined in Figure 6.6 was followed by $^{31}$P NMR spectroscopy to confirm the generation of (A) $[2-{ }^{13} \text{C}_1]$-PnAA (11 ppm) by the successive action of RhiH and PnPy decarboxylase, (B) $2-[2-{ }^{13} \text{C}_1, 2-{ }^2\text{H}_1]-\text{HEP}$ (21 ppm) via the reduction of $[2-{ }^{13} \text{C}_1]$-PnAA by DhpG in the presence of (4R)-NADP$^2$H, and (C) HMP (18 ppm) by the action of HEPD. $^{31}$P NMR chemical shifts are very sensitive to small changes in pH, and therefore the identity of 2-HEP and HMP was confirmed by spiking with an authentic synthetic standard. The additional resonance (10 ppm) in panels (B) and (C) is residual $[2-{ }^{13} \text{C}_1]$-PnAA. The additional resonance at ~19 ppm in panel (A) is an unknown byproduct of PnAA generation.

Given that FucO, another group III metal-dependent AD, has been reported to transfer the pro-$R$ hydride from C4 of the nicotinamide group of NADH to its substrate,$^{17}$ (4R)-NADP$^2$H was prepared by incubating deuterated phosphite with an engineered phosphite dehydrogenase (PtDH) that can use either NAD$^+$ or NADP$^+$ as substrate (Figure 6.6).$^{18,19}$ After (4R)-NADP$^2$H was generated, phosphite dehydrogenase was removed, and the (4R)-NADP$^2$H was incubated with DhpG and excess $[2-{ }^{13} \text{C}_1]$-PnAA. Once all (4R)-NADP$^2$H was consumed as determined by UV-Vis spectroscopy, DhpG
was removed with a centrifuge filter, and the successful formation of 2-HEP was verified by \(^{31}\)P NMR spectroscopy (Figure 6.7B). Next, purified HEPD\(^{20}\) was incubated with 2-[\(2^{-2}\)H\(_1\)]-HEP to convert the substrate into formate and HMP. Total conversion of 2-HEP into HMP was confirmed by \(^{31}\)P NMR spectroscopy (Figure 6.7C). To determine the absolute stereochemistry of the reduction of PnAA, the formate produced in the previous step was activated with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and reacted with 2-nitrophenylhydrazine to form the corresponding hydrazide, thus converting the formate into a product large enough to be analyzed by LC-MS.\(^{14}\) As shown in Figure 6.8, the vast majority of the formate product contained both the \(^{13}\)C and \(^{2}\)H labels, indicating that reduction catalyzed by DhpG of PnAA with (4\(R\))-NADP\(^2\)H results in (\(R\))-2-[\(2^{-2}\)H\(_1\)]-HEP. This finding establishes that DhpG transfers the pro-\(R\) hydride of the reduced nicotinamide to its substrate, supporting the recent finding that the group III AD FucO transfers the pro-\(R\) hydride to its substrate.\(^{17}\) Simultaneously, this result establishes that reduction by this family of group III ADs takes place on the \(Re\) face of phosphonoacetaldehyde, and therefore that the enzymes remove the pro-\(R\) hydrogen during oxidation of 2-HEP. The experimental determination that DhpG, and by extension FomC, removes the pro-\(R\) hydrogen from 2-HEP combined with the demonstration by Hammerschmidt and coworkers that feeding of (\(R\))-2-[\(2^{-2}\)H\(_1\)]-HEP results in production of unlabeled fosfomycin in \(S. fradiae\) does not rule out pathway A (Figure 6.4) of fosfomycin biosynthesis. Ultimately, definitive demonstration of the activity of the methyltransferase, Fom3, was required to determine which pathway is operative during fosfomycin biosynthesis, and after completion of this work, the in vitro activity of Fom3 was reported, demonstrating that pathway B of Figure 6.4 is correct.\(^{21}\)
Figure 6.8: Extracted ion chromatograms of formyl 2-nitrophenylhydrazide. The major product is the 2-nitrophenylhydrazide of $^{[13}\text{C},^{2}\text{H}]$-formate (182 $m/z$, blue), whereas derivatized spurious formate (180 $m/z$, red) and formate containing either $^{13}\text{C}$ or $^{2}\text{H}$ (181 $m/z$, black) are minor constituents.

6.3: Results and Discussion for the Investigation of PhnY

6.3.1: Characterization of WT and Site-Directed Mutants of PhnY

Using the PhnY crystal structures complexed to various phosphonates as guides (Sections 6.1.1-6.1.4), site-specific PhnY mutants were generated. Kinetic characterization of the wild-type and variant enzymes were carried out as described previously.\(^1\) For the wild type enzyme, the kinetic parameters determined in this study, $k_{\text{cat}} = 2.2 \pm 0.1 \text{ s}^{-1}$ and $K_M = 3.2 \pm 0.7 \mu\text{M}$ for PnAA, were in good agreement with the previously published values (Table 6.1, Figure 6.9).\(^1\) By contrast, no activity was observed when Cys291 or Glu254 was replaced by Ala, confirming the vital roles of these residues in catalysis. The Glu254Ala mutant likely catalyzed a single oxidation of PnAA to PnA with concomitant reduction of NAD$^+$ to NADH followed by very slow hydrolysis of the covalent thioester adduct; the first turnover of this conversion could
likely be observed using stopped-flow UV-Visible spectroscopy, but this technique was not employed. Instead, the PhnY Glu254Ala construct was incubated with NAD$^+$ and PnAA and the contents of the reaction were analyzed by electrospray mass spectrometry (Figure 6.10). The suppressed deacylation activity enabled the trapping of a PhnY adduct. Though the resolution of the mass spectrum did not allow distinction between the thiohemiacetal and the thioester, the higher chemical stability of the latter suggested that the trapped intermediate corresponded to the thioester.

<table>
<thead>
<tr>
<th>PhnY constructs with PnAA</th>
<th>$K_{M, PnA}$ (µM)</th>
<th>$K_{M, NAD^+}$ (µM)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>3.2 ± 0.7</td>
<td>58 ± 9</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>Arg108Ala</td>
<td>9.7 ± 0.6</td>
<td>18 ± 2</td>
<td>0.051 ± 0.001</td>
</tr>
<tr>
<td>Asn158Ala</td>
<td>29 ± 9</td>
<td>40 ± 6</td>
<td>0.010 ± 0.001</td>
</tr>
<tr>
<td>Glu254Ala</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Arg290Ala</td>
<td>5.1 ± 0.6</td>
<td>75 ± 8</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>Cys291Ala</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Glu385Ala$^a$</td>
<td>19 ± 3</td>
<td>370 ± 10</td>
<td>0.19 ± 0.03</td>
</tr>
<tr>
<td>Arg447Ala</td>
<td>150 ± 20</td>
<td>54 ± 1</td>
<td>0.076 ± 0.003</td>
</tr>
<tr>
<td>PhnY constructs with G3P</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>97 ± 7</td>
<td>530 ± 50</td>
<td>0.098 ± 0.003</td>
</tr>
<tr>
<td>Cys291Ala</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PhnY constructs with 3-OPP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>3,300 ± 100</td>
<td>ND</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Cys291Ala</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Table 6.1: ND: not determined; a: this mutant displayed substrate inhibition (see Figure 6.9), the $K_{i,PnAA} = 420 ± 70$ µM.
Figure 6.9: Michaelis-Menten curves for wild-type PhnY and variants. Reaction conditions are described in the Materials and Methods section. Data shown are for A) wild-type, B) Arg108Ala, C) Asn168Ala, D) Arg290Ala, E) Glu385Ala, and F) Arg447Ala PhnY. All lines are fits of the data to the Michaelis-Menten equation except for panel E (left), for which the data was fit using the equation for substrate inhibition. In all cases, one substrate was held at saturating conditions while the other was varied.
Figure 6.10: Covalent adduct formation in PhnY-Glu254Ala. PhnY-Glu254Ala was treated with thrombin to remove the N-terminal His6 tag; the cleaved protein was then reacted with PnAA. The reaction mixture was washed with H2O and analyzed by direct infusion into an ESI mass spectrometer. The mass difference between the two peaks corresponds to +121 Da, which is in good agreement with the expected mass shift for a thioester intermediate of PnAA (+122 Da).

The Asn158Ala mutation resulted in a 200-fold decrease in $k_{cat}$ (Table 6.1, Figure 6.9). Replacement of Arg108 by Ala led to a 40-fold decrease in $k_{cat}$ and a 3-fold increase in $K_{M,PhnAA}$, and the Arg290Ala mutation resulted in a similar 20-fold decrease in $k_{cat}$ with a slightly elevated $K_{M,PhnAA}$ relative to wild type. The Arg447Ala mutation reduced the $k_{cat}$ 30-fold and increased the $K_{M,PhnAA}$ 50-fold. Glu385 provides the only interaction with the nicotinamide ribose 2’ and 3’ hydroxyl groups and mutation of this residue to Ala resulted in a 10-fold reduction in $k_{cat}$, with an approximately 6-fold higher $K_{M,NAD^+}$. The origin of the observed substrate inhibition with the PhnY-Glu385Ala mutant is unknown.

6.3.2: Kinetics and Ternary Complex Structures of PhnY-NAD$^+$ with Non-Physiological Substrates 3-OPP and G3P

The active site of PhnY is highly similar to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Streptococcus mutans, a well-characterized non-
phosphorylating ALDH of primary metabolism that catalyzes the conversion of G3P to 3-phosphoglycerate (Figure 6.11A). A one-to-one mapping of active site side chains was observed between GAPDH and PhnY, with the exception that GAPDH-Tyr155 is His159 in PhnY. In order to explore whether this structural similarity between the two active sites translates to substrate promiscuity for PhnY, the activity of PhnY with the physiological substrate of GAPDH, G3P, was explored. Additionally, I chemically synthesized a one-carbon-atom longer mimic of PnAA (Figure 6.11B). Indeed, Dr. Agarwal demonstrated that PhnY could accept both G3P and 3-OPP as substrates. Oxidation of 3-OPP by PhnY proceeded with nearly identical $k_{cat}$, but a 1000-fold increase in $K_M$ compared to the $K_M$ for PnAA (Table 6.1). However, the oxidation of G3P proceeded with a 20-fold reduction in $k_{cat}$ for the enzyme and a 30-fold increase in $K_M$ for G3P compared to $K_M$PnAA. No activity was observed for the PhnY Cys291Ala mutant enzyme using either 3-OPP or G3P as substrates.

![Figure 6.11: Substrate analogs tested with PhnY. (A) The native reaction that GAPDH carries out with glyceraldehyde-3-phosphate to make 3-phosphoglycerate. (B) 3-OPP is a synthetic substrate analog that is not known to be naturally-occurring.](image)

In the PhnY-G3P-NAD$^+$ ternary complex, the side chains of Arg108, His159, Arg290 and Arg447 bound the phosphoryl oxygen atoms of G3P in a manner analogous to PnAA as determined by Dr. Agarwal (Figure 6.12A and 6.12B). In addition, the same ternary complex was observed from the interaction of G3P with the GAPDH active site residues Arg103, Tyr155, Arg283 and Arg437 (Figure 6.12C). Direct interactions for the
bridging phosphoryl ester oxygen atom of G3P, mediated by GAPDH residues Arg437 and Tyr155 (red dashed lines in Figure 6.12C), were absent in PhnY. However, no residues were observed in the PhnY active site that would preclude phosphorylated substrates.

Figure 6.12: (A) Stereo view of G3P and NAD$^+$ binding to PhnY. All atoms are colored as before. Superimposed is the difference electron density map contoured at 2.5σ in blue. (B) Interactions are shown as green dashes for the binding of G3P with PhnY and (C) G3P with GAPDH. The interactions for the G3P phosphoryl oxygen atom and CB hydroxyl, which are absent in PhnY, are shown in red dashes. Note the different conformations of G3P in the PhnY active site and GAPDH active site. Dr. Agarwal generated this figure with LigPlot$^+$ using refined coordinates of PhnY-G3P-NAD$^+$ complex for panel B, and PDB 2ESD for panel C.\textsuperscript{22}

6.3.3: Relaxed Substrate Specificity of PhnY and Evolution of Enzyme Function in the ALDH family

The active sites of PhnY and GAPDH are strikingly similar, apart from the replacement of GAPDH-Tyr155 by PhnY-His159. In order to determine whether this similarity in active sites would lead to substrate promiscuity within the ALDH superfamily, I tested whether PhnY could use G3P as a substrate and whether GAPDH could accept phosphonate substrates. GAPDH oxidized 3-OPP with a large decrease in catalytic efficiency, and multiple turnovers with PnAA as substrate were not detected (Table 6.1 and Figure 6.13). However, roughly one equivalent of NADPH was produced
upon incubation of GAPDH with PnAA (Figure 6.13A); the observation of burst kinetics suggested that GAPDH was competent to oxidize PnAA to PnA but that hydrolysis of the non-physiological substrate occurred slowly. In comparison, G3P was a better substrate for PhnY than PnAA had been for GAPDH, though a 20-fold reduction in $k_{cat}$ was observed for G3P oxidation by PhnY compared to GAPDH oxidation.\textsuperscript{23}

Figure 6.13: Concentration dependence of the activity of GAPDH with various substrates. (A) A time course of GAPDH incubation with PnAA. After addition of PnAA at $\sim$0.5 min, a "burst" of NADPH formation was observed followed by no significant turnover. (B) GAPDH follows normal steady-state Michaelis-Menten kinetics when incubated with varying concentrations of 3-OPP and saturating NADP$^+$ or saturating concentrations of 3-OPP and varying concentrations of NADP$^+$. (C) The oxidation kinetics with GAPDH of the physiological substrate G3P and NADP$^+$ closely match the values that were previously reported.
The observed reduction in the catalytic efficiency of G3P oxidation by PhnY as compared to GAPDH could have been a result of unfavorable electrostatic interactions. In the PhnY-G3P-NAD$^+$ ternary complex (Figure 6.12A and 6.12B), G3P was contorted so that the thiohemiacetal oxyanion was positioned only 2.9 Å away from one of the phosphoryl oxygen atoms. However, when compared to the GAPDH-G3P-NAD$^+$ ternary complex (Figure 6.12C) (PDB: 2ESD),$^{24}$ G3P was fully extended and the corresponding thiohemiacetal oxyanion would not have such destabilizing electrostatic repulsions from the phosphoryl oxygen atoms. Thus, the physical dimensions of the PhnY and GAPDH active sites seem to dictate substrate specificities. A larger GAPDH active site allowed for G3P to be fully extended in the GAPDH active site while a smaller PhnY active site distorted G3P so that the thiohemiacetal intermediate was bound in an energetically less favorable conformation. In addition, the hydroxyl group of G3P made contact with the side chain of Arg437 in GAPDH, whereas this contact was absent in the ternary complex of PhnY with G3P and cofactor.

A second postulate for the reduction in G3P oxidation activity by PhnY is provided by the orientation of the Glu254 side chain for the deacylation half reaction. In the PhnY-G3P-NAD$^+$ ternary complex, the reactive sites of the Glu254 side chain were pointed away from the thioester in all copies in the asymmetric unit. However, Glu254 showed variable positioning within the PhnY-PnAA-NAD$^+$ ternary complex. Superimposing the two structures revealed that the productive deacylation orientation of Glu254 in the PhnY-G3P-NAD$^+$ ternary complex was occluded by the G3P-$\beta$ hydroxyl due to a steric clash. This in turn hindered the deacylation half reaction, which was rate limiting for GAPDH.$^{23}$
In order to test these two postulates, I synthesized 3-OPP, and Dr. Agarwal determined the kinetic parameters for its oxidation by PhnY and GAPDH. In 3-OPP, the aldehyde is separated from the phosphoryl moiety by one more bond than in PnAA, but one bond less than in G3P. PhnY indeed accepted 3-OPP as a substrate. As expected, the \( k_{\text{cat}} \) for 3-OPP oxidation was comparable to that for PnAA as the rate limiting deacylation step was not hindered, in contrast to G3P.\(^{23}\) However, the \( K_M \) for 3-OPP oxidation was 1000-fold greater than that for PnAA (Table 6.1). This observation provided evidence that substrates larger than PnAA would be unfavorably accommodated within the constrained PhnY active site. This preference is not dependent on the substrate being a phosphate or a phosphonate. The lack of a hydroxyl group and a bridging oxygen on 3-OPP and the interactions they make with active site residues may explain the greatly reduced catalytic activity of GAPDH towards this substrate compared to G3P. These results are reflective of the diversity in catalytic activities that can be built upon the conserved ALDH superfamily structural core.

6.3.4: A Proposed Catalytic Mechanism for PhnY

Prior work has led to the characterization of a novel pathway for phosphonate degradation in \textit{S. meliloti} 1021, which utilizes PhnY to convert PnAA into PnA.\(^{1}\) Oxidation of PnAA primes the resultant carboxylic acid for subsequent enzymatic hydrolysis by PhnA.\(^{25-27}\) PhnY provides the first, and presently the only known, biochemical basis for the synthesis of PnA in the microbial metabolome. The structural data demonstrate that PhnY belongs to the family of CoA-independent non-phosphorylating aldehyde dehydrogenases. Structural comparisons of PhnY with other
ALDHs revealed how changes to the highly conserved ALDH active site could increase the substrate tolerance of the enzymes to include phosphonates and expand upon the role of ALDHs in the biodegradation of inert molecules.

Three classes of ALDHs have been characterized, and all share a common first acylation half reaction, which includes hydride transfer to the cofactor. However, variability is observed in the second deacylation half reaction, based on the deacylating nucleophile—hydroxyl anion, phosphate, or a coenzyme A (CoA) thiolate. Our understanding of the CoA independent non-phosphorylating ALDHs is based primarily on the extensive structural and mechanistic characterization of GAPDH.\textsuperscript{4,23,24,28} Based on the PhnY cocrystal structures and kinetic data presented in this study, and the structural similarity of the PhnY and GAPDH active sites, a mechanistic proposal for the conversion of PnAA to PnA by PhnY is shown in Figure 6.14.

\textbf{Figure 6.14:} A mechanism for PhnY that accounts for the various crystal structures and kinetic assays. Figure prepared by Dr. Agarwal.

In the acylation half reaction catalyzed by PhnY, PnAA is covalently bound to the side chain of Cys291 forming a \((R)\)-thiohemiacetal intermediate at the interface of the catalytic and cofactor binding domains. Residues Arg108, His159, Arg290, Thr292 and Arg447 coordinate phosphoryl oxygen atoms. The side chain of Asn158 and main chain
amide nitrogen of Cys291 constitute the ‘oxyanion hole’ to stabilize the intermediate. The cofactor is aptly positioned for hydride transfer from the thiohemiacetal to the C4 carbon atom of the nicotinamide ring from the Re-face, generating a thioester intermediate. The conformation of the cofactor that results in hydride transfer occludes the side chain of Glu254 from approaching the active site, due to steric clash between the Si-face of the nicotinamide ring and the Glu254 side chain. In the succeeding deacylation half reaction, the side chain of Glu254 is now positioned close to the thioester intermediate allowing the carboxylate to deprotonate a water molecule for nucleophilic attack at the thioester. This attack leads to the formation of a thioorthoester intermediate, which is resolved by the departure of the Cys291 side chain and generation of the product PnA.

6.4: Conclusions

Studying the oxidation of PnAA to PnA has helped elucidate the substrate scope and mechanism of the dehydrogenase responsible for the transformation. Ultimately, the insights gleaned from the study of PhnY may help enable the identification of the aldehyde dehydrogenase that is responsible for the oxidation of PnAA to PnA in the fosfazinomycin pathway. By contrast, studying the reduction of PnAA to 2-HEP helped define the facial selectivity of the hydride transfer; unfortunately, the result did not enable the exclusion of either hypothetical fosfomycin biosynthetic pathway (Figure 6.4). Taken together, these results illustrate that PnAA is a central intermediate in a wide variety of pathways including both catabolic processes and secondary metabolism.
6.5: Materials and Methods

6.5.1: Determining the Stereochemistry of Hydride Transfer by DhpG

These experiments were performed on 10/9/11 and 10/10/11. NADPH stereospecifically labeled with deuterium at C4 of the nicotinamide was synthesized by incubating NADP+ (2 mM) in 50 mM HEPES, pH 7.5 with deuterated phosphite (3 mM) and phosphite dehydrogenase (PtDH, 7 µM), which were both kindly provided by a then-graduate student in the van der Donk group, Dr. John Hung. The mixture was allowed to incubate at 30 °C for 90 min, and then PtDH was removed by centrifuge filtration (30 kDa MWCO). The presence of (4R)-NADP2H was confirmed by the generation of a peak at 340 nm in the UV-Vis spectrum. Because of its instability, the cofactor was used without further purification.

Phosphonoacetaldehyde labeled with 13C at C2 was generated by incubating thiamine pyrophosphate (TPP, 1.4 mM) with [2-13C1]-phosphoenolpyruvate (PEP, 10 mM, Sigma Aldrich) in 50 mM HEPES pH 7.5 with 1 mM Mg2+. The reaction was initiated by addition of RhiH (80 µM) and the PnPy decarboxylase (40 µM), causing turbidity. After incubating at 30 °C for 45 min, proteins were removed by centrifuge filtration (30 kDa MWCO). An aliquot was taken from the flow through, and the presence of [2-13C1]-PnAA was confirmed by 31P NMR spectroscopy.

(4R)-NADP2H (1 mM final concentration) was mixed with the prepared [2-13C1]-PnAA (4 mM final concentration) and DhpG (approximately 75 µM, expressed and purified as described previously) was added in 50 mM HEPES, pH 7.5. The reaction was allowed to proceed at room temperature for 60 min, at which point the UV-Visible spectrum indicated the consumption of the 340 nm peak associated with the (4R)-
NADP$^2$H cofactor. Protein was removed via centrifuge filtration (30 kDa MWCO), and an aliquot was taken to confirm the presence of 2-[2-$^{13}$C$_1$, 2-$^2$H$_1$]-HEP via $^{31}$P NMR spectroscopy. To the isotopically labeled 2-HEP (~0.8 mM) was added HEPD (final concentration of 20 µM) in 50 mM HEPES pH 7.5. The solution was incubated at room temperature to effect the conversion of 2-HEP to HMP and formate. Protein was removed via centrifuge filtration (30 kDa MWCO), and an aliquot of the flowthrough (10 µL) was derivatized to afford the 2-nitrophenylhydrazide adduct and analyzed by LC-MS using methodology developed previously.$^{15}$

6.5.2: General Experimental for the PhnY Investigation

G3P was obtained from Sigma-Aldrich (G5251-25MG) and used without further purification. Media components were purchased from Thermo Fisher Scientific or VWR (West Chester, PA). DNA primers were synthesized by Integrated DNA Technologies Inc (Coralville, IA). Restriction enzymes were obtained from New England Biolabs (Ipswich, MA). DNA sequencing was performed by ACGT Inc (Wheeling, IL). Phosphonoacetaldehyde was prepared before use as described elsewhere$^{29}$ and was stored at -20 °C when not in use.

Cloning, expression, and purification of recombinant PhnY from E. coli has been described previously.$^1$ The mutant constructs Arg108Ala, Asn158Ala, His159Ala, Glu254Ala, Arg290Ala, Cys291Ala, Thr292Ala, Glu385Ala and Arg447Ala were generated by Dr. Agarwal (Nair lab, University of Illinois) using standard site-directed mutagenesis protocols and confirmed by plasmid DNA sequencing. Dr. Agarwal also
performed all the crystallographic studies reported herein. Wild type and mutant enzymes were purified according to procedures described earlier.\textsuperscript{3}

6.5.3: Cloning and Purification of GAPDH

These experiments were performed on 9/17/13 and 10/3/13. GAPDH was cloned from \textit{Streptococcus mutans} Clarke ATCC\textsuperscript{®} 25175 using Failsafe Buffer A and expressed as an \textit{N}-terminal His\textsubscript{6}-fusion protein from a pET15b vector in \textit{E. coli} Rosetta2. Cells were grown to OD\textsubscript{600} \~{}0.6 in LB, expression was induced with 0.3 mM IPTG at 18 °C for 16 h, and cells were harvested by centrifugation. The cell pellet from 1 L of culture was resuspended in lysis buffer B (50 mM HEPES pH 7.5, 200 mM KCl, 10% glycerol, 5 mM DTT, 20 mM imidazole, 10 U DNAse, 25 mg lysozyme), lysed with a French press (15,000 psi), and the lysate was clarified by centrifugation. The clarified lysate was loaded onto a pre-equilibrated column with Ni-NTA resin. The column was washed with lysis buffer B, lysis buffer B supplemented with 50 mM imidazole, and lysis buffer B supplemented with 250 mM imidazole. After concentrating with an Amicon centrifuge filter, the protein buffer was exchanged to 50 mM HEPES pH 7.5, 200 mM KCl, 10% glycerol, 5 mM DTT using a NAP-25 filter. This process yielded \~{}60 mg GAPDH/L cell culture.

6.5.4: Synthesis of 3-OPP

These experiments were performed on 10/3/12 and 12/14/12. Following a modified literature procedure,\textsuperscript{30} 2.0 g (7.5 mmol) of diethyl (3,3-diethoxypropyl)phosphonate was added to a three-necked flask equipped with a
condenser under N\textsubscript{2}. TMSCl (3.8 mL, 30 mmol) was added, and the mixture was refluxed for 2 h. Although residual starting material was still present, refluxing for longer periods of time led to an increase in byproducts, which were difficult to remove. After 2 h, the mixture was cooled to 50 \degree C, and 2 mL of H\textsubscript{2}O was added to hydrolyze the residual TMSCl and deprotect the silyl groups and unmask the aldehyde. The reaction was stirred for 30 min. H\textsubscript{2}O (5 mL) was added, and the solvent was removed using a rotary evaporator to afford a yellow oil. \textsuperscript{1}H, \textsuperscript{31}P, and [\textsuperscript{1}H-\textsuperscript{31}P] gHMBC NMR analysis and ESI-MS characterization confirmed the presence of both 3-OPP with diethyl (3-oxopropyl)phosphonate as the sole byproduct. Spectral data for 3-OPP: NMR: \textsuperscript{1}H (500 MHz, D\textsubscript{2}O): δ 9.52 (s, CHO), 2.72 (dt, J = 14.6, 7.5, PCH\textsubscript{2}CH\textsubscript{2}), 2.06 (dt, J = 17.7, 7.5, PCH\textsubscript{2}CH\textsubscript{2}); \textsuperscript{31}P (202 MHz, D\textsubscript{2}O): δ 35.6 ppm. HR-MS (ESI\textsuperscript{+}): found 139.0162, calculated 139.0160 (1.4 ppm). \textsuperscript{31}P NMR analysis of a stock of the 3-OPP mixture with an internal standard of P\textsubscript{i} allowed the determination of the 3-OPP concentration.

6.5.5: Steady-State Kinetics for PhnY Variants and GAPDH

These assays were performed on 6/26/13, 6/27/13, 6/28/13, 6/29/13, 6/30/13, 7/2/13, 7/7/13, 7/21/13, 7/22/13, 9/30/13, and 10/1/13. The kinetics of substrate oxidation by wild type PhnY enzyme and site specific mutants were determined by monitoring the reduction of NAD\textsuperscript{+} by UV-Vis spectroscopy at 340 nm using procedures described previously.\textsuperscript{1} Enzyme (0.1-5 \mu M depending on the variant) was incubated with NAD\textsuperscript{+} in 50 mM HEPES, pH 7.5 supplemented with 10 mM MgSO\textsubscript{4} for 5 min at 30 \degree C. The reaction was initiated by addition of phosphonate/phosphate substrate. To obtain Michaelis-Menten kinetics, one substrate was maintained at saturating levels while the
other substrate was varied; rates were obtained in triplicate. Dr. Agarwal determined the steady-state kinetics of WT PhnY with 3-OPP; I determined all others. The Cys291Ala and Glu254Ala mutant enzymes were assayed at various substrate and enzyme concentrations. One mutant, Glu385Ala, displayed substrate inhibition and its kinetic parameters were obtained by fitting to the appropriate equation, 

\[ \frac{V}{I} = \frac{k_{cat}[E][S]}{K_{M,s} + [S] + \left(\frac{[S]^2}{K_{i,s}}\right)} \]

where [E] is the enzyme concentration, [S] is the substrate concentration, and \( K_{i,s} \) is the inhibition constant of the substrate. A similar procedure was used for the kinetic studies with by GAPDH (0.02-20 µM depending on the substrate) except that the buffer used was 50 mM HEPES pH 8.0 supplemented with 5 mM DTT and the cofactor used was NADP⁺.

**6.5.6: Detection of a Covalent Adduct with PhnY-Glu254Ala**

This assay was performed on 9/20/13. To observe the covalent thioester adduct, PhnY-Glu254Ala was treated with thrombin to remove the N-terminal His_6_ tag. The protein (2 mg/mL) was then reacted with PnAA (2 mM) in the presence of NAD⁺ (2 mM) in 50 mM HEPES pH 7.5 with 10 mM MgSO₄. The reaction mixture was washed extensively with H₂O with a 0.5 mL 30 kDa MWCO centrifuge filter, mixed with unreacted PhnY-Glu254Ala that had been similarly washed (to serve as an internal standard), and then analyzed by directly infusing into an ESI mass spectrometer. The mass difference between the two peaks corresponds to +121 Da, which is in good agreement with the mass shift for a thioester intermediate of PnAA (+122 Da).
6.6: References


(7) Shao, Z.; Blodgett, J. A.; Circello, B. T.; Eliot, A. C.; Woodyer, R.; Li, G.; van der Donk, W. A.; Metcalf, W. W.; Zhao, H. Biosynthesis of 2-


