Thioester Hydrolysis Reactivity of Metal Complexes

James Justin Danford

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THIOESTER HYDROLYSIS REACTIVITY OF METAL COMPLEXES

by

James J. Danford

A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

in

Chemistry

Approved:

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UTAH STATE UNIVERSITY
Logan, Utah

2009
ABSTRACT

Thioester Hydrolysis Reactivity of Metal Complexes

by

James J. Danford, Master of Science

Utah State University, 2009

Major Professor: Dr. Lisa M. Berreau
Department: Chemistry and Biochemistry

Glyoxalase II is one of two metalloenzymes found in the glyoxalase pathway and is responsible for catalyzing the hydrolysis of a thioester substrate. Its bimetallic active site is found to contain a variety of metal combinations, including Fe(III)Zn(II). A recent report indicates that human glyoxalase II, while containing a Fe(II)Zn(II) center, is catalytically active as a mononuclear Zn(II) enzyme. Detailed mechanistic studies of glyoxalase II enzymes are limited due to uncertainty in the metal ion content of recombinantly prepared samples. The research presented in this thesis is focused on gaining mechanistic insight into thioester hydrolysis promoted by well-characterized metal complexes.

The initial research is focused on studies involving a Fe(III)Zn(II) complex supported by the 2-{{bis(2-pyridylmethyl)amino}methyl}-6-{{[2-hydroxyphenyl)methyl]- (2-pyridylmethyl)amino}methyl]-4-methylphenol ligand. Thioester hydrolysis reactions were examined by following the loss of a deuterium-labeled thioester (hydroxyphenyl thioacetic acid S-methyl\((d_3)\) ester) over time using \(^3\)H NMR as the monitoring method.
Based on kinetic data and spectroscopic investigations (UV-vis and EPR), a reaction pathway for thioester hydrolysis promoted by the aforementioned Fe(III)Zn(II) complex has been proposed. An important feature of this pathway is the formation of a precursor complex wherein the deprotonated α-hydroxy group of the thioester coordinates to the Zn(II) center prior to nucleophilic attack by an Fe(III)-OH moiety.

Of relevance to human glyoxalase II, the thioester hydrolysis reactivity of a mononuclear zinc complex containing the \(N,N\)-bis(2-pyridylmethyl)-tert-butyamine ligand, \((\text{bpta})\text{Zn}(\text{ClO}_4)_2\cdot 0.5\text{H}_2\text{O}\), has been examined. Based on kinetic data, it is proposed that thioester hydrolysis promoted by this complex proceeds via a bimolecular pathway, with a Zn-OH moiety being the nucleophile for attack on the thioester carbonyl. Activation parameters are reported for the zinc complex-promoted thioester hydrolysis reaction and are compared to those of OH promoted thioester hydrolysis reactions.

In a separate area of investigation, the chromium chloride complex \((\text{C}_6\text{H}_{11}\text{N}_2)[\text{CrCl}_3]\)_n has been isolated and characterized by elemental analysis and X-ray crystallography. This complex has been proposed as the catalyst responsible for high yield conversion of glucose to 5-hydroxymethylfurfural (HMF), which is an important reaction toward using renewable resources as feedstock chemicals.
ACKNOWLEDGMENTS

No individual can accomplish success without the aid and assistance of friends, colleagues, and family. Over the course of my academic career I have had the opportunity to work with many individuals that have helped to shape me into the scientist I am today. Even if you are not mentioned in great detail, I do appreciate all of the help and guidance.

First off, I would like to thank my mentor, advisor, and friend Dr. Lisa M. Berreau for her never-ending assistance over the last three years. Even though I can be stubborn and impatient at times, she has helped me to channel these traits into a successful graduate career. I would not be where I am today and I would not have accomplished what I have if not for her. From the rocky beginning to the satisfying conclusion, she has been there to provide her knowledge and expertise and for that I cannot thank her enough.

To my friend and former co-worker, Dr. Kasia Rudzka, I want to thank you for all of your help. We had some good times over the 2+ years we worked together in Lisa’s lab, whether it was our quick discussions about our separate but equal greatness to the more thought provoking and detailed discussions about science, I have enjoyed every minute of it and wish you the best in the future.

To my friend and co-worker, Kasia Grubel, I want to thank you for all of your help as well. You have helped me in so many areas during my stay in graduate school that I have lost count. You have been a great colleague and even though we have had our arguments over the last three years (I still claim my innocence in all matters) I have had a great time. Unfortunately, my time here is ending but you still have many years left and will be successful, with many more publications to come.
To my buddy Danyal, these three years went by fast and we are still speaking to each other so I consider that a success. The era of racquetball and El Torro is coming to an end but you can still lose to Mark at racquetball. It has been fun, aside from the comedy of trying to do science, after working for three straight days and the extremely long EPR experiments of DOOM! Take care of yourself in the future and I see many publications to come for you.

To the entire Severinsen family (Rob, Lillian, and Libby) I want to thank you for being such a great family and true friends to the very end. I wish you the best and want you to know that I am always there if you need help.

I would like to thank Dr. Atta M. Arif for his aid in solving my crystal structures and for contributing to two of my manuscripts. I would also like to thank Dr. Piotr Dobrowolski for his assistance and for his help in teaching me how to effectively conduct and analyze $^2$H NMR spectroscopy and the kinetic experiments associated with it.

I would like to thank my committee members, Dr. Alvan C. Hengge and Dr. John L. Hubbard, for all of their help, guidance, and support throughout my entire time in graduate school. They have both provided me with useful and practical advise that has and will continue to benefit me in the future.

To my best buddy Jason (the honorary member of the Danford clan), who has been there for my family and I for years, I want to say I appreciate everything you have done. I will always be grateful to you for your years of help and support. These are traits that cannot be measured in words and your friendship has helped keep me sane over the years and will never be forgotten.
To my loving brother, Steve, I wanted to thank you for always being there for me and helping to get me through school. We have had many good times and there is still more to come (We are not done with those COW tapes yet), but you have always been there for me without question. I appreciate all of the moral support you have given me and it has helped during these last few years. You are truly a great brother and no matter where I end up I know that you will be there with open arms.

To my loving brother, Robert and his family, I wanted to send my appreciation for all of your support and for the fun times we have had. You and your family have been very gracious to me and I cannot thank you enough for all of your help. Thank you for the constant updates and for sending me all those pictures of my adorable nephew, however, I expect to get double the pictures with Katherine on the way. You are a great brother and I know you will always be there for me with an open ear and a kind word.

Finally, I need to express my utmost appreciation, thanks, and love to my Mom and Dad, for which this thesis is dedicated to. Words cannot begin to describe how much I appreciate the help, guidance, support, love, and time that the both of you have put into helping me get where I am today. There are no words that can be said to express the honor and respect I have for you two. This whole process has been easier, knowing that you two were there for me in my time of need and that support can never be forgotten. I know you are proud of what I have accomplished and I thank you for being such great parents and for taking an interest in my work. I love you both very much and enjoy the read.

James J. Danford
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<td>BPTA</td>
<td>N-N-bis(2-pyridylmethyl)-tert-butylamine</td>
</tr>
<tr>
<td>HMF</td>
<td>5-hydroxymethylfurfural</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BPAN</td>
<td>2,7-bis[2-(2-pyridylethyl)aminomethyl]-1,8-naphthyridine</td>
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<td>MAPs</td>
<td>Metalloaminopeptidases</td>
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<td>ApAP</td>
<td><em>Aeromonas proteolytica</em></td>
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<td>SgAp</td>
<td><em>Streptomyces griseus</em></td>
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<td>LNA</td>
<td>L-leucine-p-nitroanilide</td>
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<td>AP</td>
<td>Alkaline phosphatase</td>
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<td>PAPs</td>
<td>Purple acid phosphatases</td>
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<tr>
<td>H₂L/H₂BPBMP</td>
<td>2-bis[{2-pyridyl-methyl}-aminomethyl]-6-{(2-hydroxybenzyl)-(2-pyridyl-methyl)}-aminomethyl]-4 methylphenol</td>
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<tr>
<td>BpNPP</td>
<td>bis(p-nitrophenyl)phosphate</td>
</tr>
<tr>
<td>BDNPP</td>
<td>2,4-bis(dinitrophenyl) phosphate</td>
</tr>
<tr>
<td>GSH</td>
<td>Glutathione</td>
</tr>
<tr>
<td>AGE</td>
<td>Advanced glycation end-products</td>
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<td>GlxI</td>
<td>Glyoxalase I</td>
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<td>GlxII</td>
<td>Glyoxalase II</td>
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<td>SLG</td>
<td>S-(2-hydroxyacyl)glutathione thioester</td>
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<tr>
<td>DTNB</td>
<td>5,5'-dithio-bis(2-nitrobenzoic acid)</td>
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<td>CHES</td>
<td>N-Cyclohexyl-2-aminoethanesulfonic acid</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
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<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
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<td>Tp(Me&lt;sub&gt;3&lt;/sub&gt;)</td>
<td>Tris(3-cumenyl-5-methylpyrazolyl)hydroborate</td>
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<td>PATH</td>
<td>2-methyl-1-[(methyl(2-pyridin-2-ylethyl)amino)propane-2-thiolate</td>
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<td>NPA</td>
<td>4-nitrophenylacetate</td>
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<td>TE</td>
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<td>DMF</td>
<td>2,5-dimethylfuran</td>
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<td>2,5-furan dicarboxylic acid</td>
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<td>PETs</td>
<td>Polyethylene terephthalates</td>
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<td>DMA-LiCl</td>
<td>Dimethylacetamide-lithium chloride</td>
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<td>EMIM</td>
<td>1-ethyl-3-methylimidazolium</td>
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<tr>
<td>BMIM</td>
<td>1-butyl-3-methylimidazolium</td>
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CHAPTER 1
INTRODUCTION
METAL-PROMOTED HYDROLYSIS REACTIONS IN BIOLOGICAL AND MODEL SYSTEMS

I. Background of Hydrolysis Chemistry

Water ($H_2O$) is a colorless, non-toxic compound, which can exist in three different physical states, depending on temperature and pressure. Water consists of 11% hydrogen and 89% oxygen by mass and covers approximately 70% of the earth’s surface. It is essential for life, as it accounts for at least 70% of the mass of cells in living organisms.¹

Hydrolysis is defined as a reaction that involves the ability of an ion(s) or compound(s) to react with water.¹ Hydrolysis reactions are classified in several ways, depending on the area of study, including general acid-base chemistry, organic chemistry, inorganic chemistry, and biochemistry. General acid-base chemistry involves direct interaction of water with anions ($X^-$) or protons ($H^+$) (Scheme 1-1).¹ In organic chemistry, hydrolysis is defined as a reaction that involves the conversion of one compound into two different species by the addition of water.² There are numerous organic compounds, which have been shown to undergo either acid- or base-catalyzed hydrolysis, including esters,

$$X^- + H_2O \rightarrow HX + OH^-$$

$$HA + H_2O \rightarrow H_3O^+ + A^-$$

Scheme 1-1. Acid-base hydrolysis reactions.
Ester, amide, and nitrile hydrolysis reactions.

In inorganic chemistry, hydrolysis is defined as the reversible transfer of $\text{H}^+$ from an aqua species, usually a metal-aqua complex (Scheme 1-3). In biochemistry, hydrolysis is defined as the cleavage of a substrate (e.g. ATP), accompanied by the addition of the elements of water (Scheme 1-4). Hydrolysis reactions in biochemical systems are catalyzed by a variety of enzymes called hydrolases. Hydrolase enzymes possessing metal ions (metallohydrolases) are known to contain mononuclear, homobinuclear, and mixed binuclear metal centers at their active site. Among the metallohydrolase enzymes, metallo-$\beta$-lactamases, metalloaminopeptidases, alkaline phosphatases, and purple acid phosphatases are the most studied. Although Zn(II) is the most common active site metal ion in metallohydrolases, other metals found in these enzymes include Mn(II), Ni(II), Ca(II), and Mg(II).

\[
\begin{align*}
\text{R'}\text{COOR} + \text{H}_2\text{O} & \rightarrow \text{R'}\text{COOH} + \text{ROH} \\
\text{RCONH}_2 + \text{H}_2\text{O} & \rightarrow \text{RCOOH} + \text{NH}_3 \\
\text{RCN} + 2\text{H}_2\text{O} & \rightarrow \text{RCOOH} + \text{NH}_3
\end{align*}
\]

Scheme 1-2. Ester, amide, and nitrile hydrolysis reactions.

$$[\text{M(H}_2\text{O})_6]^{n+} + \text{H}_2\text{O} \rightleftharpoons \text{H}_3\text{O}^+ + [\text{M(H}_2\text{O})_5(\text{OH})]^{(n-1)+}$$

Scheme 1-3. Hydrolysis chemistry of a metal-aqua complex.
In terms of hard/soft acid base theory, the metal ions found in metallohydrolases are classified as either hard Lewis acids [Mn(II), Mg(II), Ca(II)] or borderline Lewis acids [Zn(II) and Ni(II)]. An advantage of the latter ones is related to their ability to bind both hard and soft donor atoms. In biological systems, these interactions involve histidine, glutamate/aspartate, and cysteine ligands. The Lewis acidity of the metal ion is important for biological hydrolysis as it influences the pKₐ of metal-bound water molecules.

In these enzymes, the metal center can play various roles in promoting hydrolysis reactions (Figure 1-1). The metal center can: (1) polarize chemical bonds (usually seen in the activation of carbonyls), which can lead to increased susceptibility of the carbonyl compound towards nucleophilic attack; (2) act as a Lewis acid to lower the pKₐ of a bound water molecule and produce a reactive hydroxide moiety; or (3) stabilize negative charge build-up in a transition state or intermediate formed during the hydrolysis reaction.
II. Metallohydrolases, Models, and the Use of Activated Ester Substrates

a. Metallo-β-lactamases. Metallo-β-lactamases (Class B β-lactamases) catalyze the hydrolytic ring opening of the β-lactam ring in all structural subtypes of antibiotics (penams, cephems, clavams, penems, and carbapenems) except monobactams, rendering them inactive (Scheme 1-5).\textsuperscript{10-17} These enzymes have been isolated from various bacteria including \textit{Bacteroides fragilis}, \textit{Bacillus cereus}, and \textit{Stenotrophomonas maltophilia}. X-ray crystal structures of the metallo-β-lactamase enzymes from these sources have been shown to contain a binuclear zinc active site (Figure 1-2), though recent studies have indicated that the active form may utilize a single zinc ion.\textsuperscript{18-21}

![Scheme 1-5. Hydrolysis of a β-lactam ring.](image-url)
In terms of the bimetallic form, the proposed mechanism for $\beta$-lactam hydrolysis (Scheme 1-6) involves: (1) activation of the $\beta$-lactam carbonyl carbon through interaction with one of the Lewis acidic zinc centers; (2) nucleophilic attack by a Zn-OH moiety on the carbonyl carbon of the coordinated $\beta$-lactam, forming a tetrahedral intermediate; (3) C-N bond cleavage; and (4) protonation of the ring-opened nitrogen and release of the carboxylate to give the ring-opened product.$^{22}$ In this mechanism, the role of the Zn$_2$ site is to stabilize the ring-opened, deprotonated nitrogen. In the monozinc form, the proposed mechanism for $\beta$-lactam hydrolysis is similar, but with an aspartate residue acting as a general base/acid (Scheme 1-7).$^{23}$
Scheme 1-6. Proposed mechanism of β-lactam hydrolysis by a binuclear zinc center.

In an effort to probe metal binding in metallo-β-lactamase enzymes, heterobimetallic analogues (ZnNi, ZnCo, ZnFe) from *E. coli* have been overexpressed and purified.\textsuperscript{24-26} Studies have revealed that these analogues are reactive in the hydrolysis of β-lactam substrates. Furthermore, research by Crowder and co-workers has shown that the metal content of these analogues is highly dependent on growth conditions.\textsuperscript{26} Additional research aimed at investigating inhibitors of metallo-β-lactamas has emerged and in the process, thiols have been identified as potential inhibitors of these enzymes.\textsuperscript{27-30}
b. Model studies of metallo-β-lactmases

To provide further insight into the potential mechanism(s) by which these enzymes hydrolyze β-lactams, studies of benzylpenicillin and nitrocefin hydrolysis, promoted by synthetic binuclear zinc complexes (Figure 1-3), have been employed. Of particular
interest were studies conducted using complex 3. Infrared spectroscopic studies have shown that treatment of 3 with benzylpenicillin results in formation of a hydrolyzed, ring-opened product (Scheme 1-8). To gain insight into how β-lactams interact with the binuclear zinc center of 3, X-ray crystallography, $^{13}$C NMR, and infrared spectroscopy of 3 in the presence of oxazetidinylacetate were performed. These studies provided evidence supporting the hypothesis that coordination of the β-lactam to the zinc center occurs through the carboxylate group and not the carbonyl carbon of the β-lactam ring (Figure 1-4).$^{32}$. 

**Figure 1-3.** Binuclear zinc complexes with relevance to β-lactamases.
Another investigation focused on nitrocefin hydrolysis catalyzed by the binuclear zinc complex \([\text{Zn}_2(\text{BPAN})(\mu\text{-OH})(\mu\text{-O}_2\text{PPh}_2)](\text{ClO}_4)_2\) (Scheme 1-9). This reaction results in the formation of a ring-opened product, as evidenced by a change in the \(\lambda_{\text{max}}\) of nitrocefin from 390 nm to 486 nm.\(^{33}\) Kinetic studies as a function of the concentration of \([\text{Zn}_2(\text{BPAN})(\mu\text{-OH})(\mu\text{-O}_2\text{PPh}_2)](\text{ClO}_4)_2\) revealed saturation behavior, which is consistent
Scheme 1-9. Hydrolysis of nitrocefin promoted by \([\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\mu-\text{O}_2\text{PPh}_2)](\text{ClO}_4)_2\). 

with substrate coordination to the metal complex prior to hydrolysis. The rate of hydrolysis increases with pH, yielding a kinetic \(pK_a\) of 8.7, which is identical to the thermodynamic \(pK_a\) determined by potentiometric titration. This \(pK_a\) has been assigned to the deprotonation of a terminal zinc-bound water molecule in \([\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\text{H}_2\text{O})](\text{ClO}_4)_2\).\textsuperscript{34} These results provide evidence that the active nucleophile in nitrocefin hydrolysis is a terminal Zn-OH species.\textsuperscript{33}

Spectroscopic studies revealed that treatment of \([\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\mu-\text{O}_2\text{PPh}_2)](\text{ClO}_4)_2\) with cephalothin (an analog of nitrocefin) in DMSO causes a shift in the position of the carboxylate carbon \(^{13}\text{C}\) resonance and a shift in the carboxylate \(v_{\text{C}=\text{O}}\) vibration band. These results are consistent with monodentate carboxylate coordination to the zinc center.\textsuperscript{33} Based on kinetic and spectroscopic studies, it has been proposed that nitrocefin hydrolysis promoted by \([\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\mu-\text{O}_2\text{PPh}_2)](\text{ClO}_4)_2\) (Scheme 1-10) involves: (1) coordination of nitrocefin to one of the zinc centers; (2) nucleophilic attack by a terminal Zn-OH at the \(\beta\)-lactam carbonyl carbon, leading to formation of a tetrahedral intermediate; (3) C-N bond cleavage; and (4) release of the ring-opened product and regeneration of the dizinc species.
Scheme 1-10. Proposed mechanism of nitrocefin hydrolysis promoted by $[\text{Zn}_2(\text{BPAN})(\mu-\text{OH})(\mu-\text{O}_2\text{PPh}_2)](\text{ClO}_4)_2$.

c. Metalloaminopeptidases. Metalloaminopeptidases (MAPs) are a class of metallohydrolases that catalyze the hydrolytic cleavage of the $N$-terminal amino acid residue of polypeptide chains (Scheme 1-11). Of the MAP enzymes that have been
Scheme 1-11. Cleavage of the N-terminal residue of a polypeptide chain by metalloaminopeptidases.

characterized, leucine aminopeptidase (LAP) and the aminopeptidases isolated from *Aeromonas proteolytica* (ApAP) and *Streptomyces griseus* (SgAP) have been shown to possess an active site containing either one or two zinc centers.\(^{35-37}\) The X-ray crystal structure of ApAP indicates that the co-catalytic zinc centers are coordinated by histidine, glutamate, and aspartate ligands (Figure 1-5).\(^{38}\)

Kinetic, thermodynamic, spectroscopic, and additional X-ray crystallographic studies were conducted in order to gain insight into the mechanism of ApAP. Based on these studies, a mechanism for peptide hydrolysis (Scheme 1-12) has been proposed to involve: (1) coordination of the amide carbonyl oxygen to one of the zinc centers, followed by a shift of the bridging hydroxide to a terminal position on Zn\(_1\); (2) nucleophilic attack at the amide carbonyl carbon by a Zn-OH moiety and formation of a tetrahedral intermediate; (3) breakdown of the intermediate along with protonation of the amine leaving group; (4) C-N bond cleavage; and (5) release of the products.\(^{39}\)
d. Model Studies of Metalloaminopeptidases. Model studies aimed at obtaining mechanistic information relevant to metalloaminopeptidase enzymes have concentrated on the use of binuclear zinc complexes and the model substrate L-leucine-$p$-nitroanilide (LNA). These studies have shown that model binuclear zinc complexes possessing the 2,6-bis[bis(2-methoxyethyl)aminomethyl]-4-X-phenol] ligand, where X = methyl, nitro, or chloro, mediate the hydrolysis of LNA (Scheme 1-13). The reactions were evaluated by monitoring the formation of the $p$-nitroaniline leaving group ($\lambda_{\text{max}} = 405$ nm). The rate of hydrolysis was shown to increase with pH, yielding kinetic $pK_a$ values of 9.4 and 9.1 for the methyl and chloro species, respectively. These values have been assigned to the deprotonation of a Zn-OH$_2$ to give a nucleophilic Zn-OH moiety.$^{40-42}$
Scheme 1-12. Proposed mechanism of peptide hydrolysis catalyzed by ApAP. For clarity, the zinc-coordinated amino acid ligands are shown only in the resting state (left).

e. Alkaline Phosphatase. Alkaline phosphatase (AP) catalyzes the hydrolysis of phosphate monoesters under alkaline conditions (Scheme 1-14).\textsuperscript{43-53} Alkaline phosphatase is found in almost all organisms, however, it is the enzyme isolated from \textit{Escherichia coli} (\textit{E. coli}) that has been studied in great detail.\textsuperscript{54} This enzyme contains a binuclear zinc center in the active site (Figure 1-6), as well as a third metal center [Mg(II)].\textsuperscript{48,53,55} Debate has centered around the role of magnesium in AP enzymes and it has been proposed that a magnesium hydroxide moiety may act as a general base for the deprotonation of a neighboring serine-hydroxide (Ser-OH) moiety. This would lead to the formation of a nucleophilic serine-alkoxide moiety.\textsuperscript{50}

Scheme 1-14. Hydrolysis of phosphate monoesters by alkaline phosphatase.
Mechanistic studies of the hydrolysis of phosphate monoesters catalyzed by alkaline phosphatase have been reported.\textsuperscript{7b,50,56} Based on these results, the mechanism (Scheme 1-15) is proposed to involve: (1) coordination of the phosphate monoester to the two zinc centers in a bridging fashion; (2) deprotonation of the Ser-OH; (3) nucleophilic attack by a Ser-O\textsuperscript{−} species at the phosphorus, resulting in cleavage of the P-OR bond; (4) displacement of the zinc-bound alkoxide group by a water molecule, release of ROH, and formation of a new zinc hydroxide species; (5) nucleophilic attack at the phosphorus by the Zn-OH, resulting in cleavage of the P-O-Ser bond; (6) release of [HOPO\textsubscript{3}]\textsuperscript{2−} and regeneration of the active site.

\textbf{f. Model Studies of Alkaline Phosphatase.} Literature reports of binuclear zinc complexes with phosphate monoester hydrolysis reactivity relevant to AP enzymes are limited. One example is the hydrolytic reactivity of a binuclear zinc complex supported by
Scheme 1-15. Proposed mechanism for phosphate monoester hydrolysis catalyzed by alkaline phosphatase. For clarity, the ligand environment of the zinc centers has been truncated.

a macrocyclic cryptand ligand (Figure 1-7). X-ray crystallographic studies of this complex revealed that the two metal centers exhibit distorted trigonal bipyramidal geometries and are bridged by an alkoxide anion. Treatment of this complex with 4-nitrophenyl phosphate in aqueous solution at 60 °C for two days results in the formation of the hydrolyzed product (4-nitrophenol) and a new, binuclear zinc-phosphoramide complex (Scheme 1-16).
**g. Purple Acid Phosphatases.** Purple acid phosphatases (PAPs) constitute a family of binuclear metallohydrolase enzymes that catalyze the hydrolysis of phosphate monoesters (aryl phosphate monoesters, phosphoric anhydrides, and phosphoserine residues of phosphoproteins) under acidic or neutral conditions (Scheme 1-17).\textsuperscript{58-60} X-ray crystal structures of the various forms of PAP enzymes have revealed a bimetallic iron-containing active site [Fe(III)M(II), M(II) = Fe, Zn, Mn] (Figure 1-8). The characteristic purple color found in PAP enzymes is due to a LMCT transition
Scheme 1-17. Hydrolysis of a phosphate monoester.

\[
(RO)PO_3^{2-} + H_2O \rightarrow HPO_4^- + ROH
\]

\( R = \text{Aryl, serine, etc.} \)

Figure 1-8. Active site of purple acid phosphatases.

(\( \lambda_{\text{max}} = 510-560 \text{ nm, } \varepsilon = 3000-4000 \text{ M}^{-1} \text{ cm}^{-1} \)) involving the tyrosine ligand and the Fe(III) metal center.

PAP enzymes have been isolated from numerous sources including mammals, plants, and fungi. Mammalian PAPs are monomeric proteins with a molecular mass of approximately 35 kDa and contain a redox active diiron cluster at the active site. Plant PAPs can exist as either homo or heterodimers. A homodimeric plant PAP has been isolated having a molecular mass for each subunit of approximately 55 kDa. A heterodimeric plant PAP, isolated from tomato, possesses subunits of 63 kDa and 57 kDa. Less is known about fungal PAPs, however, a monomeric fungal PAP of approximately 85 kDa has been isolated and purified from Aspergillus ficuum. Currently, the metal
The composition of this enzyme is not known, however, it is proposed that the metal center may contain zinc, copper, or manganese.  

The exact mechanism by which PAP enzymes hydrolyze phosphate monoesters is unknown, however, two mechanisms have been proposed (Scheme 1-18). In the first step of either mechanism it is proposed that the substrate coordinates to the metal center(s), yet, the mode by which coordination occurs (monodentate or bidentate) is highly debated. Spectroscopic studies of the Fe(III)Zn(II) form isolated from bovine revealed that at the pH for catalysis (~6.5) the substrate coordinates to the divalent metal center in a monodentate fashion. Spectroscopic studies of the PAP enzyme obtained from pig revealed that at the pH for catalysis (~5) the substrate coordinates to the metal centers in a bridging bidentate fashion. Following substrate coordination to the metal center(s), nucleophilic attack by either a bridging or terminal hydroxide is proposed to occur, nevertheless, the nature of the active nucleophile is still a point of speculation. Studies conducted on PAP enzymes isolated from pig and red kidney bean have shown that at lower pH values (pH <5) enzyme activity increases. This increase in activity has been assigned to the formation of a terminal Fe(III)-OH species, which has been proposed to be the active nucleophile. In contrast, ENDOR studies and fluoride inhibition studies on pig and human PAP enzymes have argued against a terminal Fe(III)-OH being the active nucleophile. These studies have provided evidence to support the notion that the bridging hydroxide is the nucleophile for phosphate monoester hydrolysis. In the final step, the bound phosphate is proposed to dissociate from the metal center(s) and water molecules bind to regenerate the active site. Depending on the pH, one or both of these new terminal water molecules may be in the hydroxide form.
Scheme 1-18. Proposed mechanisms of purple acid phosphatase enzymes.
h. Model Studies of Purple Acid Phosphatases. The active site of PAP enzymes contains two distinct metal coordination environments. Therefore, biomimetic studies of the properties of PAP enzymes have focused on the synthesis and characterization of heterobinuclear metal complexes possessing an asymmetric ligand environment. To date, the majority of model complexes of PAP enzymes have been generated using the asymmetric ligand \( \text{H}_2\text{L} \) (\( \text{L} = 2\text{-bis}\{2\text{-pyridyl-methyl}\text{-aminomethyl}\}-6\text{-}\{(2\text{-hydroxybenzyl})\text{-}(2\text{-pyridyl-methyl})\text{-aminomethyl}\}\text{-}4\text{ methyl-phenol} \)) (Figure 1-9). Addition of one equivalent of the Fe(III) and M(II) salts [M = Zn, Fe, Mn, Cu, Ni] and two equivalents of sodium acetate to \( \text{H}_2\text{L} \) in methanol generates the desired asymmetric heterobinuclear complexes (Figure 1-10). X-ray crystal structures of these complexes have shown that the Fe(III) center is coordinated to the terminal phenolate oxygen. Results of further research concerning the physical properties of these complexes are shown in Table 1-1. All complexes exhibit a characteristic purple color (\( \lambda_{\text{max}} \approx 522 - 555 \text{ nm} \), \( \varepsilon = 2600\text{-}4560 \text{ M}^{-1} \text{ cm}^{-1} \)), which occurs due to a LMCT involving the phenolate oxygen and the Fe(III) center. Potentiometric titration measurements of the manganese, zinc, copper, and nickel complexes in a mixed solvent system [water:ethanol (30:70)] have provided multiple p\( K_a \) values over the pH range of 3-9. In the case of the zinc, copper, and nickel complexes, three p\( K_a \) values were obtained, however, in the manganese case only two were identified. Recent studies of the Fe(III)Zn(II) system have revealed a shift in the three p\( K_a \) values when the solvent system was changed to water/acetonitrile (50:50).
Figure 1-9. Ligand for PAP biomimics.

Figure 1-10. Binuclear model complexes for PAP enzymes.
Table 1-1. Selected physical properties of model complexes relevant to purple acid phosphatase enzymes.

<table>
<thead>
<tr>
<th>Fe(III)M(II)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>$\varepsilon$ (M$^{-1}$ cm$^{-1}$)</th>
<th>30:70 water/ethanol</th>
<th>50:50 water/acetonitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>M = Fe</td>
<td>555</td>
<td>4560</td>
<td>p$K_{a1}$ 4.81 5.99 7.97</td>
<td>p$K_{a1}$ 2.93 4.81 8.30</td>
</tr>
<tr>
<td>Zn</td>
<td>540</td>
<td>3700</td>
<td>5.80</td>
<td>7.76</td>
</tr>
<tr>
<td>Mn</td>
<td>544</td>
<td>2680</td>
<td>5.25</td>
<td>6.20</td>
</tr>
<tr>
<td>Cu</td>
<td>546</td>
<td>3400</td>
<td>5.30</td>
<td>6.80</td>
</tr>
<tr>
<td>Ni</td>
<td>522</td>
<td>2600</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

It has been proposed that upon addition of water to each complex, the bridging acetates dissociate from the metal center(s) and water molecules bind in their place. In the case of zinc, copper, and nickel, a water molecule coordinates at the terminal position on each metal center and a third water bridges the two metals. The first protonation equilibrium (lowest p$K_a$) was assigned to the deprotonation of the bridging water molecule. The second protonation equilibrium was assigned to the deprotonation of the water molecule bound at the Fe(III) site, and finally deprotonation of the terminal water molecule bound at the M(II) site provides the final protonation equilibrium value (highest p$K_a$) (Scheme 1-19).$^{78,79,81-83,85}$

Research conducted on PAP enzymes has shown that they are unable to hydrolyze biologically relevant phosphodiesters, as evidenced by their inability to hydrolyze bis(p-nitrophenyl)phosphate (BpNPP).$^{86}$ In contrast, model complexes designed to mimic the active site of PAP enzymes are able to hydrolyze biologically relevant phosphodiesters.$^{83}$

Table 1-2. Kinetic parameters for the hydrolysis of BDNPP by PAP model complexes.60

<table>
<thead>
<tr>
<th>Fe(III)M(II)</th>
<th>$K_m$ (M)</th>
<th>$k_{cat}$ (s$^{-1}$)</th>
<th>$k_{cat}/K_m$ (s$^{-1}$ M$^{-1}$)</th>
<th>$k_{cat}/k_{uncat}$</th>
<th>pH optimum</th>
</tr>
</thead>
<tbody>
<tr>
<td>M = Zn</td>
<td>8.1 x 10$^{-3}$</td>
<td>1.1 x 10$^{-3}$</td>
<td>1.3 x 10$^{-1}$</td>
<td>6.1 x 10$^{3}$</td>
<td>6.5</td>
</tr>
<tr>
<td>Cu</td>
<td>1.1 x 10$^{-2}$</td>
<td>1.8 x 10$^{-3}$</td>
<td>1.6 x 10$^{-1}$</td>
<td>1.0 x 10$^{4}$</td>
<td>7.0</td>
</tr>
<tr>
<td>Mn</td>
<td>2.1 x 10$^{-3}$</td>
<td>7.1 x 10$^{-4}$</td>
<td>3.3 x 10$^{-1}$</td>
<td>3.9 x 10$^{3}$</td>
<td>6.7</td>
</tr>
<tr>
<td>Ni</td>
<td>3.8 x 10$^{-3}$</td>
<td>4.4 x 10$^{-4}$</td>
<td>1.2 x 10$^{-1}$</td>
<td>2.4 x 10$^{3}$</td>
<td>6.0</td>
</tr>
</tbody>
</table>

Kinetic parameters (Table 1-2) and mechanistic insight into phosphate hydrolysis has been obtained using the activated diester substrate 2,4-bis(dinitrophenyl) phosphate (BDNPP) (Figure 1-11).87 The results of these experiments indicate that the hydrolysis of BDNPP is highly dependent on the pH of the system and is optimal in the range of 6-7. In the case of
the Fe(III)Zn(II), Fe(III)Ni(II), and Fe(III)Mn(II) complexes, a terminal Fe(III)-OH is proposed to be the active nucleophile, while in the Fe(III)Cu(II) complex, the bridging hydroxide has been assigned as the active nucleophile. The debate about the exact mechanism of PAP enzymes continues and the significant differences shown in model systems, with regards to the coordination mode of BDNPP and the active nucleophile present during hydrolysis, has led to two proposed mechanisms (Scheme 1-20 and 1-21). In model complexes containing Fe(III)Zn(II), Fe(III)Mn(II), and Fe(III)Ni(II), the reaction is proposed to involve: (1) coordination of BDNPP to the M(II) metal binding site in a monodentate fashion; (2) nucleophilic attack of the Fe(III)-OH at the phosphorus, resulting in the formation of a bridging species and a free alcohol; and (3) release of the phosphate and addition of water, leading to the regeneration of the active metal complex. For the model complex containing Fe(III)Cu(II), the reaction is proposed to involve: (1) coordination of BDNPP to the metal centers in a bridging bidentate fashion; (2) nucleophilic attack by the bridging hydroxide, resulting in the release of free alcohol; and (3) release of the phosphate and addition of water, leading to regeneration of the active metal complex.
Scheme 1-20. Proposed mechanism of phosphodiester hydrolysis promoted by model Fe(III)Zn(II), Fe(III)Ni(II), and Fe(III)Mn(II) complexes.
Scheme 1-21. Proposed mechanism for hydrolysis of BDNPP by Fe(III)Cu(II) complex.
i. **Activated Substrates.** It has been shown that a convenient approach for investigating hydrolysis reactions in enzymatic and model systems is the use of “activated substrates” (Table 1-3). The term “activated” originates from the fact that the substrate(s) is more susceptible to nucleophilic attack during the hydrolysis reaction. Activation of the substrate is accomplished by introduction of strong electron withdrawing substituents. Of the various electron-withdrawing substituents available, the use of p-nitrophenyl leaving groups is preferred. These leaving groups are ideal for investigating hydrolysis reactions because: (1) their conjugate acids possess low pKₐ values; and (2) they possess an absorption feature associated with the hydrolyzed p-nitrophenolate leaving group (λ_max ~400 nm), making hydrolysis easy to monitor by UV-Vis spectroscopy.

**Table 1-3.** Hydrolysis of p-nitrophenyl substituted model substrates to the corresponding p-nitrophenolates.
III. Thioester Hydrolysis

The glyoxalase pathway (Scheme 1-22) is important for human health, as it is responsible for converting cytotoxic 2-oxoaldehydes (e.g. methylglyoxal (CH$_3$C(O)C(O)H)) into nontoxic 2-hydroxy acids, utilizing glutathione (GSH) as an essential cofactor.$^{89,90}$ Methylglyoxal is a reactive small molecule formed as a byproduct of lipid and carbohydrate metabolism.$^{91}$ The toxicity of methylglyoxal arises from the reactivity of its aldehyde group with guanine residues of DNA and amino acid nucleophiles.$^{92-96}$ Furthermore, methylglyoxal is found to be involved in the formation of advanced glycation end-products (AGE), which have been linked to aging and various diseases.$^{97-99}$

Scheme 1-22. Glyoxalase pathway.
In the glyoxalase pathway two metalloenzymes, glyoxalase I (GlxI) and glyoxalase II (GlxII) can be found. GlxI is responsible for the isomerization of a non-enzymatically formed hemithioacetal to a thioester.\textsuperscript{100,101} In the active site, GlxI contains a mononuclear metal center which, depending on the source, can be Zn(II) or Ni(II).\textsuperscript{102,103} GlxII is responsible for the hydrolysis of the enzymatically formed thioester to a 2-hydroxycarboxylic acid and free glutathione.\textsuperscript{100} GlxII contains a binuclear metal center in the active site and is classified as a member of the β-lactamase superfamily of hydrolases.\textsuperscript{104,105}

a. Glyoxalase I (GlxI). GlxI is a member of the metalloglutathione transferase superfamily and has been shown to play a crucial role in the detoxification of reactive 2-oxoaldehydes. GlxI catalyzes the glutathione dependent isomerization of hemithioacetals to the corresponding 2-hydroxy thioesters (Scheme 1-23).\textsuperscript{106} GlxI enzymes have been isolated from various sources and the active site metal content is highly dependent on the source. GlxI enzymes isolated from bacterial sources (\textit{E. coli, Y. pestis, P. aeruginosa, and N. meningitides}), as well as the enzyme isolated from the human parasite \textit{L. major} have been found to contain a mononuclear Ni(II) center.\textsuperscript{107-110} By contrast, GlxI enzymes isolated from human and yeast sources have been shown to contain a mononuclear Zn(II) center.\textsuperscript{111-114}

X-ray crystal structures of the Ni(II)-dependent GlxI enzyme from \textit{E. coli} and the Zn(II)-dependent GlxI enzyme from humans have revealed a distorted octahedral geometry around the metal centers (Figure 1-12). The Ni(II)-dependent GlxI is ligated by two
histidines, two glutamates, and two water ligands. The Zn(II)-dependent GlxI is ligated by one histidine, two glutamate, one glutamine, and two water molecules.\textsuperscript{98} Depending on the metal ion present, different mechanistic pathways have been proposed for the isomerization of a hemithioacetal substrate to the corresponding thioester. In Zn(II)-dependent GlxI enzymes (Scheme 1-24), the mechanistic pathway is suggested to first involve the loss of two water molecules, followed by a bidentate coordination of the substrate to the metal center. Deprotonation of the substrate at the C\textsubscript{1} carbon leads to the formation of an enediol. This is followed by protonation of the C\textsubscript{2} carbon leading to the formation of a bound thioester. Finally, the addition of two equivalents of water releases the thioester and regenerates the active site.\textsuperscript{98,115} In the Ni(II)-dependent GlxI enzyme (Scheme 1-25), the mechanistic pathway is suggested to involve a Ni(II)-OH species. First, one of the nickel-bound water molecules is deprotonated, leading to the formation of a Ni(II)-OH moiety. The Ni(II)-OH species extracts a proton from the C\textsubscript{1} carbon of the hemithioacetal, leading to the formation of an enolate species. Protonation of the enolate at the C\textsubscript{2} carbon yields the thioester.\textsuperscript{116,117}

\textbf{Scheme 1-23.} Isomerization of hemithioacetal to thioester.
Figure 1-12. Active site of Zn(II) and Ni(II)-dependent GlxI.

Scheme 1-24. Proposed mechanism for Zn(II) catalyzed isomerization of hemithioacetal.
b. Glyoxalase II (GlxII). GlxII is a member of the metallo-β-lactamase superfamily of hydrolase enzymes and catalyzes the final step in the glyoxalase pathway. GlxII hydrolyzes a thioester to a 2-hydroxy carboxylic acid and free glutathione (Scheme 1-26). GlxII enzymes have been isolated from various sources including human and plant. The most detailed studies reported to date on GlxII enzymes have been conducted on those isolated from human sources and the plant *Arabidopsis thaliana*. 

**Scheme 1-25.** Proposed mechanism for Ni(II) catalyzed isomerization of a hemithioacetal.
c. Human Glyoxalase II (human GlxII). X-ray crystallographic characterization of the human GlxII enzyme has shown the presence of a binuclear metal center in the active site (Figure 1-13). The two metal centers (M₁ and M₂) are separated by 3.3 to 3.5 Å and each possesses a coordination number of five. The M₁ site is ligated by three histidines (His-54, His-56, His-110), a bridging water/hydroxo moiety, and a single oxygen donor from a bridging aspartate ligand (Asp-134). The M₂ site is coordinated by two histidines (His-59, His-173), one terminal aspartate (Asp-58), one bridging aspartate (Asp-134), and a bridging water/hydroxo moiety.¹²₀ The metal binding domain in human GlxII is similar to that present in metallo-β-lactamases.¹²₀ Metal analysis of the protein used for the X-ray experiment showed the presence of ~1.5 moles of zinc and ~0.7 moles of iron per mole of protein.¹²₀ Despite this metal content, researchers initially assigned human GlxII as a dizinc metalloenzyme due to the similar metal binding domain between GlxII and metallo-β-lactamases. Based on this assumption, a mechanism was proposed for thioester hydrolysis (Scheme 1-27).¹²₀ In this mechanism, the bridging hydroxide acts as a nucleophile and

\[
\text{SG = Glutathione} \\
\text{R = CH}_3, \text{Ph, etc.}
\]

**Scheme 1-26.** Hydrolysis of a thioester to a 2-hydroxy carboxylic acid.
**Figure 1-13.** Active site of human GlxII.

**Scheme 1-27.** Proposed mechanism for a dizinc-containing human GlxII.
attacks the carbonyl carbon of the thioester and the Zn$_1$ center stabilizes the tetrahedral transition state. Cleavage of the C-S bond results in the formation of the 2-hydroxy carboxylic acid and free glutathione.

In 2009, Crowder et al. reported that human GlxII contains an Fe(II)Zn(II) center in vivo, but is catalytically active as a mononuclear Zn(II) enzyme. Therefore, it has been proposed that the second metal binding site is not essential to catalysis.$^{137}$

d. Mitochondrial and Cytoplasmic Glyoxalase II (Glx2-5 and Glx2-2) from

*Arabidopsis thaliana.* X-ray crystallographic characterization of a recombinantly produced mitochondrial GlxII (Glx2-5) from *Arabidopsis thaliana* (Figure 1-14) has shown that the coordination environment of each metal center differs from that found in human GlxII. The M$_1$ site has a tetrahedral geometry and is ligated by three histidine donors (His-54, His-56, His-112) as well as a bridging water/hydroxide ligand. The M$_2$ site has a trigonal bipyramidal geometry and is ligated by two aspartates (Asp-58, Asp-131), two histidines (His-59, His-169), and a bridging water/hydroxide.$^{119}$ Essentially, while human GlxII has a bridging aspartate ligand, this carboxylate is terminal on the M$_2$ site in Glx2-5.

Metal analysis of the recombinantly produced Glx2-5 enzyme from *Arabidopsis thaliana,* grown under conditions of excess iron and zinc (supplemented media), revealed that the enzyme contained ~1.0 mole of iron, ~1.3 moles of zinc, ~0.016 moles of manganese, and <0.001 moles of copper. However, Glx2-5 overexpressed in a non-supplemented media resulted in the enzyme containing approximately equal amounts of iron and zinc (~0.6 moles) and no detectable amounts of either manganese or copper. EPR
Figure 1-14. Active site of mitochondrial GlxII from *Arabidopsis thaliana*.

studies of Glx2-5 grown in supplemented media suggested the presence of two major species. The first was assigned to the Fe(III)Zn(II) moiety and constituted ~70% of the EPR signal. The second was assigned to the Fe(III)Fe(II) moiety and constituted the remaining 30% of the EPR signal.\(^\text{119}\)

A cytoplasmic glyoxalase II (Glx2-2) from *Arabidopsis thaliana* has been overexpressed and purified. The sequence comparison of Glx2-2 with the metal binding regions of the sequenced metallo-\(\beta\)-lactamases revealed a striking homology between these enzymes. Further studies revealed that all of the residues ligating the binuclear Zn(II) center of metallo-\(\beta\)-lactamases are conserved in Glx2-2.\(^\text{125}\) Based on these results, the active site of Glx2-2 has been proposed (Figure 1-15). The M\(_1\) site is ligated by three histidine donors (His-135, His-137, His-201), a bridging water/hydroxide, and a terminal
water molecule. The $M_2$ site is ligated by two aspartates (Asp-139, Asp-226), one histidine (His-272), a bridging water/hydroxide, and a terminal water molecule.

Metal analysis of a wild type cytoplasmic GlxII (Glx2-2) revealed the enzyme to contain $\sim$0.8 moles of zinc and $\sim$1.5 moles of iron per mole of protein. These results suggest that either zinc or iron can occupy the metal binding sites. Further studies conducted on the wild type Glx2-2 grown in rich media showed that the enzyme contains various amounts of zinc, iron, and manganese. Based on EPR, mass spectrometry, and X-ray absorption spectroscopy, Glx2-2 has been proposed to contain various heterogeneous and/or homogeneous combinations of metals in the active site, including Fe(III)Zn(II), Fe(III)Fe(II), Zn(II)Zn(II), Fe(II)Fe(II), Mn(II)Mn(II), Fe(III)Mn(II), Fe(II)Mn(II), and Zn(II)Mn(II). Based on these findings, a new mechanism for thioester hydrolysis has been proposed (Scheme 1-28). This mechanism involves: (1) coordination of the substrate to the metal center(s) (a proximal arginine (Arg-248) can help to orientate the

**Figure 1-15.** Proposed active site of Glx2-2.
thioester); (2) the bridging hydroxide between the iron and zinc centers becomes terminal on the Fe(III) site; (3) nucleophilic attack by a Fe(III)-OH at the carbonyl carbon, results in the formation of a tetrahedral transition state; (4) release of D-lactic acid; and (5) protonation of the thiolate and release of glutathione leads to the regeneration of the active site.

e. Model studies of Glyoxalase II enzymes. To date, no glyoxalase II enzyme has been produced containing only one type of bimetallic combination. This situation presents an ideal opportunity for model studies involving model complexes, in which specific metal ion content is known. In 2006, the Berreau laboratory reported the first examples of zinc hydroxide complexes (Figure 1-16) capable of mediating thioester hydrolysis. Complexes 1 and 2 are binuclear in the solid state, however, in acetonitrile solution they dissociate into mononuclear species. Treatment of either zinc complex with hydroxyphenyl-thioacetic acid S-methyl ester (Figure 1-17), at 45 °C in acetonitrile resulted in the formation of methanethiol (H₃CSH) and a zinc-bound mandelate complex. Additional studies were conducted to determine whether a binuclear zinc complex possessing a bridging hydroxide (Figure 1-18) could promote thioester hydrolysis. Treatment of this complex with hydroxyphenyl-thioacetic acid S-methyl ester at 45 °C in acetonitrile for ~3 days resulted in no hydrolysis products being observed. From these results, the authors hypothesized that a bridging hydroxide moiety is not sufficiently nucleophilic to react with a thioester substrate, and that a terminal metal-hydroxide is required in order for thioester hydrolysis to occur.
Scheme 1-28. Proposed mechanism for Fe(III)Zn(II) GlxII.
Figure 1-16. Binuclear zinc hydroxide complexes.

Figure 1-17. Hydroxyphenyl-thioacetic acid S-methyl ester.

Figure 1-18. Binuclear zinc complex with a bridging hydroxide ligand.
The research presented in Chapters 2 and 3 of this thesis centers around gaining insight into the metallohydrolase enzyme Glyoxalase II (GlxII). The research presented focuses on thioester hydrolysis reactivity studies of binuclear and mononuclear metal complexes of relevance to GlxII enzymes. Chapter 2 describes thioester hydrolysis reactivity promoted by the binuclear complex \([(BPBPMP)Fe(III)Zn(II)(\mu-OAc)_{2}]ClO_4\), which is used to mimic the Fe(III)Zn(II) form of Glyoxalase II enzymes. The results presented in this chapter provide mechanistic insight into thioester hydrolysis promoted by the mentioned metal complex in a mixed organic/aqueous solution, over the pH range of 7-9. Chapter 3 investigates thioester hydrolysis reactivity promoted by the mononuclear zinc complex \([Zn(bpta)](ClO_4)_2\), which is used to mimic the mononuclear Zn(II) form of human Glyoxalase II enzymes. Further information describing the synthesis, and characterization of this complex are outlined. Kinetic studies and activation data for the thioester hydrolysis reaction in a mixed organic/aqueous solution promoted by \([Zn(bpta)](ClO_4)_2\) are offered.

References


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CHAPTER 2

THIOESTER HYDROLYSIS REACTIVITY OF AN Fe(III)Zn(II) COMPLEX*

Abstract

Glyoxalase II enzymes catalyze the hydrolysis of a thioester substrate and have been found to coordinate a variety of dimetal combinations, including Fe(III)Zn(II), within the enzyme active site. Of relevance to these enzymes, the thioester hydrolysis reactivity of the Fe(III)Zn(II) compound \([(BPBPMP)Fe(III)Zn(II)(\mu-OAc)_{2}]ClO_{4}\) (1) was evaluated in CH$_3$CN:H$_2$O (50:50; buffered) at 26.5 °C. Thioester hydrolysis in the absence and presence of 1 was monitored using $^2$H NMR by following the loss of the thioester –SCD$_3$ signal. Two products are generated in the reaction involving the metal complex, CD$_3$SSCD$_3$ and CD$_3$SH. Kinetic studies of this reaction as a function of pH revealed maximum rate above the pK$_a$ of a Zn-OH$_2$ moiety of \([(BPBPMP)Fe(III)(OH)(\mu-OH)Zn(II)(OH)_{2}]^+\), which forms from 1 in CH$_3$CN:H$_2$O (50:50). UV-vis and EPR studies of a single turnover thioester hydrolysis reaction in the presence of one equivalent of 1 at pH = 9.0 suggest that the thioester does not initially interact with the Fe(III) center, but that changes occur at this site over the course of the reaction. The formation of a Fe(III)–SCD$_3$ moiety is proposed. This proposal is based on the observed CD$_3$SSCD$_3$ formation and a decrease in the intensity of the $g = 4.3$ feature over the course of the single turnover thioester hydrolysis reaction. The latter suggests reduction of a portion of the Fe(III) to Fe(II), which is consistent with

disulfide formation. A mechanism for thioester hydrolysis is proposed involving initial coordination of the deprotonated α-hydroxy thioester to the zinc center followed by nucleophilic attack by a terminal Fe(III)-OH moiety and thiolate leaving group stabilization by the Fe(III) center. Overall, this study outlines a novel approach of using an aliphatic thioester substrate and ²H NMR to provide mechanistic insight into thioester hydrolysis involving an Fe(III)Zn(II) complex of relevance to glyoxalase II.

**Introduction**

Metallohydrolases containing an active site heterobinuclear metal cluster are an area of intense current investigation.¹,² Falling within this classification are some glyoxalase II (GlxII) enzymes, which have been identified as containing a bimetallic iron/zinc cluster that catalyzes thioester hydrolysis.³ GlxII is a member of the metallo-β-lactamase superfamily of proteins⁴ and is one of the two metalloenzymes found in the ubiquitous glyoxalase pathway.⁵ The primary physiological substrate of the glyoxalase pathway (Figure 2-1) is believed to be methylglyoxal (CH₃C(O)CHO), a small molecule byproduct of lipid and glucose metabolism that is cytotoxic and mutagenic.⁵-⁸ GlxII catalyzes the hydrolysis of a S-(2-hydroxyacyl)glutathione thioester (SLG) to produce the corresponding S-2-hydroxyacid and free glutathione (Figure 2-1). As SLG is also cytotoxic and can inhibit DNA synthesis,⁵,⁹ the reaction catalyzed by glyoxalase II is important in cellular detoxification.¹⁰ Elevated levels of GlxI and GlxII mRNA and protein have been reported in tumor cells, and inhibitors of the glyoxalase system have been shown to affect tumor growth in vitro.¹¹ For this reason, several studies have been reported toward the
Figure 2-1. (Top) Glyoxalase pathway. (Bottom) Active site structural features in crystallographically characterized GlxII enzymes.

design of anti-tumor agents that target the glyoxalase system.\textsuperscript{10a,11,12}

Glyoxalase II enzymes from a variety of sources have been characterized by X-ray crystallography.\textsuperscript{3b,3c,13} In each structure the active site contains a binuclear metal cluster in which each metal center is coordinated by three terminal amino acid ligands, a bridging
aspartate, and a bridging water/hydroxide moiety. In a subset of the X-ray structures, a bridging carboxylate or a terminal water molecule occupies the sixth coordination position on each metal center. The active site structural features of GlxII enzymes are similar to purple acid phosphatase in that one metal site has three terminal neutral donors and the second site contains a terminal anionic oxygen donor ligand.\(^2d\)

A problem that has hampered comprehensive mechanistic studies of the GlxII-catalyzed thioester hydrolysis reaction is the variable metal ion content of GlxII enzymes which depends on the source of the enzyme and preparation conditions.\(^3\),\(^13\),\(^14\) Specifically, different amounts of iron, zinc, and manganese ions are incorporated into the active site binuclear cluster depending on conditions. Metal ion contents that have been proposed on the basis of spectroscopic studies and metal analyses in GlxII enzyme samples include Fe(III)Zn(II), Fe(II)Zn(II), Fe(III)Fe(II), Zn(II)Zn(II), Fe(II)Fe(II), and Mn(II)Mn(II).\(^3\),\(^13\),\(^14\) For the mitochondrial *Arabidopsis thaliana* GlxII produced recombinantly in *E. coli* in rich media, the predominant iron-containing form of the enzyme (\(~70\%)\) is proposed to have an Fe(III)Zn(II) metal ion content.\(^3c\)

Neves and coworkers previously reported the Fe(III)Zn(II) complex \([(BPBMP)Fe(III)Zn(II)(\mu-OAc)_2]ClO_4\) (1, Figure 2-2) which, when dissolved in CH\(_3\)CN:H\(_2\)O (50:50), exhibits three pK\(_a\) values.\(^2a\) These have been assigned to the deprotonation of a bridging water molecule, a terminal Fe(III)-OH\(_2\), and a terminal Zn(II)-OH\(_2\), indicating that the bridging acetate ligands are displaced in favor of water ligands in aqueous solution. A structurally relevant species to the proposed Fe(III)(H\(_2\)O)(\mu-
Figure 2-2. Solution properties of 1 in CH$_3$CN:H$_2$O (50:50).

OH)Zn(II)(H$_2$O) complex, [(BPBPMP)Fe(III)(H$_2$O)(µ-OH)Zn(II)](ClO$_4$)$_2$ (2), has been crystallized from a methanol:H$_2$O solution and characterized by X-ray crystallography.$^{2c}$ In 1:1 CH$_3$CN:H$_2$O, this compound exhibits pK$_a$ values of 2.93, 4.81, and 8.30. These have been assigned to similar ionizations to those depicted in Figure 2-2. X-ray absorption spectroscopic studies of 2 in CH$_3$CN:H$_2$O (70:30) provided evidence that both metal centers are six-coordinate in solution.

To our knowledge, no thioester hydrolysis reactivity studies involving an Fe(III)Zn(II) complex of relevance to GlxII have been reported. To address this deficiency, we report herein our initial studies of the thioester hydrolysis reactivity of the Fe(III)Zn(II) complex 1 in a CH$_3$CN:H$_2$O (buffered) solution. Our approach involves the use of $^2$H NMR as a monitoring technique to follow the loss of a deuterium-labeled thioester. This method
has allowed us to examine the reactivity of an aliphatic α-hydroxy thioester of structural relevance to the glutathione adduct of the glyoxalase pathway. We note that only one study of thioester hydrolysis promoted by a metal complex has been previously reported. This investigation, which involved binuclear zinc compounds and reactivity studies in organic solvent, suggested the need for a terminal Zn-OH for thioester hydrolysis.

**Experimental Section**

**General and Physical Methods.** All reagents and solvents were obtained from commercial sources and were used as received unless otherwise noted. When necessary for synthetic procedures, solvents were dried according to published procedures and were distilled under N$_2$ prior to use. IR spectra were recorded on a Shimadzu FTIR-8400 as KBr pellets. $^1$H NMR spectra for characterization purposes were recorded in CD$_3$CN at ambient temperature on a JEOL ECX-300 or Bruker ARX-400 NMR spectrometer. $^1$H NMR parameters appropriate for paramagnetic complexes were used for obtaining spectra of 1. Chemical shifts (in ppm) are referenced to the residual solvent peak in CH/D$_2$CN ($^1$H: 1.94 (quintet)). $^2$H NMR spectra were collected as previously described and were referenced to an internal standard of C$_6$D$_6$ (7.16 ppm). ESI/APCI mass spectra were obtained at the University of California, Riverside. UV-vis spectra were recorded on a Hewlett-Packard HP8453 diode array spectrophotometer at ambient temperature. EPR spectra were collected at 5(1) K using a Bruker EMXPlus spectrometer system fitted with a continuous flow liquid helium cooled cryostat. Elemental analyses were performed by Atlantic Microlabs of Norcross, GA.
Synthesis of Ligand Precursors. The organic precursors 2,6-bis-(chloromethyl)-4-methylphenol,\(^\text{18}\) bis-(2-pyridylmethyl)amine,\(^\text{19}\) and (2-hydroxybenzyl)(2-pyridylmethyl)amine\(^\text{20}\) were synthesized according to literature procedures with minor modifications.

Synthesis of Hydroxyphenyl Thioacetic Acid S-methyl\((\text{d}_3)\) Ester \((\text{PhCH(OH)}C(O)\text{SCD}_3)\). This \(\text{d}_3\)-labeled thioester was prepared according to literature procedures starting from \(\text{d}_6\)-DMSO.\(^\text{21}\) \(^1\)H NMR \((\text{CD}_3\text{CN}, 300 \text{ MHz})\): \(\delta\) 7.41-7.35 (m, 5H), 5.22-5.20 (d, \(J = 5.2\) Hz, 1H), 4.46-4.44 (d, \(J = 5.2\) Hz, 1H, O-H) ppm; \(^{13}\)C{\(^1\)H} NMR \((\text{CD}_3\text{CN}, 100.6 \text{ MHz})\) \(\delta\) 204.7, 140.4, 129.8, 129.7, 128.0, 80.3, 11.6 (7 signals expected and observed); \(^2\)H NMR \((\text{CH}_3\text{CN}, 61.4 \text{ MHz})\) \(\delta\) 1.92 (s, pwhh ~3 Hz); \(^2\)H NMR \((\text{CH}_3\text{CN}:\text{H}_2\text{O} \ (50:50, \text{buffered, pH} = 9.0))\) \(\delta\) 1.92 (s, pwhh ~4 Hz); FTIR \((\text{KBr}, \text{cm}^{-1})\) 3441 (\(\nu\text{OH}\)), 1682 (\(\nu\text{C=O}\)); Anal calcd for C\(_9\)H\(_7\)D\(_3\)SO\(_2\): C, 58.35; H+D, 5.39. Found: C, 58.48; H+D, 5.49.

Synthesis of 2-{[bis(2-pyridylmethyl)amino]methyl}-6-[[2-hydroxyphenyl)methyl]-(2-pyridylmethyl)amino]methyl]-4-methylphenol \((\text{L}_1)^\text{22}\). To a solution of 2,6-bis(chloromethyl)-4-methylphenol \((14.4\text{ g}, 0.0702\text{ mol})\) in dry CH\(_2\)Cl\(_2\) \((500\text{ mL})\) was added a solution of (2-hydroxybenzyl)(2-pyridylmethyl)amine \((9.14\text{ g}, 0.0427\text{ mol})\) in dry CH\(_2\)Cl\(_2\) \((55\text{ mL})\) and triethylamine \((19.6\text{ mL}, 0.141\text{ mol})\). The reaction mixture was stirred overnight (~12 h). At this point, bis-(2-pyridylmethyl)amine was added \((22.4\text{ g}, 0.113\text{ mol})\) and the solution was allowed to stir for an additional 24 h at room temperature. The solution was then transferred to a separation funnel and 300 mL of brine solution was added. The mixed aqueous/organic solution was extracted with CH\(_2\)Cl\(_2\) \((3 \times 100\text{ mL})\). The
combined organic fractions were then dried over sodium sulfate and the solvent was removed under reduced pressure to produce a brown solid. Purification of this solid by column chromatography on silica gel using a 4:1:1 ratio of ethyl acetate:hexanes:triethylamine as the eluent yielded a white powder ($R_f \sim 0.50$; Yield: 9.21 g, 40%); (CDCl$_3$, 400 MHz) δ 8.57-8.56 (m, 3H), 7.61-6.81 (m, 15H), 3.90 (s, 8H), 3.80 (s, 2H), 3.75 (s, 2H), 2.27 (s, 3H) ppm. The $^1$H NMR of L$^1$ matched that previously reported.$^{22}$

CAUTION! Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of material should be prepared, and these should be handled with great care.$^{23}$

[(L$^1$Fe(III)Zn(II))(µ-O$_2$C$_2$H$_3$)$_2$]ClO$_4$ (1). A procedure for the preparation of 1 has been previously reported.$^{2f}$ In our hands this procedure resulted in the formation of a significant amount of the Fe(III)Fe(II) complex as detected by $^1$H NMR.$^{24}$ To a methanol solution of L$^1$ (50.0 mg, 0.0920 mmol) was added a methanol solution of Me$_4$NOH·5H$_2$O (33.3 mg, 0.184 mmol). The reaction was stirred for ~5 minutes, at which time a methanol solution of Zn(ClO$_4$)$_2$·6H$_2$O (34.1 mg, 0.0920 mmol) was added, followed by a methanol solution of Fe(ClO$_4$)$_3$·6H$_2$O (42.4 mg, 0.0920 mmol). The resulting mixture became dark purple and was stirred for approximately 2 minutes, at which point a methanol solution of NaOAc (15.0 mg, 0.184 mmol) was added. This mixture was stirred for 1 h and then the solvent was removed under reduced pressure. The remaining solid was dissolved in CH$_2$Cl$_2$ and solution was passed through a celite/glass wool plug. The filtrate was pumped to dryness and the remaining solid was recrystallized 3-4 times from MeOH at room temperature. FTIR (KBr, cm$^{-1}$) 3441 ($\nu_{N-H}$), 1597 ($\nu_{COO}$), 1435 ($\nu_{COO}$), 1096 ($\nu_{ClO_4}$) 764
(νClO₄). ESI/APCI-MS, m/z (relative intensity), 781.15 ([M-ClO₄]⁺, 100%). Anal calcd. for C₃₈H₃₉N₅FeZnClO₁₀·1.5H₂O: C, 50.27; H, 4.67; N, 7.72. Found: C, 50.15; H, 4.65; N, 7.63.

**Thioester Hydrolysis Monitored by ²H NMR.** *Product Identification.* A 0.75 mL solution comprised of CH₃CN (0.30 mL), buffer solution (0.36 M CHES, 0.30 mL) containing NaNO₃ (I = 0.61 M), and doubly distilled H₂O (0.15 mL) was prepared. An internal chemical shift standard (C₆D₆, 1 µL) was added and the pH of this solution was adjusted to pH = 9.0 using 0.1 M NaOH. To this solution was added a 0.15 mL solution of the hydroxyphenylthioacetic acid S-methyl(d₃) ester in CH₃CN (2.0 mg dissolved in 1.0 mL, 0.011 M). The resulting mixture was transferred to an NMR tube and the tube was placed in a Bruker ARX NMR probe which had been previously optimized for ²H data collection at 26.5°C. Over the course of >450 h, the intensity of the 1.92 resonance of the thioester –SCD₃ group decreased while a new resonance appeared at 2.12 ppm. This product was confirmed as CD₃SH by identification of its volatility (purging the solution with N₂ results in loss of the 2.12 ppm resonance) and by a positive Ellman’s reagent test, which indicates the presence of a free thiol. Similar experiments were performed with appropriate buffers at pH = 7.0, 7.5, 7.9, and 8.6. In each case, the only product was CD₃SH.

For the reaction involving the metal complex, a similar reaction mixture was prepared, with 1 (variable concentration) dissolved in the CH₃CN portion. For these reactions, as the intensity of the 1.92 ppm signal decreases, two new resonances appear at 2.18 and 2.12 ppm, respectively. The resonance at 2.18 ppm was identified as being from the disulfide CD₃SSCD₃ by comparison of the chemical shift to that of CH₃SSCH₃ in 50%
D$_2$O:CD$_3$CN (2.18 ppm). The resonance at 2.12 ppm was identified as belonging to a volatile species (e.g. CD$_3$SH) by purging the reaction mixture with N$_2$, which results in the loss of this resonance. Similar product mixtures were identified at all pH values (7.0, 7.5, 7.9, 8.6, 9.0).

**Kinetic Measurements. General procedure for background reactions involving only PhCH(OH)C(O)SCD$_3$.** Reaction mixtures were prepared in an identical manner to that outlined for the qualitative NMR experiments described above. The pH of each solution was adjusted to the desired value using 0.1 M NaOH. To this solution was added a 0.15 mL solution of the hydroxyphenylthioacetic acid S-methyl($d_3$) ester in CH$_3$CN (2.0 mg dissolved in 1.0 mL, 0.011 M). The resulting mixture was transferred to an NMR tube and the tube was placed in a Bruker ARX NMR probe which had been previously optimized for $^2$H data collection. $^2$H NMR spectra were collected at 26.5 °C as a function of time and were referenced to the chemical shift of the C$_6$D$_6$ standard ($\delta = 7.16$ ppm). The progress of the reaction was monitored by observing the loss of the –SCD$_3$ resonance for the starting thioester at 1.92 ppm. Data was collected until >80% of the thioester had undergone reaction. Data was collected for a minimum of two runs at pH 8.6 and 9.0. Background data at lower pH values was collected for only one run due to the slow nature of the hydrolysis reaction ($k_{obs} \sim 10^{-7} - 10^{-8}$ s$^{-1}$). Background observed rate constants are: pH = 7.0 ($5.7 \times 10^{-8}$ s$^{-1}$), pH = 7.5 ($9.2 \times 10^{-8}$ s$^{-1}$), pH = 7.9 ($1.5 \times 10^{-7}$ s$^{-1}$), pH = 8.6 ($1.2(1) \times 10^{-6}$), pH = 9.0 ($1.1(1) \times 10^{-6}$ s$^{-1}$).

**Kinetic Measurements. General procedure for reactions containing 1 and PhCH(OH)C(O)SCD$_3$.** A 0.75 mL solution comprised of 1 in CH$_3$CN (0.30 mL), buffer
solution (0.30 mL) containing NaNO₃ (I = 0.61 M), and doubly distilled H₂O (0.15 mL) was prepared. An internal chemical shift standard (C₆D₆, 1 µL) was then added. The pH of this solution was adjusted to the desired value using 0.1 M NaOH. To this solution was added a 0.15 mL solution of the hydroxyphenylthioacetic acid S-methyl(d₃) ester in CH₃CN (2.0 mg dissolved in 1.0 mL, 0.011 M). The resulting mixture was transferred to an NMR tube and the tube was placed in a Bruker ARX NMR probe which had been previously optimized for ²H NMR data collection. Each ²H NMR spectrum was referenced to the chemical shift of the C₆D₆ standard (δ = 7.16 ppm). Each reaction was monitored by observing the loss of the -SCD₃ resonance for the starting thioester at 1.92 ppm as a function of time until the reaction was >80% complete. As the intensity of the 1.92 ppm signal decreases, two new resonances appear at 2.18 and 2.12 ppm, respectively. The resonance at 2.18 ppm was identified as being from CD₃SSCD₃ by comparison of this chemical shift to that of CH₃SSCH₃ in 50% D₂O:CD₃CN (2.18 ppm). The resonance at 2.12 ppm was identified as belonging to a volatile species (e.g. CD₃SH) by purging the reaction mixture with N₂, which results in the loss of this resonance. The initial concentration of metal complex was varied from 9.0 x 10⁻⁴ M to 0.018 M. Data was collected for a minimum of two runs at all metal complex concentrations and pH values.

**Deconvolution of ²H NMR data for the reactions involving PhCH(OH)C(O)SCD₃.** Integrated intensities of the –SCD₃ resonances in the ²H NMR kinetic data were determined using a Lorentzian-Gaussian curve fitting program provided in the Delta NMR Software v 4.3.6 package (JEOL, Ltd). For each spectrum acquired, the chemical shift of the resonance of C₆D₆ was set to 7.16 ppm. The integrated intensity of the
HDO resonance at 3.95 ppm was set to one deuteron. The concentration of –SCD₃ labeled thioester was then determined via integration of the 1.92 ppm resonance versus the HDO integrated intensity. Pseudo first-order rate constants were determined from slopes of plots of ln[thioester] versus time. Typical correlation coefficients for these plots were ≥ 0.98.

**UV-vis Experiments.** (A) A solution of 1 (0.008 mmol) was prepared in CH₃CN (15 mL), buffer solution (10 mL) containing NaNO₃ (I = 0.61 M), and doubly distilled H₂O (5 mL). The absorption spectrum of this solution was then measured. The entire solution was then added to solid PhCH(OH)C(O)SCD₃ (0.008 mmol) and the absorption spectrum was again recorded. (B) An identical solution of 1 to that described in part (A) was prepared. This solution was then added to solid mandelic acid (0.008 mmol) and the absorption spectrum was again recorded. (C) An NMR sample of the thioester hydrolysis reaction mixture, judged complete by ²H NMR, was diluted from 1.8 x 10⁻³ M to 2.67 x 10⁻⁴ M in terms of 1, and an absorption spectrum was obtained.

**EPR Experiments.** A single turnover thioester hydrolysis reaction in the presence of 1 (2.67 x 10⁻⁴ M) in 50:50 CH₃CN:H₂O (CHES buffer, pH = 9, I = 0.61 M (NaNO₃)) was monitored by removal of aliquots for EPR experiments. Each aliquot was immediately frozen at 77 K prior to EPR data acquisition at 5(1) K.

**Results**

**Investigating Thioester Hydrolysis Promoted by an Fe(III)Zn(II) complex.**

*Rationale for the use of a deuterium-labeled thioester and ²H NMR.* We have initiated our studies using an Fe(III)Zn(II) complex (1, Figure 2-2) that has previously been shown to catalyze the hydrolysis of the activated phosphate diester 2,4-bis(dinitrophenyl)phosphate
(BDNPP) in mixed organic/aqueous solution. This complex contains pyridyl and phenolate ligation to the Fe(III) center, resulting in intense ligand based absorption features below 350 nm and a broad phenolate→Fe(III) LMCT centered at ~464 nm (pH = 9.0, $\varepsilon \sim 2500$ M$^{-1}$cm$^{-1}$). Typically, hydrolysis reactions promoted by metal complexes (e.g. Zn-OH complexes) are investigated using an activated $p$-nitrolabeled substrate and UV-vis spectroscopy as the monitoring method. This approach is not feasible for investigating thioester hydrolysis reactions promoted by 1 for several reasons. First, the absorption maximum of the $p$-nitrothiophenolate leaving group of NO$_2$-C$_6$H$_4$-SC(O)CH$_3$ ($\lambda_{\text{max}} = 410$ nm) overlaps to some extent with the LMCT of 1. A more important issue is the low $pK_a$ of the $p$-nitrothiophenolate leaving group ($pK_a = 4.5$), which means that thioester bond scission in buffered aqueous solution at pH $\geq 7$ at ambient temperature is fast relative to that of the oxygen analog ($p$-nitrophenolate, $pK_a = 7.15$) in the absence of any metal complex. The presence of a methylene unit in the thioester NO$_2$-C$_6$H$_4$-CH$_2$SC(O)CH$_3$ gives a leaving group with a higher $pK_a$ value (8.87), which therefore should produce a slower thioester hydrolysis reaction. However, the absorption maximum for the $p$-nitrobenzylthiolate leaving group is at 360 nm, which is within a region that also contains intense absorption features for the Fe(III)Zn(II) complex 1. Additional methods that have previously been employed in studying thioester hydrolysis reactions in enzymatic systems also are not feasible for reactions involving 1. For example, for kinetic studies involving glyoxalase II enzymes, thioester hydrolysis is monitored using the substrate S-$D$-lactoylglutathione, which is converted to reduced glutathione and $D$-lactic acid. The rate of hydrolysis of this substrate is followed by measuring the loss in absorbance of the substrate
at 240 nm ($\varepsilon = 3100 \text{ M}^{-1}\text{cm}^{-1}$). For thioesterase enzymes, reactions are typically monitored by continuous assays based on the use of thiol derivatizing agents (e.g. Ellman’s reagent, 5,5’-dithio-bis(2-nitrobenzoic acid) DTNB) that yield colored products ($\lambda_{\text{max}} = 412$ nm). In considering thioester hydrolysis model reactions involving 1, both of these approaches used in enzymatic systems would be problematic due to overlap of the product absorption feature with absorption bands of the metal complex.

In the experiments outlined herein we have used a deuterium-labeled thioester PhCH(OH)C(O)SCD$_3$ and $^2$H NMR to examine thioester hydrolysis in the absence and presence of 1 (Scheme 2-1). The use of this particular thioester is especially relevant to the chemistry of glyoxalase II enzymes as PhCH(OH)C(O)SCD$_3$ contains an $\alpha$-hydroxy group and alkyl thiol leaving group (CD$_3$SH), both of which are found in glyoxalase II substrates (Figure 2-1). The hydrolysis products of this reaction in the pH range examined (7.0-9.0) should be mandelate anion ($pK_a = 3.85$) and CD$_3$S'/CD$_3$SH (CH$_3$SH, $pK_a = 9.7$). As outlined below, in addition to the production of CD$_3$SH we have identified the formation of the disulfide CD$_3$SSCD$_3$ in reaction mixtures involving the Fe(III)Zn(II) complex 1.

![Scheme 2-1](image)

**Scheme 2-1.** General reaction parameters for thioester hydrolysis using $^2$H NMR.

**Product Identification in Thioester Hydrolysis Reactions Involving PhCH(OH)C(O)SCD$_3$.** Mild heating of a solution of PhCH(OH)C(O)SCD$_3$ in 50:50
CH₃CN:H₂O (CHES buffer, I = 0.61 M) at pH = 9.0 at 26.5 °C results in the gradual disappearance of the \(-\text{SCD}_3\) resonance of the starting thioester at 1.92 ppm and the appearance of a new resonance at 2.12 ppm (Figure 2-3). The new species produced is volatile, as purging of the reaction mixture with N₂ at room temperature results in loss of this 2.12 ppm resonance. Based on the volatility of this product, and a positive Ellman’s reagent test for free thiol at the conclusion of the reaction, we formulate this product as CD₃SH. As can be seen in Figure 2-3, the thioester hydrolysis reaction involving PhCH(OH)C(O)SCD₃ requires >400 h to reach greater than 80% completion. The formation of mandelate as the other product in the thioester hydrolysis reaction mixture is proposed based on stoichiometry and our previous study of thioester hydrolysis involving zinc complexes using the same substrate wherein the formation of mandelate was identified using spectroscopic and analytical approaches.¹⁵ Performing the thioester hydrolysis reaction in the presence of 1 (2.5 eq relative to thioester) under identical conditions gives \(^2\)H NMR spectra containing two product resonances (2.18 and 2.12 ppm; Figure 2-4). The species at 2.18 ppm was identified as the disulfide D₃CSSCD₃ via comparison of the chemical shift of this resonance with that of H₃CSSCH₃ in 50:50 CD₃CN:D₂O. This disulfide product should be generated from CD₃S\(^-\), as alkylthiolates are known to undergo catalytic oxidation in the presence of O₂ and Fe(III).³¹ For example, Fe(III)-EDTA in the presence of O₂ has been shown to catalyze the oxidative coupling of \(n\)-butyl thiol to give the disulfide in 93% yield in aqueous methanol (pH = 9.5). Overall, the thioester hydrolysis
reaction in the presence of 0.5 eq of 1 relative to the thioester, the hydrolysis reaction was found to proceed to completion, indicating catalytic turnover occurs under the reaction conditions. Thus, pseudo first-order conditions are present even at low concentrations of metal complex.
Kinetic Studies and Effect of pH. The thioester hydrolysis reaction was monitored as a function of the concentration of the metal complex (0-10 eq relative to thioester) at pH = 7.0-9.0. The disappearance of the 1.92 ppm resonance of the thioester was monitored as a function of time. As can be seen in Figures 2-3 and 2-4, the $^2$H NMR spectra involve peak widths for the $–$SCD$_3$ resonances of the starting thioester and resulting products (pwhh ~4 Hz) that are broad relative to standard $^1$H NMR resonances. These broad peak widths required the use of curve-fitting to deconvolute peak overlap and determine accurate integration values for the 1.92 ppm thioester resonance. Fitting of the
loss of the thioester versus time to a single exponential equation yielded pseudo first-order rate constants for each reaction. In the absence of 1, the pseudo first-order rate constant for the thioester hydrolysis reaction at pH = 9.0 is 1.1(1) x 10^{-6} s^{-1}. In the presence of 2.5 equivalents of 1, the pseudo first-order rate constant is 2.3(2) x 10^{-4} s^{-1}, indicating a >200-fold rate enhancement. Rate constants for other pH values in the presence of 2.5 equivalents of 1 are given in Table 2-1. For all pH values, a rate enhancement of at least 140-fold was identified relative to the background hydrolysis reaction. As shown in Figure 2-5, at all pH values plotting of the first-order rate constant versus [1] yielded a saturation curve wherein for low concentrations of 1 the rate of the reaction initially increased linearly. However, at higher concentrations of metal complex, the rate becomes independent of [1] indicating a rate-determining step that is more complicated than a single step. This kinetic behavior is consistent with a mechanism wherein an equilibrium step (K_1) precedes an irreversible step (k_2). Fitting of the pH = 9.0 data to eq. 1 yielded K_1 = 42(24) and k_2 = 1.8(6) x 10^{-3} s^{-1}. The K_1 value indicates a weak interaction of the thioester with the metal complex. The pH dependence on the rate of thioester hydrolysis for reactions containing 2.5 equivalents of 1 is shown in Figure 2-6. The data is fit to eq. 4 derived for a monoprotic system (Figure 2-7) and yields a kinetic pK_a value of 8.8. This value is similar to the thermodynamic pK_a (8.3) previously report by Neves, et al. for the Zn-OH_2 moiety in the solution form of [(BPBPMP)Fe(III)(H_2O)(µ-OH)Zn(II)](ClO_4)_2 (2), indicating that the maximum rate is obtained upon formation of a Zn-OH unit. We note that data cannot be collected at higher pH values using the ^2H NMR approach as the reaction proceeds too rapidly at 26.5 °C.
Table 2-1. Observed rate constants for thioester hydrolysis promoted by 1.\(^a\)

<table>
<thead>
<tr>
<th>pH</th>
<th>(k_{\text{obs}}) ((\text{s}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.0</td>
<td>(1.10(2) \times 10^{-5})</td>
</tr>
<tr>
<td>7.5</td>
<td>(1.69(6) \times 10^{-5})</td>
</tr>
<tr>
<td>7.9</td>
<td>(2.49(26) \times 10^{-5})</td>
</tr>
<tr>
<td>8.6</td>
<td>(1.63(9) \times 10^{-4})</td>
</tr>
<tr>
<td>9.0</td>
<td>(2.31(20) \times 10^{-4})</td>
</tr>
</tbody>
</table>

\(^a\)Conditions: 50:50 \(\text{CH}_3\text{CN}:\text{H}_2\text{O}\), 0.36 M MOPS or CHES buffer, \(I = 0.61\ \text{M (NaNO}_3\text{)}, [\text{PhCHC(O)SCD}_3]= 1.8 \times 10^{-3}\ \text{M}

Figure 2-5. Plot of [I] versus \(k_{\text{obs}}\) for the thioester hydrolysis reaction. Conditions: 50:50 \(\text{CH}_3\text{CN}:\text{H}_2\text{O}\), pH = 7.0-9.0 (0.36 M MOPS or CHES buffer), \(I = 0.61\ \text{M (NaNO}_3\text{)}, [\text{PhCHC(O)SCD}_3]= 1.8 \times 10^{-3}\ \text{M}, 26.5 \degree\text{C.}
Figure 2-6. Dependence of $k_{obs}$ on the pH of the reaction mixture. Conditions: 50:50 CH$_3$CN:H$_2$O, [buffer] = (0.36 M MOPS or CHES), $I = 0.61$ M (NaNO$_3$), [PhCHC(O)SCD$_3$] = $1.8 \times 10^{-3}$ M, [1] = $4.5 \times 10^{-3}$ M, 26.5 °C.

$$k_{obs} = \frac{k_2 K_1 [1]}{1 + K_1 [1]} \quad (1)$$

![Graph showing the dependence of $k_{obs}$ on pH](image)

Figure 2-7. Equations describing the pH dependence of the thioester hydrolysis reaction involving 1.

$$([\text{BPBMP}][\text{Fe(III)}(\mu-\text{OH})\text{Zn(II)}(\text{H}_2\text{O})]^+) \xrightarrow{K_a}$$

$$[([\text{BPBMP}][\text{Fe(III)}(\text{OH})](\mu-\text{OH})\text{Zn(II)}(\text{OH})) + \text{H}^+] \quad (2)$$

$$([\text{BPBMP}][\text{Fe(III)}(\text{OH})(\mu-\text{OH})\text{Zn(II)}(\text{OH})]) + \text{PhCH(OH)C(O)SCD}_3 \xrightarrow{k_{2\max}^\text{max}} \text{thioester hydrolysis products} \quad (3)$$

$$k_{obs} = \frac{k_{2\max}^\text{max} K_a}{K_a + [\text{H}^+]} \quad (4)$$
Spectroscopic Investigations of the Thioester Hydrolysis Reaction. In order to gain further insight into the reaction pathway of thioester hydrolysis promoted by 1, a single turnover reaction was monitored by UV-vis and EPR.

UV-vis Experiments. A solution of 1 (1.8 x 10^{-3} M) identical to that used in the kinetic studies at pH = 9.0 and containing one equivalent of thioester was prepared and immediately diluted using CH$_3$CN:H$_2$O (0.36 M CHES buffer, I = 0.61 M NaNO$_3$) to [1] = 2.67 x 10^{-4} M for UV-vis spectral analysis. The features of the spectrum in the region of 300-850 nm are identical to that found for 1 in the absence of thioester under identical conditions (Figure 2-8). Of particular importance, no change is seen in the position or intensity of the terminal phenolate-to-Fe(III) LMCT at ~464 nm, which indicates that the thioester does not interact with the Fe(III) center.

The absorption spectrum obtained at the conclusion of the single turnover thioester hydrolysis reaction differs from the starting reaction mixture, with an increase in intensity at ~400 nm and the phenolate–to-Fe(III) LMCT being less distinct. To evaluate whether the mandelate product interacts with the metal complex, a solution of 1 (2.67 x 10^{-4} M in CHES buffer (pH = 9), I = 0.61 M (NaNO$_3$)) was prepared containing one equivalent of mandelic acid. This produces an absorption maximum at ~440 nm, which is somewhat similar to that found for the solution of the reaction products. As disulfide is generated in the thioester hydrolysis reaction mixture, which suggests that some reduction of Fe(III) to Fe(II) could be occurring, we also examined the absorption spectra of analytically pure 1 in the presence of equimolar amounts of sodium dithionite and mandelic acid. This produced a shift in the absorption maximum similar to the solution containing mandelic acid, but
with a slightly lower intensity. Overall, the UV-vis experiments suggest that changes are occurring at the Fe(III) center over the course of the reaction, with coordination of mandelate being a possibility.

The other product of the thioester hydrolysis reaction, CD$_3$S$^-$, may also coordinate to the metal complex. For this reason, we introduced one equivalent of NaSCH$_3$ into a solution of 1 in CH$_3$CN:H$_2$O (2.67 x 10$^{-4}$ M in CHES buffer (pH = 9.0), $I = 0.61$ M (NaNO$_3$)). This produced no shift in the absorption maximum of the complex. Addition of another 6 equivalents of NaSCH$_3$ also produced no change in the absorption spectrum. $^2$H NMR analysis of a solution of 1 and one equivalent of NaSCD$_3$ in CH$_3$CN:CHES buffer at pH = 9.0 ($I = 0.61$ M) revealed the formation of CD$_3$SH. Thus, free thiolate is rapidly converted to thiol in the reaction mixture.
**EPR experiments.** A reaction mixture was prepared as outlined for the $^2$H NMR kinetic studies. This mixture was then immediately diluted to $[\text{I}] = 2.67 \times 10^{-4}$ M using CH$_3$CN:H$_2$O (0.36 M CHES buffer, $I = 0.61$ M NaNO$_3$). Using UV-vis to evaluate reaction progress (at this concentration the single turnover reaction takes $\sim 120$ h to go to completion), aliquots were removed at specified intervals and frozen at 77 K. EPR spectra were then collected at 5(1) K. Selected spectra are shown in Figure 2-9. Upon introduction of the thioester (90 sec) a spectral change is identifiable wherein the intensity of the $g = 9$ and $g = 4.3$ features increased, whereas a decrease in intensity is found for the feature at $g = 6.6$. We note that it has been previously reported that the addition of 2,4-BDNPP to $[(\text{BPBPMP})\text{Fe(III)}(\text{H}_2\text{O})(\mu-\text{OH})\text{Zn(II)}](\text{ClO}_4)^2$ (2) in acetonitrile:HEPES buffer at pH = 7.0 produces an increase in the $g = 9$ and $g = 4.3$ features similar to that identified here.$^{2f}$

The data for the phosphate diester 2,4-BDNPP was interpreted as indicating that the phosphate ester monoanion did not directly interact with the Fe(III) center, and instead coordinated in a monodentate fashion to the Zn(II) center. One possible explanation for the initial EPR features of both systems is that in the absence of a substrate molecule there is a portion of the compound that exists as a dimer ([Fe(III)Zn(II)]$_2$ with $J > \nu$ for Fe(III)...Fe(III) coupling) and is EPR silent and a portion that is a monomer ([Fe(III)Zn(II)]) which gives an EPR signal. Upon reaction with the thioester or phosphate diester the proportion of the monomeric species is enhanced. Alternatively, the changes in the spectra upon addition of thioester could be due to changes in the zero field splitting parameters (i.e. $D$) for the $S = 5/2$ Fe(III) center.$^{32}$ Overall, the similarity of the EPR
Figure 2-9. EPR spectra obtained at various intervals in the single turnover thioester hydrolysis reaction promoted by 1. All spectra were recorded at 5(1) K using 1.0 mW microwave power, 100 kHz modulation frequency, 0.814 mT field modulation amplitude, and a 20.3 mT/s sweep rate.

Spectral changes for the thioester and phosphate diester reactions suggests similar interactions with the metal complex.

In the thioester hydrolysis reaction a second intermediate maximizes in intensity at 49 h (Figure 2-9). This species is characterized by a feature at ~850 G (g ~8), with a residual feature still present at g ~9. Overall, the EPR spectrum did not cleanly convert
Figure 2-10. EPR spectrum of 1 in the presence of one equivalent of mandelic acid. The spectrum was recorded at 5(1) K using 1.0 mW microwave power, 100 kHz modulation frequency, 0.814 mT field modulation amplitude, and a 20.3 mT/s sweep rate.

back to exactly that of the starting complex after 168 h (g ~8 feature still present). In addition, the g = 4.3 signal in the final spectrum is less intense and broadened compared to that of the starting material. This may be due to the reduction of a portion of the Fe(III) to Fe(II) over the course of the reaction.

An EPR spectrum of analytically pure 1 in the presence of one equivalent of mandelic acid ([1] = 2.67 x 10^{-4} M using 50:50 CH₃CN:H₂O (0.36 M CHES buffer, I = 0.61 M NaNO₃)) revealed a spectrum with g ~6.4, 4.3, and 2.0 (Figure 2-10). The distinctly different nature of this spectrum versus that of 1 under identical conditions (Figure 2-9) indicates that the mandelate anion interacts with the Fe(III)Zn(II) complex in the absence of thioester or its hydrolysis products. However, for the single turnover thioester hydrolysis reaction mixture, a spectrum containing a significant g ~6.4 feature is not produced at any
time interval (Figure 2-9). Importantly, this indicates that following thioester hydrolysis the coordination properties (e.g. available coordination sites) of the Fe(III)Zn(II) complex have been altered. We hypothesize that this is due to coordination of \( \text{CD}_3\text{S}^- \), or structural changes resulting from a Fe(III)-SCD\(_3\) interaction. When produced near the Fe(III) center, the thiolate anion could be stabilized via metal coordination. As noted herein, formation of a Fe(III)-SCD\(_3\) adduct is a logical precursor to the observed formation of the disulfide D\(_3\)CSSCD\(_3\).

**Proposed Mechanism.** Based on literature precedent\(^{2e,2f}\) and the kinetic and mechanistic data presented above, we propose a mechanism for thioester hydrolysis promoted by 1 as outlined in Scheme 2-2. Saturation kinetic behavior is found for the thioester hydrolysis reaction at pH = 7.0-9.0, with maximum rate being produced above the pK\(_a\) of the Zn-OH\(_2\) moiety (pH > 8.3).\(^{2e}\) Saturation kinetic behavior was also found for the phosphate diester hydrolysis reaction promoted by 1, which occurs with a pH optimum of 6.5. The lack of change in the UV-vis and EPR spectral features found upon the introduction of thioester to 1 at pH = 9.0 is similar to the data reported for the introduction of phosphate diester to 1 at pH = 7.0.\(^{2e}\) This suggests that for both types of substrates (thioester or phosphate diester monoanion) similar chemistry is occurring, albeit at different pH values. On this basis, we propose that at pH = 9.0 ligand exchange occurs at the zinc center via deprotonation of the \( \alpha \)-hydroxy group of the thioester to give an equilibrium amount of a zinc alkoxide species.\(^{33}\) Formation of such an alkoxide adduct is proposed based in part on the fact that at pH = 9.0 no coordination position is available on
the Zn(II) center in the absence of reaction with the Zn(II)-OH moiety. Importantly, an alkoxide type structure is also proposed to form in the borate-catalyzed hydrolysis of an α-hydroxy thioester (Scheme 2-3), wherein a borate-substrate complex is formed followed by intramolecular attack of a boron-coordinated OH\(^{-}\) to form a tetrahedral transition state.\(^{34}\) For the Fe(III)Zn(II) complex, attack of the terminal Fe(III)-OH moiety on the thioester carbonyl could lead to the formation of a tetrahedral transition state from which the \(–\text{SCD}_3\)
Scheme 2-3. Proposed mechanism for borate-catalyzed α-hydroxythioester hydrolysis.\textsuperscript{34}

leaving group could be stabilized via coordination to the Fe(III). The formation of a Fe(III)-SCD\textsubscript{3} unit can serve as the precursor for the observed formation of the disulfide D\textsubscript{3}CSSCD\textsubscript{3}. Specifically, the proposed mechanistic pathway for disulfide formation catalyzed by Fe(III) complexes involves Fe(III)-SR ligation prior to a redox process that results in the formation of Fe(II) and RS\textsuperscript{-}.\textsuperscript{31} Coupling of two RS\textsuperscript{-} species gives the disulfide product, and oxidation of the Fe(II) to Fe(III) via reaction with O\textsubscript{2} could regenerate \textbf{1}. Alternatively, the Fe(III)-SR species may undergo protonation and ligand displacement to give CD\textsubscript{3}SH (pK\textsubscript{a} ~ 9.7) and \textbf{1}. Based on independent EPR experiments, the mandelate anion does not interact with the Fe(III)Zn(II) complex following thioester hydrolysis. We hypothesize that the coordination properties of the Fe(III)Zn(II) complex are affected by the formation of the Fe(III)-SCD\textsubscript{3} entity. However, the observed turnover, as reaction mixtures containing 0.5 eq of \textbf{1} promote thioester cleavage in >80% yield, is consistent
with regeneration of at least a portion of the Fe(III)-OH moiety either by protonation and release of CD$_3$SH, or oxidation of an Fe(II)Zn(II) complex by O$_2$.

**Discussion**

The glyoxalase pathway, by mediating the chemical modification of methylglyoxal, is directly involved in preventing the biosynthesis of advanced glycation end-products (AGEs), which are associated with aging and disease mechanisms. Both enzymes of the glyoxalase pathway (Figure 2-1) are metalloenzymes, with glyoxalase I containing either Zn(II) or Ni(II) depending on the source, and glyoxalase II containing a variety of bimetallic combinations as described herein. Despite the health relevance of this pathway, very few studies involving synthetic complexes of relevance to the glyoxalase pathway have been reported.

The active site of the Fe(III)Zn(II) form of glyoxalase II has similarity to the active site of purple acid phosphatase (PAP). Specifically, they have in common a divalent metal binding site comprised of three neutral donors, and a second metal binding site with one terminal carboxylate ligand. In both glyoxalase II and PAP the metal centers are bridged by a carboxylate and a hydroxide ligand. Due to this structural similarity, [(BPBPMP)Fe(III)Zn(II)(µ-OAc)$_2$ClO$_4$](1), which has been used in model studies of PAP enzymes, is an appropriate starting point for an investigation of thioester hydrolysis reactivity.

At pH values below the pK$_a$ of the Zn-OH$_2$ in 1, the observed saturation kinetic behavior (pH = 7.0, 7.5, 7.9; Figure 2-5) is consistent with a reaction pathway that involves
Scheme 2-4. Proposed mechanism for thioester hydrolysis promoted by 1 at pH values below the pKₐ of the Zn-OH₂ moiety.

Coordination of the thioester carbonyl moiety to the Zn(II) center via displacement of water (Scheme 2-4). Attack of the Fe(III)-OH on the thioester could then lead to a tetrahedral transition state and subsequent product formation. This reaction pathway is very similar to that proposed by Zang et al. for the Fe(III)Zn(II) containing form of the cytoplasmic glyoxalase II from Arabidopsis. In this mechanism, Zang and coworkers suggested that the (His)₃-ligated metal site (M1, Figure 2-1) activates the metal carbonyl, with the M2 site (Figure 2-1) being the source of a terminal hydroxide nucleophile and also assists in
stabilizing the transition state and thiolate leaving group. Our results support the notion that the Fe(III) site (M2 in the Fe(III)Zn(II) form) could provide the nucleophilic hydroxide and stabilize the thiolate leaving group in the thioester hydrolysis reaction. Reaction mechanisms proposed for the dizinc form of human glyoxalase II based on the X-ray structure\textsuperscript{13a} and computational studies\textsuperscript{38} suggest that a bridging hydroxide can serve as the nucleophile to attack the thioester, with the M2 site serving to stabilize the transition state and the thiolate leaving group.\textsuperscript{38}

We propose that the increased rate found above the p\textsubscript{K\textsubscript{a}} of the Zn-OH\textsubscript{2} moiety in reactions involving I is associated with anionic coordination of the α-hydroxy thioester to the Zn(II) center. The reactivity of Zn-OH species with alcohols has been previously investigated.\textsuperscript{39} Using mononuclear zinc complexes supported by a hydrophobic hydrotris(pyrazolyl)borate ligand, Parkin and coworkers found that more acidic alcohol hydroxyl groups produce equilibria that lie further toward the Zn-OR species. For example, p-methoxyphenol (p\textsubscript{K\textsubscript{a}} = 10.2)\textsuperscript{40} produces an equilibrium that lies toward the Zn-OR product ($K = 4.2(9)$ at 300 K), whereas aliphatic alcohols (p\textsubscript{K\textsubscript{a}} > 15) produce equilibria that lie significantly toward the reactants ($K = 10^{-3}$-$10^{-5}$ at 300 K). The hydroxyl group of PhCH(OH)C(O)SCD\textsubscript{3} should have a lower p\textsubscript{K\textsubscript{a}} than benzyl alcohol (p\textsubscript{K\textsubscript{a}} = 15) due to the presence of the electron withdrawing thioester functionality. The $K_1$ values determined from fitting of the saturation curves at pH = 7.0 – 9.0 (Figure 2-5) have large error bars (e.g. $K_1 = 42(24)$ at pH = 9.0) which precludes a detailed interpretation. However, the similarity of this proposed pathway to that outlined for borate-promoted thioester hydrolysis lends additional support to the proposed Zn-OR formation.
With regard to possible Fe(III)-SCD<sub>3</sub> interactions, for all of the pH values examined for the thioester hydrolysis reaction promoted by 1, the disulfide D<sub>3</sub>CSSCD<sub>3</sub> is produced in the reaction mixture. This suggests the formation of a Fe(III)-SCD<sub>3</sub> species that can undergo redox reactivity. A decrease in the intensity of the \( g = 4.3 \) signal observed at pH = 9.0 (Figure 2-9) suggests the possible reduction of a portion of the Fe(III) to Fe(II) during the course of the single turnover reaction,\(^{41}\) albeit this change in intensity may also indicate a perturbation in the electronic structure of the iron center, as the intensity of the \( g = 4.3 \) line is sensitive to the precise value of the axial ZFS parameter (\( D \)). We propose that the formation of the Fe(III)-SCD<sub>3</sub> species provides a rationale for why mandelate anion does not coordinate to the Fe(III)Zn(II) complex following thioester hydrolysis. Our control experiments indicate that free CD<sub>3</sub>S<sup>-</sup> anion in the reaction mixture at pH = 9.0 is quickly protonated to produce CD<sub>3</sub>SH. However, during the thioester hydrolysis reaction, we propose that CD<sub>3</sub>S<sup>-</sup> is generated in close proximity to the Fe(III) center thus enabling the formation of Fe(III)-SCD<sub>3</sub>, which subsequently undergoes either redox to produce CD<sub>3</sub>SSCD<sub>3</sub>, or protonation to release CD<sub>3</sub>SH.

**Conclusions and Prospects**

We have demonstrated that the Fe(III)Zn(II) complex 1 promotes thioester hydrolysis in a pH dependent reaction that achieves maximal rate above the pKa of the Zn-OH<sub>2</sub> moiety. Formation of a novel zinc alkoxide species via deprotonation of the \( \alpha \)-hydroxythioester is proposed at pH values wherein the maximum rate of thioester hydrolysis is obtained. Products generated in the reaction include both a thiol and a
disulfide, the latter of which indicates the involvement of redox activity that is likely derived from a Fe(III)-SCD$_3$ species.

The studies outlined herein have particular relevance to glyoxalase II enzymatic reactions, as the thioester substrate employed contains a $\alpha$-hydroxy group and an aliphatic leaving group (-SCD$_3$) akin to the aliphatic glutathione-containing substrate hydrolyzed by glyoxalase II enzymes. In this regard, this model study is rare in terms of the use of a substrate that is not an activated nitro-substituted compound.$^{25}$

This initial study sets the stage for follow up investigations using a similar approach (-SCD$_3$ labeled thioester and $^2$H NMR as a monitoring tool) to examine the thioester hydrolysis reactivity of complexes having other binuclear metal combinations, or mononuclear zinc complexes. With regard to the latter, Crowder, et al. recently reported that human glyoxalase II contains an Fe(II)Zn(II) center but that it is active as a mononuclear Zn(II) enzyme.$^{3a}$ The monozinc form would have (His)$_3$ ligation for the Zn(II) center (occupying the M1 site) and thus may have multiple solvent-occupied coordination positions for possible nucleophile and/or thioester substrate coordination. While activated ester hydrolysis reactivity involving mononuclear zinc complexes has been extensively investigated,$^{25}$ thioester reactions have been only minimally investigated to date.$^{16}$ Overall, our novel approach of using a deuterium-labeled aliphatic thioester and $^2$H NMR to monitor hydrolysis reactions opens a window of opportunity into studies of metal complex-promoted thioester hydrolysis reactions of relevance to glyoxalase II enzymes. Understanding the mechanism of catalysis of glyoxalase II enzymes is an important goal as
the glyoxalase pathway is being actively investigated with regard to its role in the clinical complications of diabetes, Alzheimer’s disease, oxidative stress, and cancer.42

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CHAPTER 3

THIOESTER HYDROLYSIS PROMOTED BY A MONONUCLEAR ZINC COMPLEX

Abstract

The mononuclear zinc complex [(bpta)Zn](ClO₄)₂·0.5H₂O promotes the hydrolysis of the thioester PhCH(OH)C(O)SCD₃ when dissolved in CH₃CN:H₂O (50:50 buffered at pH = 9.0). This reaction results in the formation of a mixture of CD₃SH and a zinc thiolate complex, the latter of which can be protonated to generate additional CD₃SH. Kinetic studies revealed an overall second-order reaction with an activation energy that is similar to that found for aqueous OH⁻ promoted thioester hydrolysis. These studies represent the first investigation of chemistry relevant to that occurring in the monozinc-containing form of human glyoxalase II.

Introduction

The glyoxalase pathway is ubiquitous in biological systems and involves two metalloenzymes, glyoxalase I (GlxI) and glyoxalase II (GlxII), with glutathione as an essential cofactor.¹ GlxI catalyzes the isomerization of a hemithioacetal of methyl glyoxal to a thioester, and GlxII then catalyzes the hydrolysis of the thioester to produce non-toxic products. The glyoxalase pathway is involved in cellular detoxification and is important in preventing the formation of advanced glycation end products, which are linked to aging and various diseases.² Because of its critical role in cellular detoxification, the glyoxalase system is under investigation as a possible antitumor and antibacterial target.³

¹ Coauthored by James J. Danford, Atta M. Arif, and Lisa M. Berreau.
Crowder and coworkers recently reported that human glyoxalase II contains an Fe(II)Zn(II) center, but is catalytically active as a mononuclear zinc enzyme.\textsuperscript{4} This is similar to the reactivity found for some metallo-\(\beta\)-lactamases, which catalyze the hydrolytic ring-opening of \(\beta\)-lactam antibiotics.\textsuperscript{5} In the monozinc form of both enzymes, a (His)\textsubscript{3}Zn site catalyzes the hydrolysis of the substrate. While the reactivity of a mononuclear Zn-OH complex with the \(\beta\)-lactam-containing nitrocefin has been investigated,\textsuperscript{6} there are no reports in the literature of detailed studies of a thioester hydrolysis reaction involving a mononuclear zinc complex. Such studies would have relevance to human glyoxalase II. Additionally, these investigations have relevance to a recently reported Zn-OH-promoted hydrolysis of the thioester compound thiocoumarin within the active site of carbonic anhydrase (CA). This reaction results in the formation of a ring-opened product that is a nanomolar inhibitor of three CA isozymes.\textsuperscript{7}

**Experimental**

**General Synthetic Methods.** All reagents and solvents were obtained from commercial sources and were used as received unless otherwise noted. The ligand \(N,N\)-bis(2-pyrindylmethyl)-tert-butylamine (bpta) was prepared as previously described with minor modifications.\textsuperscript{8} The model thioester substrate hydroxyphenyl-thioacetic acid-S-\textsubscript{d\textsubscript{3}}-methyl ester (PhCH(OH)C(O)SC\textsubscript{d\textsubscript{3}}) was prepared according to literature procedures.\textsuperscript{9}

**Physical Methods.** \(^1\text{H}\) and \(^{13}\text{C}\{^1\text{H}\}\) NMR spectra were recorded on a JEOL ECX300 or a Bruker ARX400 spectrometer. Chemical shifts (in ppm) are referenced to the residual solvent peaks(s) in CD\textsubscript{3}CN (\(^1\text{H}\): 1.94 (quintet); \(^{13}\text{C}\{^1\text{H}\} 1.39\) (heptet)). \(^2\text{H}\) NMR spectra for characterization purposes were recorded on a Bruker ARX400 at 61.4 MHz as
previously described¹⁰ and were referenced to an internal standard of C₆D₆ (²H: 7.16 (singlet)). IR spectra were recorded on a Shimadzu FTIR-8400 spectrometer as KBr pellets. MALDI mass spectra were obtained from the University of California, Riverside. Elemental analysis was performed by Atlantic Microlabs of Norcross, GA.

**Caution!** Perchlorate salts of metal complexes with organic ligands are potentially explosive. Only small amounts of material should be prepared and these should be handled with great care.¹¹

\[
[(\text{bpta})\text{Zn}]\text{(ClO}_4\text{)}_2\cdot0.5\text{H}_2\text{O} \ (1)
\]

To a methanol solution (~1 mL) of bpta (18 mg, 0.071 mmol) was added a methanol solution (~1 mL) of Zn(ClO₄)₂·6H₂O (26 mg, 0.070 mmol). The resulting clear, colorless solution was stirred at room temperature for ~1 hour. To this solution was added excess Et₂O. The resulting solution was cooled to 4 °C for 24 hours. The clear solid that had deposited was dried and then recrystallized from CH₃CN via Et₂O diffusion. This yielded clear, colorless plates of 1 suitable for single X-ray diffraction analysis (22 mg, 60%). Anal Calc. for C₁₆H₂₂N₃O₈.5Cl₂Zn: C, 36.50; H, 4.22; N, 7.99. Found: C, 36.32; H, 4.30; N, 8.01. ¹H NMR (CD₃CN, 300 MHz) δ 8.73-8.71 (d, J = 5.4 Hz, 2H), 8.11-8.09 (td, J₁ = 1.7 Hz, J₂ = 7.5 Hz, 2H), 7.66-7.61 (t, J = 6.3 Hz, 2H), 7.58-7.55 (d, J = 8.3 Hz, 2H), 4.57-4.51 (d, J = 17.2 Hz, 2H), 3.94-3.88 (d, J = 17.5 Hz, 2H), 1.13 (s, 9H); ¹³C{¹H} NMR (CD₃CN, 100 MHz): δ 158.0, 148.0, 142.6, 125.7, 124.8, 61.5, 55.9, 26.3 (8 signals expected and observed); FTIR (KBr, cm⁻¹): 3440 (br, vO-H), 1620, 1103 (vClO₄), 633 (vClO₄). MALDI-MS (MeOH) [m/z (relative intensity)]: 418 ([M-H₂O-ClO₄])⁺.

**X-ray Crystallography.** A crystal of 1·2CH₃CN·0.5H₂O was mounted on a glass fiber using a viscous oil. The sample was then transferred to a Nonius KappaCCD
diffractometer with Mo Kα radiation (\(\lambda = 0.71073\ \text{Å}\)) for data collection at 150(1) K. An initial set of cell constants was obtained from ten frames of data that were collected with an oscillation range of 1 deg/frame and an exposure time of 20 sec/frame. Indexing and unit cell refinement based on all observed reflections from those ten frames indicated an orthorhombic \(P\) lattice. Final cell constants were determined from a set of strong reflections from the actual data collection. These reflections were indexed, integrated, and corrected for Lorentz, polarization, and absorption effects using DENZO-SMN and SCALEPAC.\(^{12}\) The structure was solved by a combination of direct methods and heavy atom using SIR 97.\(^{13}\) All of the non-hydrogen atoms were refined with anisotropic displacement coefficients. The hydrogen atoms of the coordinated water molecule were located and refined independently. All other hydrogen atoms were assigned isotropic displacement coefficients \(U(H) = 1.2U(C)\) or \(1.5U(C_{\text{methyl}})\), and their coordinates were allowed to ride on their respective carbons using SHELXL97.\(^{14}\) Three oxygen atoms of one perchlorate anion are each disordered over two positions (0.64:0.36).

**Product Identification in Reactions of 1 with PhCH(OH)C(O)SCD\(_3\).** Solutions comprised of acetonitrile (0.30 mL), CHES buffer (0.36 M, 0.30 mL), distilled water (0.15 mL), and 1 (0.0019-0.027 M) were prepared. To each solution was added hydroxyphenyl-thioacetic acid S-\(d_3\)-methyl ester (0.15 mL of a 0.011 M solution). Each reaction was monitored by \(^2\)H NMR until the thioester was completely consumed. In reaction mixtures having [1]:thioester ratios of >5:1, primarily one product with a –SCD\(_3\) resonance at 1.77 ppm was generated. For reaction mixtures having lower [1]:thioester ratios (1.05:1 and lower), two products were identified, having chemical shifts of 2.12 and 1.77 ppm,
respectively. The species at 2.12 is CD₃SH as previously determined. The 1.77 ppm resonance has been assigned to the formation of a zinc thiolate complex, with a proposed formulation of \([(\text{bpta})\text{Zn-SCD₃(Sol)}ₙ]X\) (Sol = CH₃CN or H₂O). The formation of a zinc thiolate species is supported by the fact that reducing the pH of the final reaction mixture wherein this species predominates results in the production of CD₃SH. For example, treatment of a final reaction mixture derived from an initial [I]:thioester ratio of 10:1 with HNO₃ to lower the pH to 8.6 resulted in the formation of CD₃SH, which was identified by $^2$H NMR. Lowering the pH further to 8.3 results in the production of additional CD₃SH. Alternatively, treatment of a similar reaction mixture with excess CH₃I results in the formation of \([(\text{CD₃})\text{S(CH₃)}₂]X\) (\(^2\)H NMR 2.59 ppm; X is possibly NO₃⁻, ClO₄⁻, I⁻ or another anion present in the mixed aqueous:organic reaction mixture). To verify the identity of this proposed sulfonium salt, CD₃SCD₃ was treated with excess CH₃I under identical reaction conditions to those employed for the thioester hydrolysis reactions. This control reaction also gave a $^2$H NMR signal at 2.59 ppm.

We note that isolation and/or further characterization of the proposed zinc thiolate complex \([(\text{bpta})\text{Zn-SCD₃(Sol)}ₙ]X\) is precluded by the nature of the reaction conditions, particularly the mixed solvent environment (CH₃CN:H₂O 50:50), and the presence of buffer (CHES) and salt (NaNO₃ (0.61 M)). For example, mass spectrometry of the reaction mixture is not an option due to the high salt content of the solution. In situ NMR experiments (e.g. $^{13}$C NMR) are complicated by the presence of signals from the CH₃CN solvent and CHES buffer.
By stoichiometry, the other product generated in the reaction of 1 with \( \text{PhCH(OH)C(O)SCD}_3 \) must be mandelate \( (\text{PhCH(OH)C(O)O}^-) \).\textsuperscript{16} This product has been previously identified in reactions of zinc hydroxide complexes with \( \text{PhCH(OH)C(O)SCD}_3 \) performed in an organic solvent.\textsuperscript{16} In these reactions, \( \text{CD}_3\text{SH} \) is released and the mandelate product coordinates to the Zn(II) center.

**Kinetic Studies of the Reactivity of 1 with \( \text{PhCH(OH)C(O)SCD}_3 \) in \( \text{CH}_3\text{CN}:\text{H}_2\text{O} \).** Kinetic studies of the thioester hydrolysis reaction promoted by 1 were monitored and the data was processed as previously described.\textsuperscript{15}

**Results and Discussion**

We have previously shown that use of an aliphatic, deuterium-labeled thioester \( (\text{PhCH(OH)C(O)SCD}_3) \), with \(^2\text{H} \) NMR as the monitoring method, is a feasible approach for investigating thioester hydrolysis reactions promoted by dizinc and Fe(III)Zn(II) complexes.\textsuperscript{15,16} These studies revealed that the presence of a terminal Zn-OH species enhanced thioester hydrolysis reactivity in both types of complexes. In the research outlined herein, we have studied thioester hydrolysis promoted by the mononuclear zinc complex \([\text{bpta}Zn]\)(\text{ClO}_4)\textsubscript{2}\cdot0.5\text{H}_2\text{O} \text{ (1)}\). Nitrate and triflate analogs of this complex have been previously reported.\textsuperscript{6} However both were prepared in solution, and no structural or spectroscopic data was reported. We have fully characterized 1 using elemental analysis, \(^1\text{H} \) and \(^{13}\text{C} \) NMR, FTIR, mass spectrometry, and X-ray crystallography.\textsuperscript{17} When crystallized from \( \text{CH}_3\text{CN}/\text{Et}_2\text{O} \), the cationic portion exhibits facial coordination of the bpta ligand, with two acetonitrile donors and one water molecule completing the coordination sphere.
Figure 3-1. Cationic portion of [(bpta)Zn(CH$_3$CN)$_2$(H$_2$O)](ClO$_4$)$_2$ (1·2CH$_3$CN·0.5H$_2$O).

Hydrogen atoms other than those of the coordinated water molecule have been omitted for clarity. Selected bond distances (Å) and angles (°): Zn(1)-N(3) 2.055(2), Zn(1)-O(1) 2.1103(3), Zn(1)-N(2) 2.130(2), Zn(1)-N(5) 2.185(2), Zn(1)-N(4) 2.2213(3), Zn(1)-N(1) 2.2542, N(1)-Zn(1)-N(2) 77.48(9), N(2)-Zn(1)-N(3) 100.02(9), N(1)-Zn(1)-N(3) 80.66(9).

(Figure 3-1). The water ligand is positioned trans to a pyridyl nitrogen, with a Zn-O distance of 2.110(3) Å. The two coordinated acetonitrile ligands have Zn-N distances of 2.221(3) and 2.185(2) Å, respectively. Three distinct Zn-N distances are found involving the bpta ligand, with the shortest being Zn(1)-N(3) (2.055 Å), which is trans to the coordinated water molecule. Upon crushing and drying of the crystals, the acetonitrile
ligands and half of the water is lost, leading to an analytical formulation for the dried solid as \([(\text{bpta})\text{Zn}](\text{ClO}_4)_2\cdot0.5\text{H}_2\text{O}\) (I).

Kinetic studies of nitrocefin hydrolysis promoted by \([(\text{bpta})\text{Zn}](\text{NO}_3)_2\cdot\text{nH}_2\text{O}\) yielded a kinetic pK\(_a\) of 7.84(2) for the Zn-OH\(_2\) moiety.\(^{6}\) For the thioester hydrolysis studies reported herein we have performed all reactions at pH = 9.0 to ensure that a Zn-OH species is present. Admixture of 1.05 equivalents of I and one equivalent of PhCH(OH)C(O)SCD\(_3\) in 50:50 CH\(_3\)CN:H\(_2\)O (buffered at pH = 9.0), followed by heating of this solution at 329.5 K for ~12 h, results in the formation of two –SCD\(_3\)-containing products as identified by \(^2\)H NMR (Figure 3-2). The more abundant product has a \(^2\)H NMR signal at 1.77 ppm,\(^{18}\) and a smaller signal is found at 2.12 ppm. The latter signal corresponds to CD\(_3\)SH (pK\(_a\) = 10.4).\(^{15}\) We note that in the absence of I, the hydrolysis of PhCH(OH)C(O)SCD\(_3\) under identical conditions results in the formation of CD\(_3\)SH as the only sulfur-containing product.\(^{15}\) We propose that the species at 1.77 ppm is a zinc thiolate complex, with an analytical formulation such as \([(\text{bpta})\text{Zn-SCD}_3\text{(Sol)}\text{n}]\text{X}\) (sol = H\(_2\)O or CH\(_3\)CN).\(^{19}\) The relative amounts of CD\(_3\)SH and the zinc thiolate complex produced in the reaction mixture change as a function of the I:thioester ratio (Figure 3-2), with more of the zinc thiolate species being produced in solutions having a higher initial concentration of zinc complex. Using the 10:1 ([I]:thioester) reaction mixture, which following stoichiometric thioester hydrolysis contains only a trace amount of CD\(_3\)SH (Figure 3-2), lowering of the pH to ~8.6 resulted in the formation of additional CD\(_3\)SH (2.12). Further lowering of the pH to 8.3 resulted in additional CD\(_3\)SH being generated. This data is consistent with the presence of a Zn-SCD\(_3\)
Figure 3-2. $^2$H NMR spectra obtained following hydrolysis of PhCH(OH)C(O)SCD$_3$ promoted by 1 at pH = 9.0. The species at 2.12 ppm has been identified as CD$_3$SH. The resonance at 1.77 ppm is proposed to be the signal for $[(bpta)Zn(SCD_3)(Sol)_n]X$ (Sol = CH$_3$CN or H$_2$O; X = anion present in solution).

complex that can undergo protonation to release CD$_3$SH. Additional evidence that the 1.77 ppm species contains a Zn-SCD$_3$ moiety comes from treatment of the reaction mixture with excess CH$_3$I, which results in the formation of the sulfonium salt $[(CD_3)S(CH_3)_2]X$ ($^2$H NMR 2.59 ppm).
Figure 3-3. Plot of $k_{obs}$ (s$^{-1}$) versus [1] for 299.5 – 329.5 K.

At pH = 9.0, the thioester hydrolysis reaction promoted by 1 is catalytic, with 10 turnovers requiring ~12 days to go to completion at 329.5 K. Monitoring of the loss of thioester as a function of time at specific concentrations of 1 yielded plots from which pseudo-first-order rate constants ($k_{obs}$) were determined.$^{20}$ Varying the concentration of 1 from 1.9 to 27 mM, with [PhCH(OH)C(O)SCD$_3$] = 1.8 mM, produced linear plots of $k_{obs}$ versus [1] from which second-order rate constants ($k_2$) were determined (Figure 3-3; Table 3-1). Variable temperature studies in the range of 299.5 - 329.5 K, and construction of an Eyring plot (Figure 3-4), yielded $\Delta H^\neq = 13.6(6)$ kcal/mol, $\Delta S^\neq = -25.5(2.0)$ cal/mol·K, and $E_a = 14.1(6)$ kcal/mol.
Table 3-1. Rate constants for the hydrolysis of PhCH(OH)C(O)SCD₃ promoted by 1.

<table>
<thead>
<tr>
<th>Temperature (K)</th>
<th>k₂ (M⁻¹s⁻¹)</th>
</tr>
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<tbody>
<tr>
<td>299.5</td>
<td>1.87(26) x 10⁻³</td>
</tr>
<tr>
<td>309.5</td>
<td>4.69(24) x 10⁻³</td>
</tr>
<tr>
<td>319.5</td>
<td>1.06(11) x 10⁻²</td>
</tr>
<tr>
<td>329.5</td>
<td>2.44(6) x 10⁻²</td>
</tr>
</tbody>
</table>

[a][PhCH(OH)C(O)SCD₃] = 0.0018 M; [1] = 0.0019 – 0.027 M; 50:50 CH₃CN:H₂O (CHES buffer, 0.36 M, I = 0.61 M (NaNO₃))

Figure 3-4. Eyring plot for the hydrolysis of PhCH(OH)C(O)SCD₃ promoted by 1 in the temperature range of 299.5 – 329.5 K.
The kinetic data for the hydrolysis of PhCH(OH)C(O)SCD$_3$ promoted by 1 revealed that the reaction is second-order overall with an associative type mechanism that likely involves nucleophilic attack of the Zn-OH moiety on the thioester, with no formation of a precursor complex (Scheme 3-1). This differentiates thioester hydrolysis promoted by the mononuclear zinc complex from that found for a Fe(III)Zn(II) complex, which mimics a common binuclear core found in glyoxalase II enzymes.$^{15}$ In reactions involving the Fe(III)Zn(II) complex, saturation kinetic behavior was interpreted as indicating coordination of the deprotonated α-hydroxy group of the thioester to the Zn(II) prior to nucleophilic attack by an Fe(III)-OH moiety (Scheme 3-1). We propose that the lack of thioester coordination to 1, and the observation of a simple second-order reaction, is due to differences in the p$K_a$ values of the Zn-OH moieties of 1 and the Fe(III)Zn(II) complex, with the latter being a better base (Zn-OH$_2$, kinetic p$K_a$ = 8.8),$^{15}$ thus enabling deprotonation of the thioester hydroxyl group. The thioester hydrolysis reaction promoted by the Fe(III)Zn(II) complex is >15-fold faster than the reaction performed under identical conditions using 1.$^{15}$ Thus, the equilibrium coordination of the deprotonated thioester to the Zn(II) center of the Fe(III)Zn(II) complex facilitates the hydrolysis reaction by positioning the thioester carbonyl for nucleophilic attack by the Fe(III)-OH moiety. In terms of leaving group stabilization, for the reaction involving the Fe(III)Zn(II) complex, thiolate coordination was proposed to occur at the Fe(III) center on the basis of the formation of a disulfide side product that likely results from redox chemistry involving an Fe-SCD$_3$ moiety. For the thioester hydrolysis reaction promoted by 1, the thiolate leaving group is stabilized via zinc coordination. For both systems, catalytic turnover presumably occurs via
**Scheme 3-1.** Comparison of the thioester hydrolysis reaction pathways involving binuclear Fe(III)Zn(II) and mononuclear Zn(II) complexes.

Protonation of the M-SCD₃ species and generation of the reactive metal-hydroxide species. The proposed pathway for the thioester hydrolysis reaction promoted by 1 is shown in Scheme 3-2.

We note that the activation energy for the thioester hydrolysis reaction promoted by 1 is similar to that found for the alkaline hydrolysis of *n*-butylthioacetate.¹¹ This suggests that both reactions proceed via rate-determining nucleophilic attack of hydroxide (either free or zinc-bound) on the thioester carbonyl carbon. Similar results have been reported for the hydrolysis of 4-nitrophénylacétate promoted by OH⁻ or mononuclear zinc complexes (Table 3-2).²²,²³
Scheme 3-2. Thioester hydrolysis promoted by 1.

Table 3-2. Activation energies for OH⁻ and Zn-OH promoted thioester and ester hydrolysis reactions.

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Thioester or ester</th>
<th>$E_a$(kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OH&lt;a&gt;</td>
<td>$n$-BTA&lt;b&gt;</td>
<td>13.9&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>TE&lt;sup&gt;e&lt;/sup&gt;</td>
<td>14.1(6)</td>
</tr>
<tr>
<td>OH&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4-NPA&lt;sup&gt;f&lt;/sup&gt;</td>
<td>10.3(1)</td>
</tr>
<tr>
<td>[(12)aneN₃]Zn-OHClO₄&lt;sup&gt;g&lt;/sup&gt;</td>
<td>4-NPA</td>
<td>11.7(1)</td>
</tr>
<tr>
<td>[(12)aneN₄]Zn-OHClO₄&lt;sup&gt;h&lt;/sup&gt;</td>
<td>4-NPA</td>
<td>10.8(1)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ref. 21. <sup>b</sup>n-BTA = n-butylthioacetate. <sup>c</sup>Temperature = 298 K. <sup>d</sup>This work. <sup>e</sup>TE = PhCH(OH)C(O)SCD₃. <sup>f</sup>4-NPA = 4-nitrophenylacetate. <sup>g</sup>Ref. 22. <sup>h</sup>Ref. 23.
In summary, we report studies of thioester hydrolysis promoted by a mononuclear zinc complex in a mixed organic-aqueous environment. This reaction is relevant to the chemistry of the monozinc-containing form of human GlxII. In considering the results of this study in combination with those derived from an investigation involving a Fe(III)Zn(II) complex, it appears that the reaction pathway for the hydrolysis of an α-hydroxythioester akin to the GlxII substrate is influenced by the pKₐ of the Zn-OH₂ moiety. Further investigations to test this hypothesis are currently in progress. Additionally, our results provide evidence to support the notion that Zn-OH promoted ester and thioester hydrolysis reactions can proceed via similar reaction pathways. This is relevant to the hydrolysis of coumarin and thiocoumarin derivatives within the active site of CA enzymes.

References


17. $\textbf{1}$: $C_{20}H_{29}Cl_2N_5O_9Zn$, $M = 619.75$, orthorhombic, $Pbca$, colorless plate, $a = 10.8255(2)$ Å, $b = 14.0872(2)$ Å, $c = 32.7227(8)$ Å, $V = 5245.30(18)$ Å$^3$, $Z = 8$, $T = \ldots$
150(1) K, 10038 total reflections, 5736 independent [\( R_{int} = 0.0295, R1 (I > 2\sigma(I)) = 0.0443, wR2 (all data) = 0.1160 \)].

18. The CDH₂CN signal is positioned under the 1.77 ppm resonance.


20. Rates for the buffer promoted thioester hydrolysis reaction were determined at temperatures of 299.5 – 329.5 K. These values were subtracted from those obtained for the reactions involving 1 as described in the literature. diTargiani, R. C.; Chang,


CHAPTER 4

FUNDAMENTAL INVESTIGATIONS INTO THE EFFECT OF METAL HALIDES ON THE CHEMISTRY OF GLUCOSE IN IONIC LIQUIDS

I. Background

A sustainable future requires chemical feedstocks which can be obtained from renewable resources rather than steadily depleting existing ones (e.g. petroleum).\(^1\) In 2002, the US DOE Office of Energy Efficiency and Renewable Energy combined its various biochemical programs (biofuels, biopower, etc.) into a single biomass program. One of the goals of this program was to identify, screen, and test bio-derived chemicals as the new feedstocks for the chemical industry. Researchers at the National Renewable Energy Laboratory (NREL) and Pacific Northwest National Laboratory (PNNL) evaluated over 300 compounds, and based on this study, formulated a list of twelve key sugar-derived building block molecules (Table 4-1).\(^2\) An important molecule for 2,5-furan dicarboxylic acids generation, which are polymer building blocks, is 5-hydroxymethylfurfural (HMF, Figure 4-1).\(^3,4\) Notably, HMF is also of interest for its role in the synthesis of 2,5-dimethylfuran (DMF, Figure 4-2), a potentially new biofuel.\(^5\)

![Figure 4-1. Structure of 5-hydroxymethylfurfural (HMF).](image-url)
Table 4-1. Twelve key sugar-derived building block molecules.

<table>
<thead>
<tr>
<th>#</th>
<th>Building Block Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,4 diacids (succinic, fumaric and malic)</td>
</tr>
<tr>
<td>2</td>
<td>2,5 furan dicarboxylic acid (HMF)</td>
</tr>
<tr>
<td>3</td>
<td>3-hydroxy propionic acid</td>
</tr>
<tr>
<td>4</td>
<td>aspartic acid</td>
</tr>
<tr>
<td>5</td>
<td>glucaric acid</td>
</tr>
<tr>
<td>6</td>
<td>glutamic acid</td>
</tr>
<tr>
<td>7</td>
<td>itaconic acid</td>
</tr>
<tr>
<td>8</td>
<td>levulinic acid</td>
</tr>
<tr>
<td>9</td>
<td>3-hydroxybutyrolactone</td>
</tr>
<tr>
<td>10</td>
<td>glycerol</td>
</tr>
<tr>
<td>11</td>
<td>sorbitol</td>
</tr>
<tr>
<td>12</td>
<td>xylitol/arabinitol</td>
</tr>
</tbody>
</table>

Figure 4-2. Structure of 2,5-dimethylfuran (DMF).

II. 5-hydroxymethylfurfural (HMF)

a. Synthesis of HMF. HMF is synthesized by acid-catalyzed triple dehydration of hexoses (cellulose, glucose, fructose) (Scheme 4-1). The difficulty in the synthesis of HMF lies in the numerous side products formed during dehydration process (levulinic acid, formic acid, HMF ethers, and insoluble humic acids). The exact mechanism for HMF
formation from hexoses is not known, however, two pathways for this process have been proposed. The first one involves dehydration without ring opening (Scheme 4-2) and the second one involves formation of ring-opened intermediates (Scheme 4-3).
Scheme 4-3. Proposed mechanism for HMF formation from fructose involving ring-opened intermediates.

b. Industrial Uses of HMF. Oxidation of HMF leads to the formation of 2,5-furan dicarboxylic acid (FDA) (Scheme 4-4), which is proposed to be an alternative for terephthalic acid (Scheme 4-4), a building block molecule used in the synthesis of polyethylene terephthalates (PETs). The structural similarities of FDA and terephthalic acid make FDA a promising starting material for a new generation of polyesters and nylon polymers.

The hydrogenolysis of HMF (Scheme 4-5) leads to formation of 2,5-dimethylfuran (DMF), a potentially new biofuel. The appeal of this compound is due to its: (1) high energy density relative to ethanol (40% higher); (2) stability in air; and (3) insolubility in water.
c. Conversion of Biomass to HMF. Currently, the industrial use of HMF as a chemical feedstock is limited by its high production cost and poor yield. Extensive research has been conducted to develop new, higher yield catalysts for the synthesis of HMF. Over the years, hundreds of catalysts have been evaluated for the aforementioned purpose (representative examples are shown in Table 4-2). Unfortunately, the use of these catalysts has either led to low yields of HMF, or the production of undesired by-products. Traditionally, the synthesis of HMF has been focused on acid-catalyzed dehydration of simple hexose sugars (glucose and fructose) (Scheme 4-6), however, recent studies have shown that lignocellulose biomass (untreated corn stover) and cellulose can be converted to
HMF in respectable yields (~48%).\textsuperscript{9,10} In an effort to minimize the amount of by-products formed during the synthesis of HMF, acidic ionic liquids have been utilized.\textsuperscript{1,10-13} In 2007, researchers at PNNL reported the discovery of a new chemical system (Scheme 4-7) which is capable of converting glucose into HMF with little to no by-products. It was found that the treatment of glucose with 1-ethyl-3-methylimidazolium chloride (EMIMCl) and chromium(II) chloride (CrCl\textsubscript{2}) gave good yields of HMF (~70%). Interesting was the fact that replacement of CrCl\textsubscript{2} with CrCl\textsubscript{3} still yielded significant amounts of HMF (~45%).\textsuperscript{1} Considering the kinetic inertness of chromium(III), these results appear counterintuitive. Nevertheless, some researchers have argued that the interconversion between the two oxidation states of chromium could account for these results. The incorporation liquids as

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Organic Acids & Inorganic Acids & Salts & Lewis Acids & Others \\
\hline
Oxalic Acid & Phosphoric Acid & (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}/SO\textsubscript{3} & ZnCl\textsubscript{2} & Ion-exchange resins \\
Levulinic Acid & Sulphuric Acid & Pyridine/PO\textsubscript{4}\textsuperscript{3-} & AlCl\textsubscript{3} & Zeolites \\
Maleic Acid & Hydrochloric Acid & Pyridine/HCl & BF\textsubscript{3} & \\
\textit{p}-Toluencesulfonic acid & Hydroiodic Acid & Aluminum salts & Zr\textsubscript{3}(PO\textsubscript{4})\textsubscript{4} & \\
& & & ZrOCl\textsubscript{2} & \\
& & & VO(SO\textsubscript{4})\textsubscript{2}, TiO\textsubscript{2} & \\
& & & Zr, Cr, Ti, V porphyrine & \\
\hline
\end{tabular}
\caption{Catalysts used for the synthesis of HMF.}
\end{table}
Scheme 4-6. Formation and decomposition reactions of HMF.

Scheme 4-7. Conversion of glucose to HMF.
Scheme 4-8. Conversion of cellulose to HMF in the absence of ionic liquids.

Solvents for the manufacturing of HMF has become a common approach, of ionic however, in 2009, researchers at the University of Wisconsin-Madison reported the conversion of cellulose into HMF in the absence of ionic liquids (Scheme 4-8).\textsuperscript{10} These reports have shown that a solution of dimethylacetamide-lithium chloride (DMA-LiCl) is a suitable substitute for ionic liquids.

**III. Fundamental Chemistry of Ionic Liquids**

**a. Ionic Liquids.** Ionic liquids (Figure 4-3) were originally described as air and water sensitive liquid electrolytes composed entirely of ions, with a melting point below 100 °C. In the early 1980’s, room-temperature ionic liquids based on the 1-alkyl-3-methylimidazolium salts were reported.\textsuperscript{14,15} Later, researchers sought to solve the problems of air and water sensitivity by replacing the less stable chloroaluminate anion with the more stable tetrafluoroborate.\textsuperscript{16} The latest generation of ionic liquids has included substituted appendages for performing task specific reactions.\textsuperscript{17}
Over the years, ionic liquids have been used as both solvents and catalysts.\textsuperscript{14} According to the transition-state theory, solvents can modify the Gibbs energy of activation by differential solvation of the reactants and intermediate(s).\textsuperscript{14} Work conducted by Hughes and Ingold showed that changes in the solvent polarity result in either an increase or decrease of the reaction rate. This change is dependent on whether the intermediate(s) is more or less polar than the initial reactants.\textsuperscript{18,19} It is believed that the increase in solvent polarity leads to decrease in the activation energy.\textsuperscript{14} Hence, the use of these highly polar ionic liquids allows for an increase in the reaction rate. Of interest to the research described herein is the ability of ionic liquids to act as a catalyst. The next section focuses on how ionic liquids affect the stereochemistry of glucose during its conversion to HMF.

**b. Stereochemistry of Glucose in Ionic Liquids.** In order to gain insight into the chromium-catalyzed conversion of glucose to HMF, investigations of the stereochemistry of glucose in ionic liquids have been undertaken. It has been shown that: (1) glucose, in the presence of the ionic liquid [EMIM]Cl, exists primarily in the $\alpha$-anomer form; (2) the presence of a metal halide can alter the $\alpha/\beta$ anomeric ratio; and (3) the presence of an additional solvent can influence the $\alpha/\beta$ anomeric ratio.\textsuperscript{1,20-22}

![Figure 4-3. Three generations of ionic liquids.](image)
Researchers at PNNL reported that when glucose is dissolved in [EMIM]Cl only, ~90% of glucose exists in its α-pyranose form (~92 ppm) and the remaining ~10% is in the β-pyranose form (~98 ppm), as determined by $^{13}$C NMR. $^1$ Interesting is that upon addition of CrCl$_2$ to the reaction mixture, there is a significant change in the α/β ratio to approximately 1:1. In 2001, researchers at the University of Notre Dame showed that at 30 °C and in the presence of water, the composition of D-glucose is ~38% α-pyranose and ~62% β-pyranose. $^{20}$ Further research published in 2006, showed that by changing the solvent system from water to [BMIM]Cl:DMSO (BMIM = 1-butyl-3-methylimidazolium and DMSO being present at 15 wt%) results in a minimal shift in the α/β ratio to ~41% α and ~57% β. $^{22}$ These results show that the α/β anomic ratio of glucose is highly dependent on the reaction conditions.

c. Role of [EMIM]CrCl$_3$ in Conversion of Glucose to Fructose. It has been proposed that during the conversion of glucose to fructose, the metal catalyst [EMIM]CrCl$_3$ will perform two different reactions: mutarotation and isomerization. First, [EMIM]CrCl$_3$ is expected to initiate mutarotation of α-glucopyranose to β-glucopyranose via proton transfer (Scheme 4-9). $^1$ This is consistent with the observed change in the α/β anomic ratio upon addition of CrCl$_2$. Next, [EMIM]CrCl$_3$ will affect a formal hydride transfer leading to the isomerization of glucose to fructose, followed by dehydration to HMF (Scheme 4-10). $^1$ Prior to the work outlined in Chapter 5, no structural data for the complex formed when CrCl$_2$ is mixed with [EMIM]Cl has been reported. Since the nature of the active catalyst is of interest, we have determined the solid state crystal structure of [EMIM]CrCl$_3$. 

Scheme 4-9. Mutatoration of α-glucopyranose to β-glucopyranose.

Scheme 4-10. Isomerization of glucose to HMF.
References


CHAPTER 5
SYNTHESIS AND CRYSTALLIZATION OF POLY[1-ETHYL-3-METHYLIMIDAZOLIUM[TRI-\(\mu\)-CHLORIDO-CHROMATE(II)]]

Abstract

The title compound, \(\{(C_6H_{11}N_2)[CrCl_3]\}_n\), was generated via mixing of the ionic liquid 1-ethyl-3-methylimidazolium chloride with CrCl\(_2\) in an ethanol/ethyl acetate mixture. Crystals were obtained by diffusion of diethyl ether into the solution. In the crystal structure, the anions form one-dimensional chains of chloride-bridged, Jahn-Teller distorted chromium(II) centers extending along the \([1,0,0]\) direction. The imidazolium cations are positioned between the chains.

Introduction

Recently, it was shown that at 100°C a solution of CrCl\(_2\) in the ionic liquid 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) will catalyze the conversion of glucose to 5-hydroxymethylfurfural (HMF) in 70% yield.\(^1\) The proposed active catalyst in this system is a compound formulated as [EMIM]CrCl\(_3\). While alkali metal, ammonium, and tetramethylammonium chromium(II) trihalides have been previously reported in the literature, the title compound is the first structurally characterized imidazolium analog.\(^2-7\)

The research described herein is focused on the synthesis and crystallization of CrCl\(_2\) in [EMIM]Cl. This work has relevance to research dealing with renewable energy and HMF.

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production, as we have determined the solid state structure of the proposed active catalyst [EMIM]CrCl₃.₁

**Experimental Section**

**General and Synthetic Methods.** All reagents and solvents were obtained from commercial sources and were used as received, unless otherwise noted. Elemental analyses were performed by Atlantic Microlabs Inc., Norcross, GA.

[EMIM]CrCl₃ (1). Under a N₂ atmosphere, a solution of anhydrous CrCl₂ (23 mg, 0.19 mmol) in ethanol (2 ml) was added to solid 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) (23 mg, 0.16 mmol). The resulting, teal colored solution was stirred at ambient temperature until the entire solid had dissolved. Addition of ethyl acetate (2 ml), followed by Et₂O diffusion, produced pale yellow crystals suitable for single crystal X-ray diffraction analysis. Yield 51.4%. Anal Calcd. for C₆H₁₁N₂Cl₃Cr: C, 26.87; H, 4.14; N, 10.45. Found: C, 26.50; H, 4.16; N, 10.26.

**X-ray Crystallography.** A crystal of 1 was mounted on a glass fiber using a viscous oil. The sample was then transferred to a Nonius KappaCCD diffractometer with Mo Kα radiation (λ = 0.71073 Å) for data collection at 150(1) K. An initial set of cell constants was obtained from ten frames of data that were collected with an oscillation range of 1 deg/frame and an exposure time of 20 sec/frame. Indexing and unit cell refinement based on all observed reflections from those ten frames, indicated a monoclinic P₂₁/a lattice and the space group P2₁/a. Final cell constants were determined from a set of strong reflections from the actual data collection. These reflections were indexed, integrated, and corrected for Lorentz, polarization, and absorption effects using DENZO-
The structure was solved by a combination of direct methods and heavy atom using SIR 97. All H atoms were located and refined isotropically using SHELXL97.

Results

Synthesis and Crystallization of Poly[1-ethyl-3-methylimidazolium [tri-µ-chlorido-chromate(II)]] (1). The title compound, [(C₆H₁₁N₂)CrCl₃]ₙ, was generated via mixing of the ionic liquid 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) with CrCl₂ in an ethanol/ethyl acetate mixture. Pale yellow crystals suitable for X-ray crystallographic analysis were obtained by diffusion of diethyl ether into the solution. Details of the X-ray data collection and refinement are given in Table 5-1. Selected bond distances and angles are given in Table 5-2. The structure consists of infinite, linear chains of Jahn–Teller-distorted chromium centers (Figure 5-1) bridged by a facial array of chloride ligands (Figure 5-2). Each Cr(II) has four Cr-Cl bonds of approximately 2.39-2.45 Å and two longer Cr-Cl interactions (2.87-2.91 Å). The Cr-Cr distance is 3.33 Å and the shortest Cr-Cr distance between chains is 9.19 Å. The Cl-Cr-Cl bond angles are in the range of 87-90°. A number of differences are evident in the structures of [EMIM]CrCl₃ (collected at 150 (1) K) and the previously reported [N(CH₃)₄]CrCl₃ (collected at room temperature). Specifically, the chromium center in [EMIM]CrCl₃ has a pseudo D₄h site symmetry, whereas [N(CH₃)₄]CrCl₃ contains trigonally distorted chromium centers (C₃v site symmetry) positioned in alternating compressed and elongated face-sharing octahedra. Similar site symmetry to that found in [N(CH₃)₄]CrCl₃ was identified in the room temperature structure of α-CsCrCl₃. This C₃v site symmetry is described as resulting from randomly distributed
elongation of Cr-Cl bonds along three principal axes of the octahedron.

Conclusion

A sustainable future requires chemical feedstocks which can be obtained from renewable resources rather than steadily depleting ones (e.g. petroleum). Recently, HMF (Figure 5-3) has become an extremely important feedstock molecule because of its potential to act as both a biofuel and a polymer precursor. Industrial use of HMF as a chemical intermediate is limited by high production costs and poor yields. However, extensive research has been conducted toward the development of new methods for converting renewable biomass into HMF, with increased yields and decreased costs. In 2007, researchers at Pacific Northwest National Laboratories (PNNL) reported the discovery of a novel chemical system (Scheme 5-1) which is capable of converting glucose into HMF with little to no by-products. The proposed active catalyst in this system is a compound formulated as [EMIM]CrCl$_3$. It was found that treatment of glucose with the ionic liquid 1-ethyl-3-methylimidazolium chloride ([EMIM]Cl) and chromium(II) chloride (CrCl$_2$) gives good yields of HMF (~70%). However, the exact composition present during the conversion of glucose to HMF remains unidentified. Our goal was to synthesize and crystallize this proposed active catalyst. We were successful in providing a solid state crystal structure of [(C$_6$H$_{11}$N$_2$)CrCl$_3$]$_n$. In the crystal structure, anions form one-dimensional chains of chloride-bridged Jahn-Teller distorted chromium(II) centers extending along the [1,0,0] direction. The imidazolium cations are positioned between these chains. Further studies aimed at elucidating how the metal halide and ionic liquid affect the structure of this complex are being pursued.
Table 5-1. Summary of X-ray data collection and refinement of 1.

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<tr>
<th></th>
<th>1</th>
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<tbody>
<tr>
<td>Empirical formula</td>
<td>C₆H₁₃N₂Cl₃Cr</td>
</tr>
<tr>
<td>Formula weight</td>
<td>269.52</td>
</tr>
<tr>
<td>Crystal system</td>
<td>Monoclinic</td>
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<tr>
<td>Space group</td>
<td>P2₁/a</td>
</tr>
<tr>
<td>a (Å)</td>
<td>6.66150 (10)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>16.4317 (4)</td>
</tr>
<tr>
<td>c (Å)</td>
<td>9.5258 (2)</td>
</tr>
<tr>
<td>α (deg)</td>
<td>90.</td>
</tr>
<tr>
<td>β (deg)</td>
<td>95.6881 (14)</td>
</tr>
<tr>
<td>γ (deg)</td>
<td>90.</td>
</tr>
<tr>
<td>V (Å³)</td>
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</tr>
<tr>
<td>Z</td>
<td>4</td>
</tr>
<tr>
<td>Density (calcd), Mg m⁻³</td>
<td>1.725</td>
</tr>
<tr>
<td>Temp (K)</td>
<td>150 (1)</td>
</tr>
<tr>
<td>Crystal size (mm)</td>
<td>0.25 x 0.20 x 0.15</td>
</tr>
<tr>
<td>Diffractometer</td>
<td>Nonius KappaCCD</td>
</tr>
<tr>
<td>Abs. coeff. (mm⁻¹)</td>
<td>1.82</td>
</tr>
<tr>
<td>Θ range for data collection (deg)</td>
<td>2.48 to 27.49</td>
</tr>
<tr>
<td>Completeness to Θ(%)</td>
<td>99.7</td>
</tr>
<tr>
<td>Reflections collected</td>
<td>4056</td>
</tr>
<tr>
<td>Indep. Reflections</td>
<td>2384 [R(int) = 0.0181]</td>
</tr>
<tr>
<td>Variable parameters</td>
<td>154</td>
</tr>
<tr>
<td>R1 / wR2[^b]</td>
<td>0.0321 / 0.0642</td>
</tr>
<tr>
<td>Goodness-of-fit (F²)</td>
<td>1.084</td>
</tr>
<tr>
<td>Largest diff. (e Å⁻³)</td>
<td>0.417 / 0.484</td>
</tr>
</tbody>
</table>

[^a]Radiation used: Mo Kα (λ = 0.71073 Å).
[^b]R1 = Σ(∥ |F₀| - |Fᵣ|∥) / Σ |F₀|^2|, wR2 = [Σ [w(F₀^2 - Fᵣ^2)^2]/Σ (F₀^2)^2]^{1/2}
where w = 1/[(σ²(F₀^2) + (aP)^2 + bP)].


Table 5-2. Selected bond lengths (Å) and angles (°) for 1 characterized by X-ray crystallography.ª

<table>
<thead>
<tr>
<th>Bond or Angle</th>
<th>Length/Distance</th>
<th>Symmetry Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cr(1)-Cl(1)</td>
<td>2.3876 (5)</td>
<td></td>
</tr>
<tr>
<td>Cr(1)-Cl(2)</td>
<td>2.3898 (5)</td>
<td></td>
</tr>
<tr>
<td>Cl(2)-Cr(1)-Cl(1)</td>
<td>177.976 (19)</td>
<td></td>
</tr>
<tr>
<td>Cl(2)-Cr(1)-Cl(3)</td>
<td>87.073 (15)</td>
<td></td>
</tr>
<tr>
<td>Cl(1)-Cr(1)-Cl(3)</td>
<td>91.904 (16)</td>
<td></td>
</tr>
<tr>
<td>Cl(2)-Cr(1)-Cl(3)i</td>
<td>91.906 (16)</td>
<td></td>
</tr>
<tr>
<td>Cr(1)-Cl(3)</td>
<td>2.4431 (5)</td>
<td></td>
</tr>
<tr>
<td>Cr(1)-Cl(3)i</td>
<td>2.4476 (5)</td>
<td></td>
</tr>
<tr>
<td>Cl(1)-Cr(1)-Cl(3)i</td>
<td>89.027 (15)</td>
<td></td>
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<tr>
<td>Cl(3)-Cr(1)-Cl(3)i</td>
<td>176.95 (2)</td>
<td></td>
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<tr>
<td>Cr(1)-Cl(3)-Cr(1)i</td>
<td>85.856 (13)</td>
<td></td>
</tr>
</tbody>
</table>

ªEstimated standard deviations in the last significant figure are given in parentheses. Symmetry codes: (i) x - 1/2, -y + 1/2, z; (ii) x + 1/2, -y + 1/2, z.

Figure 5-1. Coordination environment of [(C6H11N2)CrCl3]n.
Figure 5-2. One-dimensional chain structure of [(C₆H₁₁N₂)CrCl₃]ₙ extending along [1,0,0].

Figure 5-3. Structure of 5-hydroxymethylfurfural (HMF).

Scheme 5-1. Conversion of glucose to HMF catalyzed by [EMIM]CrCl₃.
References


CHAPTER 6

CONCLUSIONS

The work presented in Chapters 2 and 3 of this thesis summarizes our efforts to gain mechanistic insight into the thioester hydrolysis reactivity of metal complexes of relevance to glyoxalase II enzymes. Glyoxalase II (GlxII) is one of two metalloenzymes found in the glyoxalase pathway and is responsible for catalyzing the hydrolysis of a \( S-(2\text{-hydroxyacyl}) \) glutathione thioester (SLG) to produce the corresponding \( S\text{-2-hydroxyacid} \) and free glutathione (Scheme 6-1). The primary physiological substrate of the glyoxalase pathway is methylglyoxal, \((\text{CH}_3\text{C(O)CHO})\), a small, cytotoxic, and mutagenic byproduct of lipid and glucose metabolism. Methylglyoxal is toxic because it is involved in the formation of advanced glycation end-products (AGE), which have been linked to aging and various diseases.\(^1\)-\(^3\)

\[
\begin{align*}
\text{O} & \quad \text{O} \\
\text{R} & \quad \text{C} & \quad \text{C} & \quad \text{H} & \quad + & \quad \text{GSH} & \quad \overset{\text{Non-enzymatic}}{\longrightarrow} & \quad \text{O} & \quad \text{OH} \\
& & & & & & \quad \text{R} & \quad \text{C} & \quad \text{C} & \quad \text{SG} \\
& & & & & & \quad \text{H} \\
\text{R} = \text{CH}_3, \text{Ph}, \text{etc.} \\
\text{GSH} = \text{glutathione}
\end{align*}
\]

\[
\begin{align*}
\text{OH} & \quad \text{O} \\
\text{R} & \quad \text{C} & \quad \text{C} & \quad \text{OH} & \quad + & \quad \text{GSH} & \quad \overset{\text{GlxII}}{\longrightarrow} & \quad \text{OH} & \quad \text{O} \\
& & & & & & \quad \text{R} & \quad \text{C} & \quad \text{C} & \quad \text{SG} \\
& & & & & & \quad \text{H} \\
\text{H}
\end{align*}
\]

Scheme 6-1. The Glyoxalase pathway.
Figure 6-1. Active site of glyoxalase II enzymes.

GlxII enzymes from a variety of sources have been characterized by X-ray crystallography.\textsuperscript{4,7} In each structure the active site contains a binuclear metal cluster wherein which each metal center is coordinated by three terminal amino acid ligands, a bridging aspartate, and a bridging water/hydroxide ligand (Figure 6-1). The main problem associated with mechanistic studies of the GlxII-catalyzed thioester hydrolysis is the variable metal ion content of the active site of the GlxII enzymes, which depends on metal ion availability and the source of the enzyme.\textsuperscript{4-12} This situation presented an ideal opportunity for studies of thioester hydrolysis involving metal complexes of known metal ion content.

We began our investigations by testing the thioester hydrolysis reactivity of the known binuclear Fe(III)Zn(II) complex \([\text{BPBMPM}]\text{Fe(III)Zn(II)(\mu-OAc)}_2\text{ClO}_4\cdot\text{H}_2\text{O}\). The ability of this complex to promote thioester hydrolysis was evaluated in CH\textsubscript{3}CN:H\textsubscript{2}O.
(50:50; buffered) at 26.5 °C over the pH range of 7-9. A deuterium labeled thioester (hydroxyphenyl thioacetic acid S-methyl(d₃) ester) was used, as it possess an α-hydroxy group and an aliphatic leaving group (-SCD₃) similar to the aliphatic glutathione-containing substrate hydrolyzed by GIxII enzymes. Thioester hydrolysis was monitored by following the loss of the thioester –SCD₃ resonance signal over time, using ²H NMR as a monitoring method. The products generated were identified as CD₃SSCD₃ and CD₃SH, with the formation of CD₃SSCD₃ resulting from a proposed redox reaction involving a Fe(III)-SCD₃ moiety.

Based on kinetic data and the use of other spectroscopic techniques (UV-vis and EPR), a mechanism for thioester hydrolysis promoted by the Fe(III)Zn(II) complex was put forth wherein: (1) a Zn(II)-OH abstracts a hydrogen atom from the α-hydroxy group of the thioester substrate, leading to the formation of a zinc-alkoxide species; (2) nucleophilic attack by terminal a Fe(III)-OH results in cleavage of the C-S bond; (3) leaving group stabilization by the Fe(III) center occurs via the formation of a Fe(III)-SCD₃ species; and (4) protonation of Fe(III)-SCD₃ moiety occurs to give CD₃SH, or a redox process is initiated involving the Fe(III)-SCD₃ species which leads to the formation of CD₃SSCD₃.

Based on a recent report by Crowder and co-workers that human GIxII contains an Fe(II)Zn(II) center but is catalytically active as a mononuclear Zn(II) enzyme,⁸ we investigated the thioester hydrolysis reactivity of a mononuclear Zn(II) complex. We discovered that the thioester hydrolysis reaction promoted by [(bpta)Zn](ClO₄)₂•0.5H₂O leads to formation of two products (CD₃SH and a zinc-thiolate complex). Interestingly, the
product distribution is highly dependent on the concentration of the metal complex present, with higher amounts of CD$_2$SH being generated at lower concentrations of metal complex.

Variable temperature kinetic studies enabled the determination of activation parameters for the thioester hydrolysis reaction promoted by [(bpta)Zn](ClO$_4$)$_2$·0.5H$_2$O. Our data indicates that the activation energy ($E_a$) for thioester hydrolysis involving an aliphatic leaving group is similar to the $E_a$ for hydrolysis reactions for aqueous OH$^-$ promoted thioester hydrolysis.

In sum, the research presented in Chapters 2 and 3 provides the first detailed mechanistic insight into thioester hydrolytic reactivity promoted by metal complexes of relevance to GlxII enzymes. These studies contribute to a better understanding of GlxII enzymes and the area of metal complex promoted hydrolysis reactions.

In Chapters 4 and 5 of this work, the importance of [EMIM]CrCl$_3$ is discussed in terms of its role in promoting the conversion of glucose to HMF, a small molecule of emerging importance in the area of renewable feedstock chemicals. We were successful in providing the first X-ray crystallographic characterization of a complex of the [EMIM]CrCl$_3$ analytical formulation.

References


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