Metallopeptides As Model Systems For The Study Of Cu(II)-Dependent Oxidation Chemistry

by

William Maung Tay

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
Department of Chemistry
College of Arts and Sciences
University of South Florida

Major Professor: Li-June Ming, Ph.D.
Steven H. Grossman, Ph.D.
Kirpal S. Bisht, Ph.D.
Peter Zhang, Ph.D.
Jun Tan, M.D., Ph.D.

Date of Approval:
April 1, 2008

Keywords: amyloid-β, bacitracin, histatin 5, copper (II), oxygen, catechol oxidation, antimicrobial peptides, Alzheimer's disease, hydrogen peroxide, kinetics, NMR spectroscopy

© Copyright 2008, William Maung Tay
DEDICATION

Overall, my graduate career had been a special journey and a very rewarding experience. In addition to the academic enrichment, I had the opportunity to gain life experiences and make life-long friends. None of these would have been possible without the strong support of my family. First and foremost, I would like to dedicate this work to my parents Ma Khin Win and Kian Chwan Tay, who sacrificed a lot to come to the United States in search of a better life for their children. Their strong will and dedication have been an inspiration all throughout my academic career. With this accomplishment, I hope to set an example in perseverance to my siblings as well as to future generations. I would also like to dedicate this accomplishment to my girlfriend Jennifer Wellborn for always being there during difficult times and loving me for who I am.

Next, I would like to make a special dedication to my grandfather, who is currently suffering from end-stage Alzheimer’s disease. He had always been a loving and caring family man of great character as well as a strong supporter of education. I would also like to extend my dedication to Jen’s late grandmother Mary Wellborn, who also succumbed to this debilitating disease. “Granny” was a devoted grandmother and is greatly missed by us.
ACKNOWLEDGEMENT

First, I would like to acknowledge Dr. Li-June Ming for being a great mentor and providing me with the opportunity to do research in his lab. Next, I must acknowledge my big sister Dr. Vasiliki Lykourinou and big brother Dr. Giordano F. Z. da Silva for their emotional support as well as professional guidance. I must also acknowledge my other sisters Dr. Brianne O’Leary, Dr. Brenda Held, and Erin for being supportive during difficult times. I would also like to acknowledge Kashmir Juneja, who is like a younger brother, for always providing a good laugh and being a great opponent in basketball. William Wagner, Alaa Hashim, and Justin Moses must be acknowledged for being great friends and colleagues, who have been very supportive in times of need. Finally, I would like to acknowledge my committee members Dr. Steven Grossman, Dr. Kirpal Bisht, Dr. Peter Zhang, and Dr. Jun Tan for their patience and guidance.
NOTE TO THE READER

Note to Reader: The original of this document contains color that is necessary for understanding the data. The original dissertations is on file with the USF library in Tampa, Florida.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>iv</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIST OF FIGURES</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>ix</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>xii</td>
</tr>
<tr>
<td>CHAPTER 1: COPPER-OXYGEN CHEMISTRY: A BRIEF OVERVIEW</td>
<td>1</td>
</tr>
<tr>
<td>1.1 Introduction</td>
<td>2</td>
</tr>
<tr>
<td>1.2 Cu Proteins</td>
<td>3</td>
</tr>
<tr>
<td>1.2.1 Dinuclear Copper Proteins</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 Mononuclear Copper Proteins</td>
<td>6</td>
</tr>
<tr>
<td>1.3 Biomimetic Synthetic Models Complexes</td>
<td>16</td>
</tr>
<tr>
<td>1.4 Metallopeptides</td>
<td>21</td>
</tr>
<tr>
<td>REFERENCES</td>
<td>24</td>
</tr>
<tr>
<td>CHAPTER 2: ANTIOXIDATIVE PROPERTIES OF A NATURALLY OCCURRING FLAVONOID, QUERCETIN: IMPLICATIONS TOWARD TREATMENT STRATEGIES FOR ALZHEIMER’S DISEASE</td>
<td>38</td>
</tr>
<tr>
<td>2.1 Amyloid–β</td>
<td>39</td>
</tr>
<tr>
<td>2.1.1 Flavonoids</td>
<td>49</td>
</tr>
</tbody>
</table>
CHAPTER 3: ELUCIDATION OF THE IN VITRO OXIDATION CHEMISTRY OF COPPER(II)–BACITRACIN COMPLEX

3.1 Bacitracin

3.1.1 Structure of Bacitracin

3.1.2 Metal Binding and Antibacterial Mechanism

3.2 Materials and Methods

3.2.1 Kinetic Studies

3.2.2 Optical Studies

3.2.3 Anaerobic Studies

3.2.4 DNA Cleavage Assay

3.3 Results and Discussion

3.4 Conclusion

REFERENCES
CHAPTER 4: ELUCIDATION OF THE IN VITRO OXIDATION CHEMISTRY OF COPPER(II)–HISTATIN 5 COMPLEX BY MEANS OF REACTIVITY STUDIES

4.1 Traditional Antibiotics

4.1.1 Antimicrobial Peptides

4.1.2 Candida albicans

4.1.3 Histatin 5

4.2 Materials and Methods

4.2.1 Kinetic Studies

4.2.2 Optical Studies

4.2.3 NMR Studies

4.3 Results and Discussion

4.4 Conclusion

REFERENCES

ABOUT THE AUTHOR
LIST OF TABLES

Table 2.1: Full assignment of the $^1$H NMR spectrum of the 1:1 Co$^{2+}$–Qr complex from 1D saturation transfer and 2D EXSY experiments and their corresponding T$_1$ relaxation times. 78

Table 2.2: Full assignment of the $^1$H NMR spectrum of the 1:1 Yb$^{3+}$–Qr complex from 1D saturation transfer and 2D EXSY experiments and their corresponding T$_1$ relaxation times. 89
LIST OF FIGURES

Figure 1.1: Proposed Mechanism for tyrosinase. 7

Figure 1.2: Crystal structure of two oxygen-binding functional units of Octopus hemocyanin (PDB ID: 1JS8). 8

Figure 1.3: Crystal structure of Cu,Zn-superoxide dismutase with the two metal center shown in ball and stick configuration (PDB ID: 2c9v). 10

Figure 1.4: Mechanism of Cu,Zn-superoxide dismutase. 12

Figure 1.5: Crystal structure of peptidylglycine α-hydroxylating monooxygenase (PDB ID: 3PHM). 14

Figure 1.6: Proposed mechanism for peptidylglycine α-hydroxylating monooxygenase and dopamine β-monooxygenase. 15

Figure 1.7: End-on versus side-on binding mode (top) and a bidentate (L^{Py1}) versus a tridentate (L^{Py2}) ligand (bottom). 19

Figure 1.8: Ligands used for the preparation of Cu complexes: TPA^{R} (Karlin); tren^{RR'} (Tolman); HB(3-tBu-5-iPrpz)_{3} (Kitajima). 20

Figure 2.1: Proposed mechanism for Cu^{II}-centered oxidation of catechol-containing substrates in the presence (A–D) and absence (F–H and B–D) of H_{2}O_{2}. 46

Figure 2.2: Proposed mechanism for the Cu^{II}-centered hydroxylation and oxidation of phenol-like substrate 47

Figure 2.3: Structure of quercetin (I), 5-hydroxyflavone (II), and catechin (III). 51

Figure 2.4: Quercetin inhibition of catechol oxidation by Cu^{II}Aβ_{1–20}. 60
Figure 2.5: Lineweaver-Burk analysis of quercetin inhibition of catechol oxidation by Cu$^{II}$$\alpha$$\beta_{1-20}$ and replot of the slope *versus* inhibitor concentrations.

Figure 2.6: Optical titration of Cu$^{II}$ to quercetin in DMSO.

Figure 2.7: Optical Job plot of Cu$^{II}$ binding by quercetin in DMSO.

Figure 2.8: Optical titration of Co$^{II}$ to quercetin in DMSO.

Figure 2.9: Optical Job plot of Co$^{II}$ binding to quercetin in DMSO.

Figure 2.10: Optical Co$^{II}$ titration of catechin and 5-hydroxyflavone.

Figure 2.11: 1D NMR spectrum of Co$^{II}$-quercetin-triethylamine in a 1:5:1.5 ratio in $d_6$–DMSO.

Figure 2.12: The 2D $^1$H EXSY spectrum of the 1:1 Co$^{II}$–quercetin complex in $d_6$-DMSO.

Figure 2.13: 1D saturation-transfer results for the Co$^{II}$-quercetin complex.

Figure 2.14: Optical titration of Ca$^{II}$ to quercetin in DMSO.

Figure 2.15: Calcium influence on quercetin inhibition of catechol oxidation by Cu$^{II}$$\alpha$$\beta_{1-20}$.

Figure 2.16: 1D NMR spectrum of Yb$^{III}$-quercetin-triethylamine in a 1:1:0.7 ratio in $d_6$–DMSO.

Figure 2.17: The 2D $^1$H EXSY spectrum of the 1:1 Yb$^{III}$–quercetin complex in $d_6$-DMSO.

Figure 2.18: The 2D $^1$H EXSY spectrum of the expanded region of ~12 to –4 ppm for Fig. 2.17 of the 1:1 Yb$^{III}$–quercetin complex in $d_6$-DMSO.

Figure 2.19: Optical titration of Yb$^{III}$ to quercetin at 20.0 $\mu$M in DMSO.

Figure 2.20: Optical Job plot of Yb$^{III}$ binding to quercetin in DMSO.
Figure 2.21: Ytterbium influence on quercetin inhibition of catechol oxidation by CuII-Aβ1-20.

Figure 3.1: Structures of bacitracin congeners (adopted from ref. 2).

Figure 3.2: Oxidative cleavage of 225 ng of plasmid DNA by 25.0 μM CuII-bacitrcin with 0.05% H2O2 in 100.0 mM HEPES buffer at pH 7.0 and 25 ºC.

Figure 3.3: Catechol oxidation by CuII-bacitracin in 100.0 mM HEPES at pH 7.0 and 25 ºC.

Figure 3.4: 4,5–dichlorocatechol (DCC) binding study of CuII-bacitracin in DMF.

Figure 3.5: Optical Job binding study of DCC toward the CuII-bacitracin complex.

Figure 3.6: Interaction between the CuII-bacitracin and catechol using the Job method.

Figure 3.7: The oxidation of 2.5 mM catechol by 100.0 μM CuII-bacitracin under anaerobic condition.

Figure 3.8: Addition of air into anaerobic sample containing 100.0 μM CuII-bacitracin and 2.5 mM catechol.

Figure 3.9: Hydrogen peroxide influence on catechol oxidation by 2.0 μM CuII-bacitracin at pH 7.0 in 100.0 mM HEPES buffer at 25ºC.

Figure 3.10: Hanes analysis of oxidation of catechol by CuII-bacitracin at different concentrations of H2O2 (kinetic data from Figure 3.9 right).

Figure 3.11: ZnII (A), CoII (B), and NiII (C) dilution of CuII for the analysis of mononuclear versus dinuclear metal center in the catalysis of catechol oxidation by CuII-bacitracin.

Figure 3.12: Proposed mechanism for catechol oxidation by CuII-bacitracin
through a mononuclear Cu$^{II}$-centered catalysis.

Figure 4.1: Optical Cu$^{II}$ binding study of histatin 5 (200.0 μM) in 100.0 mM HEPES buffer at pH 7.0 and 25 ºC.

Figure 4.2: The continuous-wave (CW) EPR spectra of Cu$^{II}$-histatin 5 complexes in DMF.

Figure 4.3: NMR spectra of Co$^{II}$-histatin 5 at 2:1 (bottom) and 6:1 (top) ratios with 8k scans.

Figure 4.4: Catechol oxidation by Cu$^{II}$-histatin 5 (4:1) in 100.0 mM HEPES at pH 7.0 and 25 ºC.

Figure 4.5: Cu$^{II}$ binding of histatin 5 monitored with catechol oxidation activity.

Figure 4.6: Cu$^{II}$ binding of Hn5 monitored with catechol oxidation activity.

Figure 4.7: 4,5-dichlorocatechol binding study of Cu$^{II}$-Hn5 in DMF.

Figure 4.8: Hydrogen peroxide influence on catechol oxidation by 0.2 μM Cu$^{II}$-histatin 5 at pH 7.0 in 100.0 mM HEPES buffer at 25 ºC.

Figure 4.9: Hanes analysis of oxidation of catechol by Cu$^{II}$-Hn5 at different concentrations of H$_2$O$_2$ (kinetic data from Figure 4.7).
LIST OF ABBREVIATIONS

Cu$^{II}$: Copper(II)
Co$^{II}$: Cobalt(II)
Ni$^{II}$: Nickel(II)
Fe$^{III}$: Iron(III)
Zn$^{II}$: Zinc(II)
ROS: Reactive Oxygen Species
AD: Alzheimer’s Disease
Aβ: Amyloid-β
Ca$^{II}$: Calcium
Yb$^{III}$: Ytterbium
AMPs: Antimicrobial Peptides
Qr: Quercetin
Cat: Catechin
$V_{max}$: Maximum Velocity
$K_M$: Michaelis-Menten Constant
$K_A$: Affinity Constant
\( K_D: \) Dissociation Constant
\( K_i: \) Inhibition Constant
\( k_{cat}: \) Catalytic Turnover
ATP: Adenosine 5' Triphosphate
EDTA: Ethylenediaminetetraacetic acid
\( \text{H}_2\text{O}_2: \) Hydrogen Peroxide
\( \text{O}_2: \) molecular oxygen or dioxygen
Be: Bacitracin
Hn: Histatins
Hn5: Histatin 5
UV-Vis: Ultraviolet-Visible
nm: Nanometer
ppm: Parts-per-million
Hz: Hertz
\(^1\text{H} \text{NMR:}\) Proton Nuclear Magnetic Resonance
EPR: Electron Paramagnetic Resonance
ORD: Optical Rotatory Dispersion
EXAFS: Extended X-ray Absorption Fine Structure
DFT: Density Functional Theory
\( d_6\)-DMSO: deuterated Dimethyl Sulfoxide
HEPES: N-(2-HydroxyEthyl)-Piperazine-N'-2-EthaneSulfonic Acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>MES:</td>
<td>2-Morpholino-Ethanesulfonic Acid</td>
</tr>
<tr>
<td>MBTH:</td>
<td>3-Methyl-2-BenzoThiazolinone Hydrazone Hydrochloride</td>
</tr>
<tr>
<td>CA:</td>
<td>Catechol</td>
</tr>
<tr>
<td>DTBC:</td>
<td>Di-Tert-Butyl Catechol</td>
</tr>
<tr>
<td>DCC:</td>
<td>Di-Chloro-Catechol</td>
</tr>
<tr>
<td>MALDI-TOF:</td>
<td>Matrix Assisted Laser Desorption Mass Spectrometry Time Of Flight</td>
</tr>
<tr>
<td>CD:</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>DI:</td>
<td>Deionized Water</td>
</tr>
<tr>
<td>TEA:</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>5HF:</td>
<td>5-hydroxyflavone</td>
</tr>
<tr>
<td>CN:</td>
<td>Catechin</td>
</tr>
</tbody>
</table>
Copper is one of the essential metal ions for aerobic organisms. Two well known functions of copper in the biological systems are electron transfer and molecular oxygen interaction. Thus, this metal can be found in haemocyanin, an oxygen carrier protein, and superoxide dismutase, an enzyme that involves in electron transfer. In addition, having a positive redox potential allows copper to be involved in redox chemistry. It is the redox properties of copper that are responsible for many important biochemical processes. Although the copper-containing oxidases have been well studied over the years, certain mechanistic details such as reaction intermediates remain to be elucidated. Several research groups have been trying to study this by trying to mimic the native systems, synthesizing bulky organic molecules with copper-binding and oxidative capabilities. However, these model systems are only applicable in organic solvents at low temperatures. In this study, three naturally occurring peptides, amyloid-β, bacitracin, and
histatin 5, have been shown to display the oxidative chemistry when complexed with Cu$^{II}$. A combination of spectroscopic (UV-Vis and NMR) and reactivity was used in studying their metal-binding properties as well as in elucidating their catalytic mechanism.
1.1 Introduction

Copper is essential for various biological processes, but can be very toxic when presence in excess. Some of the biological processes that involve Cu are antioxidant defenses, pigmentation, mitochondrial respiration, neurotransmitter synthesis, connective tissue formation, peptide amidation, and iron metabolism.\(^1\) As a result, it is strictly regulated. Disturbances in this delicate balance can lead to detrimental disorders such as Menkes (i.e. Cu deficiency)\(^2\) and Wilson (i.e. Cu excess) disease.\(^3\) In the case of Cu deficiency, the inability to incorporate this metal into important enzymes like dopamine \(\beta\)-monooxygenase and Cu,Zn-superoxide dismutase reduces the body’s ability to synthesize neurotransmitters and defend against oxidative stress, respectively. Thus, the clinical symptoms of Menkes disease are neurological defects (such as severe metal retardation and neurodegeneration), growth retardation, hypothermia, and hypopigmentation.\(^4\) On the other extreme, the complications in Wilson disease are due to inability to remove Cu, leading to accumulation of Cu in the liver and brain. As a result, the victim typically suffers from liver failure and neurodegeneration.\(^5\)

The toxicity of Cu is partially associated with its potential to bind and activate dioxygen (\(O_2\)), leading to generation of reactive oxygen species (ROS).\(^6\) Ironically, the same harmful chemical property of Cu is the one that has enabled us to survive and evolve into multicellular organisms under the oxidizing conditions of the Earth’s atmosphere.\(^7\) This is evident in the essential involvement of Cu in \(O_2\) transfer (i.e. hemocyanin), energy production (i.e. cytochrome oxidase), and antioxidation (i.e. superoxide dismutase).
Cu,Zn-superoxide dismutase). The versatility and reactivity of Cu is largely owing to its flexibility in ligand binding by both possible oxidation states (i.e. Cu$^I$ and Cu$^{II}$). Thus, it is essential to understand the redox chemistry of Cu with respect to how the O$_2$ is activated in order to better understand its pathological roles.

In this respect, a general overview of Cu-O$_2$ chemistry on selected dinuclear and mononuclear Cu centers in biological systems will be discussed herein. Then, a brief discussion of progresses in the study of Cu-O$_2$ chemistry using biomimetic approach through synthetic model complexes will follow. Finally, the chapter is concluded with an introduction to metallo-peptides with a relevance to Cu oxidation chemistry.

1.2 *Cu Proteins*

Copper in biological systems can be classified into three groups named type I, type II, and type III.$^{20c}$ Type I Cu consists of a group of small mononuclear Cu-containing proteins known as the “blue Cu proteins”, owing to their visible intense blue color. This intense color is a result of a ligand-to-metal charge transfer between sulfur of Cys to Cu$^{II}$.\textsuperscript{8} A typical ligand environment of type I Cu consists of two His, one Cys, and an axial Met through a distorted tetrahedral geometry. These blue Cu proteins are mainly involved in a single electron transfer processes. Type II Cu proteins contain a regular tetragonally distorted Cu ceter and can bind to a variety of amino acids to give different coordination geometry. These Cu proteins can participate in oxidation and oxygenation chemistry.\textsuperscript{19c} Type III Cu systems are composed of two Cu$^{II}$ bridged by
H$_2$O or OH$^-$, and the two Cu$^{II}$ ions are antiferromagnetically coupled. Type III Cu proteins can also be involved in oxidation and oxygenation chemistry.$^9$

1.2.1 **Dinuclear Cu Proteins**

*Tyrosinase, Catechol Oxidase, and Hemocyanin.* Tyrosinase is a type III di-Cu$^{II}$ protein ubiquitously expressed in nature.$^{10}$ The molecular weight of tyrosinase can vary from 14 to 43 kDa depending on the source.$^{11}$ In plants, sponges, arthropods, and many invertebrates, they are involved in wound healing and primary immune response.$^{12}$ In mammals, they are expressed in melanocytes of the retina and skin and are responsible for melanin formation.$^{13}$ Furthermore, this enzyme is the cause of fruit and vegetable browning.

By activating molecular oxygen, tyrosinase can exhibit two types of activities toward phenol-containing substrates: cresolase or monophenolase activity and catecholase or diphenolase activity. The di-Cu center can be in four different states: Cu$^I$Cu$^I$ (*deoxy*); Cu$^I$Cu$^{II}$ (*half met*); Cu$^{II}$-OH$^-$-Cu$^{II}$ (*met*); and Cu$^{II}$-O$_2$$^{2-}$-Cu$^{II}$ (*oxy*).$^{11}$ In the *met* state, the Cu$^{II}$ centers are bridged by OH$^-$; whereas, the O$_2$$^{2-}$ serves as a bridge in the *oxy* state. The cresolase activity requires the *oxy* state, while the catecholase activity can occur through either the *met* in the presence of H$_2$O$_2$ or *oxy* state. In the cresolase activity, the phenol substrate is hydroxylated at the *ortho* position to give a catechol, which can be further oxidized to *o*-quinone (catecholase activity).$^{11}$ A crystal structure of tyrosinase from *Streptomyces Castaneoglobisporus* is recently proposed with a small
protein bound to the active site.\textsuperscript{14} This protein named ORF378 is suggested to be important for crystallization and insertion of Cu\textsuperscript{II} ions into the active site of tyrosinase. The overall structure of tyrosinase is $\alpha$-helical and both Cu\textsuperscript{II} ions are coordinated by 3 histidine residues. From the crystal structure, the distances between different states are 4.1, 3.3–3.9, and 3.4 Å for the deoxy, met, and oxy states, respectively.\textsuperscript{14}

According to the proposed mechanism for tyrosinase (Figure 1.1),\textsuperscript{15} oxygen initially binds to the deoxy di-Cu\textsuperscript{II} center in a side-on binding mode, forming a peroxo-bridged di-Cu\textsuperscript{II} center. In the monophenolase or cresolase activity, the phenolic substrate binds to one of the Cu\textsuperscript{II} atoms in the axial position (Figure 1.1). The Cu\textsuperscript{II} center is then rearranged to place the ortho position of the substrate closer to one of the peroxo oxygen for hydroxylation.\textsuperscript{15} The phenolic substrate is proposed to be subsequently hydroxylated by mechanism consistent with electrophilic aromatic substitution, forming a diphenol or catechol. In the diphenolase or catecholase activity, the catechol substrate binds to both Cu\textsuperscript{II} at the same time and oxidized and released as o-quinone (Figure 1.1). Finally, the deoxy di-Cu\textsuperscript{I} site is regenerated by the release of bridging OH as water.\textsuperscript{15}

Catechol oxidase, also known as o-diphenol oxidase and 1,2-benzenediol oxygen oxidoreductase, catalyzes the conversion of catechol to o-quinone.\textsuperscript{16} This enzyme can be found in plant tissues, insects, and crustaceans.\textsuperscript{17} Like tyrosinase, the molecular weight of catechol oxidase ranges between 30 and 60 kDa, depending on the source. Based on extended X-ray absorption fine structure (EXAFS) and X-ray absorption near edge
structure (XANES) studies, the distances between the Cu\textsuperscript{II} ions are 2.9 and 3.8 Å for met and oxy states, respectively.\textsuperscript{18}

Hemocyanin, a structurally related protein to tyrosinase and catechol oxidase, is an oxygen carrier in arthropods and mollusks. Hemocyanin has also been proposed to form a large protein aggregate with up to 8 MDa.\textsuperscript{19} Although the di-Cu\textsuperscript{II} center is conserved among all three proteins, each protein has a different function. While hemocyanin is involved in O\textsubscript{2} transfer, both catechol oxidase and tyrosinase can perform the oxidation of catechol to o-quinone, whereas tyrosinase also has a monophenolase activity. The presence of an amino acid residue (which varies among different species) in the active site of hemocyanin and catechol oxidase has been proposed to be correlated to the inability to perform cresolase activity of tyrosinase.

1.2.2 Mononuclear Copper Proteins

Cu,Zn-Superoxide dismutase. Copper-zinc superoxide dismutase (Cu,Zn-SOD) is an enzyme containing a type-II mononuclear Cu center, essential for catalyzing the decomposition of the highly reactive superoxide (O\textsubscript{2}\textsuperscript{−}) to O\textsubscript{2} and H\textsubscript{2}O\textsubscript{2}.\textsuperscript{20} It is expressed in both bacteria and eukaryotes.\textsuperscript{21} In bacteria, this enzyme is found in the periplasmic space.\textsuperscript{21e,f} In eukaryotes, Cu,Zn-SOD is largely found in the cytosol, nuclei, lysosomes, peroxisomes, and intermembrane space of mitochondria.\textsuperscript{21a-d} However, most structural and mechanistic studies are done on the eukaryotic forms (yeast, bovine, and human) of the enzyme.\textsuperscript{22}
Figure 1.1. Proposed Mechanism for tyrosinase. The monophenolase activity (or phenol o-hydroxylation) only occurs through the oxy state of di-Cu center; whereas, the diphenolase (or catecholase activity) can occur through both the met and oxy states of the di-Cu center. This illustration is adopted from ref. 15.
Figure 1.2. Crystal structure of two oxygen-binding functional units of *Octopus* hemocyanin (PDB ID: 1JS8). The giant protein is composed of 10 subunits, and each subunit is consisted of 7 oxygen-binding functional units. The blue spheres represent Cu atoms, and the black spheres are oxygen atoms bound in a side-on mode.
The human form of Cu,Zn-SOD is composed of homodimers of 32 kDa, in which each has one Cu$^{II}$ and one Zn$^{II}$ atom$^{24}$. Each domain is composed of eight stranded $\beta$-sheets forming a Greek key $\beta$-barrel and three extended loop regions (Figure 1.3). In its oxidized form, the Cu$^{II}$ is penta-coordinated by four histidine residues (i.e. His-46, His-48, His-63, and His-120 in human form) in a distorted square planar coordination and a weakly axial-coordinated water at 2.5 Å away from the metal. The Zn$^{II}$ ion is located approximately 6.6 Å from the Cu$^{II}$, and the two metals are bridged by the imidazole nitrogens of His-63. The Zn$^{II}$ atom is coordinated by His-63, His-71, His-80, and Asp-83 in a nearly tetrahedral geometry$^{20}$. Furthermore, the active site is stabilized by extensive network of hydrogen bonds. The dismutation of O$_2^-$ to occur at the Cu$^{II}$ center.

However, the Zn$^{II}$ binding has been proposed to help position the amino acid residues Glu-132, Glu-133, and Lys-136 in the correct conformation for substrate recognition$^{25}$. In general, the dismutation process can be explained in two parts (Eq. 1 and 2). In the first part, Cu$^{II}$ is reduced to Cu$^{I}$ by one O$_2^-$ molecule. According to a proposed mechanism, the superoxide is initially non-specifically protonated to give a hydroperoxyl radical (HO$_2^\cdot$) (Figure 1.4)$^{26}$. Then, the bond between Cu$^{II}$ and the imidazole group is broken upon HO$_2^\cdot$ binding to the Cu$^{II}$ center. The hydroperoxyl radical is deprotonated upon binding, followed by the protonation of the imidazole nitrogen. Next, an electron is transferred to the Cu$^{II}$ center to afford Cu$^{I}$ and O$_2$ (Figure 1.4 c). In the second part, Cu$^{I}$ is reoxidized to Cu$^{II}$ by another HO$_2^\cdot$ molecule$^{26}$. Upon binding to the Cu$^{I}$,
Figure 1.3. Crystal structure of Cu,ZnSOD with the two metal center shown in ball and stick configuration (PDB ID: 2c9v).\textsuperscript{27} Zn\textsuperscript{II} and Cu\textsuperscript{II} are displayed in green and orange, respectively.
HO$_2^*$ is reduced to hydroperoxide while Cu$^I$ is oxidized to Cu$^{II}$. Finally, the hydroperoxide is protonated to give hydrogen peroxide by the proton from the imidazole group of His-63 (Figure 1.4 f). The deprotonated imidazole group then can bind to Cu$^{II}$ and bridge the two metal centers.

\[
\text{Cu}^{II}\text{Zn}^{II}\text{SOD} + O_2^- \rightarrow \text{Cu}^{I}\text{Zn}^{II}\text{SOD} + O_2 \quad (1)
\]

\[
\text{Cu}^{I}\text{Zn}^{II}\text{SOD} + O_2^- + 2H^+ \rightarrow \text{Cu}^{II}\text{Zn}^{II}\text{SOD} + H_2O_2 \quad (2)
\]

Amyotrophic lateral sclerosis (ALS) is a debilitating neurodegenerative disorder where 20–25% of familial ALS, has been associated with mutations in the Cu,Zn-SOD gene, sod1. Over 100 mutations of sod1 have been identified, which ranges from amino acid substitutions to frameshifts.$^{28}$ Furthermore, protein aggregates containing mutant Cu,Zn-SOD have been found from ALS patients, ALS transgenic mice, and cell culture model systems.$^{29}$ Currently, there is no cure for this devastating disease.

**Peptidylglycine α-Hydroxylating Monooxygenase (PHM) and Dopamine β-Monooxygenase (DβM).** Peptidylglycine α-hydroxylating monooxygenase (PHM) and dopamine β-monooxygenase (DβM) are another group of proteins with mononuclear Cu center.$^{30}$ While PHM hydroxylates C-terminal glycine-extended peptides, DβM converts dopamine to norepinephrine by hydroxylating the β carbon. Both enzymes are exclusively expressed in higher eukaryotes, and their primary sequences are 27% identical and 40% similar.$^{31}$ PHM and DβM can be found in the secretory vesicles of the pituitary gland$^{32}$ and the chromaffin granules of the adrenal gland,$^{33}$ respectively.
Figure 1.4. Mechanism of Cu,Zn superoxide dismutase. This illustration is adapted from ref. 4. Steps a–c constitute disproportionation on one O$_2^-$ molecule, where the oxidation state of Cu is 1+ and the imidazole nitrogen is protonated. Steps d–f account for catalysis of a second O$_2^-$ molecule.
Each enzyme contains two Cu\textsuperscript{I} sites, which are 11 Å apart (Figure 1.5).\textsuperscript{34} In PHM, the copper centers are named Cu\textsubscript{H} and Cu\textsubscript{M} (i.e. in DβM, Cu\textsubscript{H} = Cu\textsubscript{A} and Cu\textsubscript{M} = Cu\textsubscript{B}), and Cu\textsubscript{M} has been proposed to be the site of substrate and oxygen interaction. On the other hand, Cu\textsubscript{H} is suggested to be involved in electron storage and transfer. As a result, PHM and DβM are considered as mononuclear Cu proteins during their catalysis. Furthermore, the amino acids involved in Cu binding are highly conserved between them. The Cu\textsubscript{H} atom is coordinated by three histidine residues, whereas Cu\textsubscript{M} is bound by two histidine and a methionine residue.\textsuperscript{30, 34}

According to the proposed hydroxylation mechanism (Figure 1.6), the substrate binds close to the Cu\textsubscript{M} site and not at the metal center.\textsuperscript{30} Upon O\textsubscript{2} binding to Cu\textsubscript{M}, an electron is transferred from the Cu atom to O\textsubscript{2} to afford Cu\textsuperscript{II}O\textsubscript{2}\textsuperscript{−} species. Then, a proton-tunneling occurs from the substrate to the Cu\textsuperscript{II}O\textsubscript{2}\textsuperscript{−} complex to give Cu\textsuperscript{II}OOH (hydroperoxo) complex. At this stage, two pathways have been proposed. In the first proposal, the electron from Cu\textsubscript{H} reductively cleaves the hydroperoxo complex to give Cu\textsubscript{M}\textsuperscript{II}oxo radical, which reacts with the substrate-derived radical to give alcohol product.\textsuperscript{35} The second route involves a direct hydroxyl radical abstraction by the substrate-derived radical to afford Cu\textsubscript{M}\textsuperscript{II}oxo, which is then reduced by the electron transfer from the Cu\textsubscript{H} atom to afford Cu\textsubscript{M}\textsuperscript{II}OH.\textsuperscript{36} Finally, Cu\textsuperscript{I} is regenerated by ascorbic acid.\textsuperscript{30}
Figure 1.5. Crystal structure of peptidylglycine α-hydroxylating monooxygenase (PDB ID: 3PHM). The two Cu atoms are displayed in orange, and they are ~11 Å from each other. In addition, they are fully exposed to solvent and yet the electron transfer from CuH (right orange sphere) occurs to CuM (left orange sphere).
Figure 1.6. Proposed mechanism for peptidylglycine α-hydroxylating monooxygenase and dopamine β-monooxygenase. This illustration is adapted from ref. 30.
1.3 **Biomimetic Synthetic Model Complexes**

Even among the type III di-Cu proteins with a very well conserved active site, such as hemocyanin, catechol oxidase, and tyrosinase, nature has fine-tuned them to function differently. While the same di-Cu center is used for reversible O$_2$ transfer in hemocyanin, this active site is also applicable in both $\alpha$-hydroxylation of phenol to catechol and oxidation of catechol to $\alpha$-quinone in tyrosinase and the latter in catechol oxidase. These three are only a few of many Cu-containing proteins that are involved in oxidation and oxygenation chemistry. The interactions between O$_2$ and different types of Cu centers vary, and the reaction mechanism can become complicated with respect to the active site environment.

These structural and mechanistic complexities have long interested inorganic chemists such as the late Nobumasa Kitajima, William B. Tolman, Shinobu Itoh, and Kenneth D. Karlin just to name a few. Their approaches have been to elucidate the O$_2$ intermediates during catalysis by Cu proteins through synthetic organic complexes that mimic the protein active site. Since histidine is the most common ligand in Cu proteins, most of these synthetic ligands contain nitrogen, involved in Cu binding. Their findings have made significant contributions in understanding the O$_2$ chemistry of Cu proteins. A classic example is the very first crystal structure of a di-Cu$^{II}$-peroxo complex supported by the [$(\text{HB}(3,5\text{-iPr}_2\text{pz})_3)]_2$ ligand with O$_2$ bound in a side-on binding mode determined by Kitajima and co-workers in 1989.$^{38}$ Prior to his discovery, the end-on binding between the two Cu centers had been well accepted for O$_2$ binding to the active site of
hemocyanin. He revealed that oxygen binds between the two Cu centers in a side-on mode. His finding was also demonstrated 5 years later on oxy-hemocyanin from arthropod with X-ray structural determination. Since then, many ($\mu$-$\eta^2:\eta^2$-peroxo)Cu$_2^{II}$ complexes of different bidentate and tridentate nitrogen ligands have been synthesized and studied.

Although a catalytic mechanism of tyrosinase has been proposed, the O$_2$ transfer from the dinuclear Cu$_{II}$-peroxo center to phenol (i.e. o-hydroxylation or monophenolase activity) still has not been well explained. This step of the mechanism has been long investigated with dinuclear Cu$_{II}$ model systems. One common finding is the formation of C-C coupled dimer at the ortho position of phenol. This is the indication that phenoxy radicals have been generated. Since there are two possible O$_2$ binding modes, end-on bis($\mu$-oxo) and side-on ($\mu$-$\eta^2:\eta^2$), a bidentate (L$^{Py1}$) and a tridentate (L$^{Py2}$) ligand that give end-on and side-on complexes (Figure 1.7), respectively, are used to determine the hydroxylation step of the mechanism in a recent study. Using phenolate as a substrate in organic solvent at –94 °C, the dinuclear Cu$_{II}$-peroxo complex gives the oxygenated product catechol, whereas, using neutral phenol only resulted in the dimer product. However, the $\mu$-oxo complex does not give catechol regardless of the type of substrate used. By correlating the kinetic data to the oxidation potential of the substrate, the authors to propose the hydroxylation to occur through an electrophilic aromatic substitution mechanism.
Unlike dinuclear Cu-O2 complexes, the studies of mononuclear Cu-O2 complexes have a big short coming. This is due to a higher tendency of CuI and O2 to form stable dinuclear Cu-O2 complexes. However, a number of mononuclear Cu-O2 complexes have been prepared based on spectroscopic evidence and structure determined. Transient mononuclear end-on CuII-superoxo complexes with tripodal tetradeionate ligands such as TPA\textsuperscript{R} and tren\textsuperscript{R′R′} (Figure 1.8) have been observed to show a strong absorption at ~410 nm prior to assembling into (trans-\(\mu\)-1,2-peroxo)Cu\textsuperscript{II} \(_2\) complexes. The resonance Raman results from isotope-labeling experiments indicate these intermediates have stretching vibrations at ~1120 cm\(^{-1}\) (\(\nu = \text{^{16}O} - \text{^{16}O}\)) and ~60 cm\(^{-1}\) (\(\nu = \text{^{16}O} - \text{^{18}O}\)), which have been suggested to be consistent with the formation of CuII-superoxo. Kitajima and co-workers have successfully isolated and characterized a mononuclear side-on Cu\textsuperscript{II}-superoxo complex with the ligand HB(3-tBu-5-iPrpz)\textsuperscript{−} (Figure 1.8), a monoanionic tridentate ligand having a bulky alkyl substituents.

Other mononuclear Cu-O2 complexes have been proposed, but spectroscopic results alone are not enough to unambiguously assign the oxygen intermediates in some cases. In general, both the size of the attached groups and the hydrogen bonding interaction between the oxygen and ligand help stabilize the complex. Nevertheless, most of these mononuclear Cu-O2 complexes are inactive toward external substrates. Although slow, progress has been made in characterization of mononuclear Cu-O2 complexes with the help of a low-temperature stopped-flow technique.
Figure 1.7. End-on versus side-on binding mode (top) and a bidentate ($L^{Py1}$) versus a tridentate ($L^{Py2}$) ligand (bottom).43
Figure 1.8. Ligands used for the preparation of Cu complexes: \( \text{TPA}^R \) (Karlin);\(^{45b}\) \( \text{tren}^{R,R'} \) (Tolman);\(^{40b}\) \( \text{HB}(3\text{-}t\text{Bu}-5\text{-}i\text{Prpz})_3^- \) (Kitajima).\(^{46}\)
1.4  **Metallopeptides**

Recently, there is a new interest in the study of peptides with metal binding capabilities, owing to their versatile nature, in which *de novo* sequences can be generated to exhibit various functions. Some of their well known functions are DNA/RNA recognition and cleavage, heavy metal binding, antibacterial and antifungal activities, and fluorescent probes. These peptides contain one or more metal binding sites which increase the stability of the overall complex or provide the active site for various catalyses.

Cowan and co-workers have shown one of many potential applications of designed metallopeptides in DNA and RNA recognition and cleavage. In a recent study, they have constructed polypeptides ranging from 21–27 amino acid residues containing N-terminal ATCUN (Amino Terminal Cu\(^{II}\) and Ni\(^{II}\) binding) metal binding motif and C-terminal HIV Rev response element (RRE) RNA recognition peptide with a glycine linker with different lengths. Then, a green fluorescent protein is co-expressed with RRE RNA in the N-terminus of the DNA oligonucleotide sequence in pET-21 plasmid vector in *E. coli*. The Cu\(^{II}\) complexes of ATCUN-RRE RNA recognition peptides are included during the expression. The damage of RRE RNA is determined based on the decrease in fluorescence intensity, which reflects the population of translatable mRNA. Significant reduction in the fluorescence intensity is observed only in the presence of Cu\(^{II}\)-ATCUN-RRE RNA complexes and not ATCUN or RRE RNA sequences alone. In addition, Cowan and co-workers have shown the damage of
polynucleotides by Cu\textsuperscript{II} complexes of short ATCUN peptides is through oxidative cleavage.\textsuperscript{57}

In a study by Imperiali et al., the versatility and specificity of synthetic peptidyl templates are well exploited.\textsuperscript{55} A family of peptides is designed, modeled after the Zn finger domain,\textsuperscript{60} to be used as a fluorescent sensor specifically toward Zn\textsuperscript{II} ions. The chemosensor is composed of a Zn finger domain, an L-\(\beta\)-amino alanine, and a fluorophore. The L-\(\beta\)-amino alanine is essential for the incorporation of the fluorophore. Before removing the newly synthesized polypeptide, the L-\(\beta\)-amino alanine can be selectively deprotonated with a Pd\textsuperscript{II} catalyst under mild conditions.\textsuperscript{61} Then, the fluorophore can be coupled to L-\(\beta\)-amino alanine. Thus, incorporation of L-\(\beta\)-amino alanine in any part of the sequence also enables the attachment of the fluorophore at any desired part of the peptidyl sequence. Since Zn fingers have been known to undergo metal-induced reversible folding,\textsuperscript{62} the chelation can be sensitively detected by placing the fluorophore close to the hydrophobic residues, which potentially involve in the folding process. As a result, the binding of Zn\textsuperscript{II} ions to the Zn finger motif is reported to show great sensitivity. Due to the high Zn\textsuperscript{II} binding affinity of the Zn finger domain, the specificity can be achieved.\textsuperscript{55}

The above two studies clearly illustrate the versatility, specificity, and applicability of peptide templates in studies involving metal ions. In the same respect, I have proposed the use of three naturally occurring peptidyl systems in studying the Cu\textsuperscript{II}-O\textsubscript{2} chemistry with relevance to their biological activities in the following chapters.
peptides of interests are short N-terminal amyloid-β fragment, $\text{A}\beta_{1-20}$ (the full length peptide found in the plaques isolated from the brain of Alzheimer’s patients.), bacitracin (a metal binding peptide antibiotic with specificity toward Gram positive bacteria.), and histatins 5 peptide (a salivary peptide with high potency toward the opportunistic yeast *Candida albicans*). In chapter 2, the possible therapeutic effect of a naturally occurring flavonoid, quercetin, toward metal-centered oxidation chemistry of Cu$^{II}\text{A}\beta_{1-20}$ is presented. Then, in chapters 3 and 4 the oxidation chemistry of Cu$^{II}$ complexes of bacitracin and histatin 5 are discussed.
References


14 Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M.


38 Kitajima, N.; Fujisawa, K.; Moro-oka, Y. $\mu$-$\eta^2:\eta^2$-peroxo binuclear copper complex, $\left[\text{Cu(HB(3,5-pPr_2pz)}_3\right]_2(O_2)$. *J. Am. Chem. Soc.* **1989**, *111*, 8975–8976.


(e) Mahadevan, V.; Henson, M. J.; Solomon, E. I.; Stack, T. D. P. Differential reactivity between interconvertible side-


62 Eis, P. S.; Lakowicz, J. R. Time-resolved energy transfer measurements of donor-
acceptor distance distributions and intramolecular flexibility of a CCHH zinc finger
CHAPTER 2

ANTIOXIDATIVE PROPERTIES OF A NATURALLY OCCURRING FLAVONOID, QUERCETIN: IMPLICATIONS TOWARD TREATMENT STRATEGIES FOR ALZHEIMER’S DISEASE
2.1  *Amyloid-β*

Alzheimer’s disease (AD) is a debilitating neurodegenerative disorder that mainly affects the elderly population and currently without a cure. The average age-onset is 60, and the risk increases with aging. According to the National Institute of Health, as many as 4.5 million Americans suffer from this disorder.¹ While approximately only 5% of the population between the ages 65–74 has this disease, up to 50% have been estimated for the age group 85 and older. AD is characterized by a progressive deterioration of the brain; the average life-span of the diagnosed individuals is 5–10 years, although some may live up to 20 years. It is a memory disorder, initially characterized by inability to recall recent events. As the disease progresses, memory loss and cognitive impairments become a part of the patient’s daily life. AD was initially recognized by Dr. Alois Alzheimer in 1906. He discovered a noticeable amount of tissue masses accumulated in the brain of a female patient, suffering from a strange mental illness. These masses were later termed *amyloid-β plaques*.¹

Amyloid-β (Aβ) is a proteolytic product of amyloid precursor protein (APP), consisting of 39–42 amino acids, and aggregation of this peptide in the brain is the hallmark of Alzheimer’s disease.² APP is a membrane-bound synaptic protein, where the N- and C-terminal ends are extracellularly and intracellularly situated, respectively.³ In addition, APP has two evolutionarily related cousins, APLP-1 and APLP-2, in mammals.⁴ All three synaptic proteins are essential for developmental and postnatal functions, as have been shown by the double and triple knock-out studies in mice.
Cranial abnormalities and early postnatal lethality are two phenotypes common among all knock-out studies. Different proteolytic cleavage of APP by secretases decide the fate of Aβ. The sequential processing of APP by β- and γ-secretases results in the amyloidogenic and toxic form of Aβ, while the proteolytic product of α-secretase is considered non-toxic since this enzyme cleaves APP within the Aβ peptide. The full length peptide can be further cleaved into small soluble fragments (e.g. Aβ₃₋₁₆) from the N-terminal end by α- and β-secretases as well as insulin-degrading enzyme.

Despite extensive studies, the physiological function of Aβ is still unclear. Nevertheless, a commonality in the brains of postmortem AD patients is the presence of plaques, resulted from the aggregated Aβ peptide. The two most common forms of Aβ peptide are 1–40 and 1–42. While the former is found in larger quantity, the latter is suggested to be more toxic. The primary sequence of Aβ is as follows:

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV.

The Aβ peptide is amphipathic in nature, and its conformational state is influenced by a few factors, such as pH, temperature, and solvent environment. At pH < 4 or > 7, the peptide is suggested to remain as a monomeric form in α-helical or random coiled conformation. Within the pH range 4–7, however, it becomes less stable and tends to form oligomers, which eventually lead to aggregation. The N-terminal 20 amino acids are more hydrophilic and can adopt α-helical or random coil conformation in aqueous environment, depending on the conditions. The C-terminus is quite hydrophobic and
is suggested to form stable β-sheet structures with a high tendency to aggregate, regardless of the conditions and environment.\textsuperscript{10g}

Furthermore, the plaques have been shown to contain a high concentration of metal ions such as Cu\textsuperscript{II}, Fe\textsuperscript{III}, and Zn\textsuperscript{II}, determined to be 390, 940, and 1055 μM, respectively.\textsuperscript{11} Comparing to the age-matched controls, the concentration of these metal ions in the brain of AD patients have increased 6, 3, and 3 times for Cu\textsuperscript{II}, Fe\textsuperscript{III}, and Zn\textsuperscript{II}, respectively. All three metal ions can cause Aβ aggregations \textit{in vitro}; a process that is reversed by metal chelator such as EDTA.\textsuperscript{12} Thus, it is important to determine the interaction of Aβ with metals.

Contrary to exhaustive metal binding studies using numerous powerful spectroscopic techniques,\textsuperscript{13} the coordination environment is still under debate. The matter is complicated by experimental variations such as solvent system, pH, and the length of peptide. Nevertheless, it is generally accepted that only the N-terminal region (1–16 residues) of the Aβ peptide is involved in metal binding and that the affinity for the metal ion is in the micromolar range,\textsuperscript{13e, h} although an earlier study suggested an attomolar affinity, which mistakenly combined a coagulation constant into the affinity constant.\textsuperscript{13g} The N-terminus contains three histidine (at positions 6, 13, and 14) and several acidic residues (Asp-1, Glu-3, Asp-7, and Glu-11) that can potentially involve in metal binding. The involvement of all three His residues in Cu\textsuperscript{II} and Zn\textsuperscript{II} binding is universally accepted.\textsuperscript{13, 63} The lack of AD in rats clearly demonstrates the involvement of these histidine residues in AD, since the rat variant of Aβ peptide contains the following
three mutations: Arg-5 to Gly, Tyr-10 to Phe, and His-13 to Arg. The ability to bind metal is greatly reduced by the H13R mutation, and thus, rats do not suffer from AD. In addition to the histidine residues, several candidates have been proposed to be the fourth ligand in metal binding, including the amino group of the N-terminal Asp-1, the phenolate group of Tyr-10, and the carboxylate group of Glu-11.

According to the amyloid cascade hypothesis, the plaques are the culprits of neurodegeneration. However, this theory was quickly invalidated by postmortem brain analysis of AD victims. There is a discrepancy between the amount of aggregates present and the severity of deterioration. Closer analysis of the plaques led to evidence of a possible oxidative stress in the brain.

Metal ions in biological systems are closely regulated, especially redox-active metals such as Cu and Fe. The availability of Fe in cell involves two transporters, DMT1 (influx) and Ireg1 (efflux), and the extracellular transport and storage are responsible by high affinity binding transferrin and highly efficient ferritin, respectively. Similarly, the influx of Cu ions is performed by Ctr1, DCT1, and DMT1, while the excretion is controlled by Menkes (MNK) and Wilson (WND) ATPases. In addition, the intracellular Cu concentration is highly regulated by multiple chaperone proteins. However, these regulatory processes lose efficiency with aging, leading to accumulation of the unbound metal ions. Redox-active metals such as Cu and Fe can generate highly reactive hydroxyl (OH) radical through Fenton chemistry in the presence of molecular oxygen. This radical can attack and chemically
modify biological molecules such as lipid molecules in membranes, amino acids in proteins, and nucleic acids (DNA and RNA), rendering them dysfunctional.\textsuperscript{17}

A recent study displaying the ability of Aβ to generate hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), a reactive oxygen species (ROS), in the presence of biological reducing agents and Cu\textsuperscript{II} confirms the involvement of redox chemistry in the etiology of AD.\textsuperscript{25} In fact, there is direct evidence of oxidative damages in the Aβ plaques.\textsuperscript{17} It is possible that oxidative stress leads to neurodegeneration, and the plaques are the end result of a protective role played by the Aβ peptide.\textsuperscript{26} The same has been proposed for other aggregate-forming neurodegenerative disorders, such as Prion-related and Parkinson’s diseases.\textsuperscript{27} In this proposed theory, the Aβ peptide attempts to chelate and remove the accumulated free pool of metal ions. The situation is exacerbated instead when the Cu\textsuperscript{II}/Fe\textsuperscript{III} Aβ complexes themselves can act as the redox centers to generate ROS. The free and complexed peptides may be chemically modified and subsequently aggregated in the process.\textsuperscript{28} Recent findings show the ability of APP to bind and reduce Cu\textsuperscript{II}, suggesting a role in Cu\textsuperscript{II} regulation of this protein.\textsuperscript{29} In addition, APP has been shown to be neuroprotective \textit{in vivo}, further supporting the protective role of its C-terminal Aβ fragment.\textsuperscript{30} If the aggregated form of Aβ is the aftermath, the soluble or oligomeric form of the peptide is truly responsible for the redox chemistry. In fact, recent studies have shown the oligomeric Aβ peptide to be more toxic.\textsuperscript{31} This explains the lack of consistency between the disease stage and the amount of Aβ aggregate.
In addition to oxidative stress, interference in the intracellular Ca\textsuperscript{II} homeostasis by Aβ was also suggested to contribute to the pathology of AD.\textsuperscript{32} Even though the dysfunction in Ca\textsuperscript{II} homeostasis does not play a role in the overall neurodegeneration, it is responsible for the cognitive impairments in AD.\textsuperscript{33} In the hippocampus, Aβ aggregates have been shown to span lipid membrane, creating channels with poor ion selectivity.\textsuperscript{34} Moreover, the soluble forms of Aβ can cause membrane depolarization and impair Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, which can lead to an increase in Ca\textsuperscript{II} influx.\textsuperscript{35,36} The rise in the Ca\textsuperscript{II} level defects neurons and synapses, deters long-term potentiation in certain brain regions, and leads to impairments in learning and memory.\textsuperscript{37} Thus, regulation of redox-active metals as well as Ca\textsuperscript{II} influx in the hippocampus is essential toward possible prevention and treatment of AD.

We have recently reported that the Cu\textsuperscript{II} complexes of the shorter proteolytic fragments, Aβ\textsubscript{1–16} and Aβ\textsubscript{1–20}, are able to perform two-electron oxidation of polyphenol, catechol, and phenol to their respective o-quinone products consistent with Type-3 copper oxidases.\textsuperscript{38,63,64} In the proposed catechol oxidase-like mechanism (Figure 2.1),\textsuperscript{63} the reducing substrate catechol can bind directly to the di-Cu\textsuperscript{II} center bridged by a hydroxyl group and readily oxidized into the corresponding ortho-quinone (Figure 2.1 steps F and G). This process involves 2e\textsuperscript{-} transfers, and the di-Cu\textsuperscript{II} is reduced to 2Cu\textsuperscript{I}. In the presence of dioxygen, the metals can resume its oxidized states by forming a dinuclear dioxygen-bound Cu\textsuperscript{II} complex, where the species present can be one of the three isoelectric states:
\( \mu - \eta^2 : \eta^2 - \text{peroxo} \rightarrow \text{Cu}^{2+}_2, \mu - \eta^1 : \eta^1 - \text{peroxo} \rightarrow \text{Cu}^{2+}_2, \text{or} \left( \mu - \text{oxo} \right)_2 \rightarrow \text{Cu}^{3+}_2 \) (B in Fig. 2.1). This peroxo-bridged di-Cu\(^{2+}\) center can go through another cycle of oxidation, before returning to its hydroxyl-bridged di-Cu\(^{II}\) following the concomitant release of one of the bound-oxygen as water (Figure 2.1 steps C and D). In addition, in the presence of the peroxo-bridged di-Cu\(^{II}\) can form directly from the hydroxyl-bridged species (Figure 2.1 steps A–D).\(^{63}\)

In the phenol-oxidase-like mechanism (Fig. 2.2),\(^{64}\) the phenol substrate initially binds to a Cu\(^{II}\) center, followed by a transfer of an electron to give a Cu\(^I\)-(phenol radical) intermediate (step a). The radical is stabilized with the unpaired e\(^-\) situated on the ortho and para positions. Next, the binding of dioxygen to the Cu\(^I\)-phenol radical results in another electron transfer to afford a Cu\(^{II}\) superoxide structure (step b), which is potentially stabilized by binding of a second Cu\(^{II}\) center. Finally, the \(o\)-quinone product is formed by the transfer of an electron and an oxygen atom to the semi-quinone (Figure 2.2 steps c, d). The possible involvement of di-Cu\(^{II}\) center was shown by diluting redox-active Cu\(^{II}\) with the redox-inactive Zn\(^{II}\).\(^{64}\)

We also observed catecholamine neurotransmitters can be effectively oxidized by Cu\(^{II}\)-Aβ with and without H\(_2\)O\(_2\).\(^{38}\) Thus, in the presence of Cu\(^{II}\) the Aβ peptide can potentially oxidize and deplete the catechol- and phenol-containing neurotransmitters (i.e. dopamine, serotonin, epinephrine, and norepinephrine) in the brain, causing neuronal death. The fact that oxidative stress is a metal-dependent process raises a new interest in metal-chelation therapy as a treatment for AD.
Figure 2.1. Proposed mechanism for Cu$^{ll}$-centered oxidation of catechol-containing substrates in the presence (A–D) and absence (F–H and B–D) of H$_2$O$_2$.\textsuperscript{63}
Figure 2.2. Proposed mechanism for the Cu$^{II}$-centered hydroxylation and oxidation of phenol-like substrate(s): (a) binding and electron transfer to the phenol substrate from the Cu$^{II}$ center to give a Cu$^{I}$-phenol radical; (b) dioxygen and a second Cu$^{II}$ center binding; (c) transfer of an electron and an oxygen atom to the phenol to result in an $\alpha$-quinone product (d).
One of the current treatment strategies toward AD rely on metal chelation as a key step in preventing the aggregated Aβ plaques from forming, such as the use of Cu\textsuperscript{II} chelator clioquinol (5-chloro-7-iodo-8-hydroxyquinoline).\textsuperscript{39} This drug was banned from its original usage in 1970 as an antibiotic due to a severe side effect, subacute myelo-optic neuropathy, in the central nervous system.\textsuperscript{40} This toxicity has been explained to be the result of Co\textsuperscript{II} chelation by clioquinol from Cobalamine, leading to vitamin B\textsubscript{12} deficiency. In a transgenic mice treatment study, clioquinol has been shown to alleviate up to 49% Aβ deposition, accompanied by a small increase in the concentration of soluble Aβ.\textsuperscript{41} A recent Phase II clinical trial involving 36 patients suggests this drug to be more effective toward those with greater disease severity. In this study, one of the clioquinol-treated subjects suffered from dysfunction in visual acuity and color vision with the symptoms go away upon discontinued treatment; two other patients suffered from leg numbness.\textsuperscript{42}

In addition, an earlier study by Yagi et. al. has shown clioquinol can induce lipid peroxidation in cultured chick retinal cells in the presence of iron.\textsuperscript{43} A recent study supports the pro-oxidant activity of this drug by the killing of cultured neuronal cells in the presence of redox-active Fe\textsuperscript{III} and Cu\textsuperscript{II}, and 90% cell viability was recovered in the presence of vitamin C and Trolox C.\textsuperscript{44} Thus, clioquinol, possessing these intrinsic toxicities, may not be suitable for use as a therapeutic treatment toward AD. Since oxidative stress in different regions of the brain is the underlying theme among the neurological pathologies, the possibility of using naturally occurring powerful
antioxidants from plants has become an increasingly popular interest. One family of these antioxidative secondary plant metabolites are flavonoids.

2.1.1 Flavonoids

Flavonoids are polyphenolic compounds ubiquitously distributed in plants. They display antimicrobial and antifungal properties, which largely contribute toward growth and development of plants. They show various beneficial biological activities such as antihepatotoxic, antiinflammatory, antiatherogenic, antiallergenic, antiosteoporotic, anticancer, and neuroprotective properties. These biological activities can be credited to their involvements in signaling, interaction with enzymes, and ability to scavenge free radicals and chelate metal ions. Flavonoids have gained intense interest because of their consumptions through fruits, vegetables, and related food products, which can be correlated to a better health (e.g., reduce the risk of cardiovascular diseases). Many in vitro studies have shown antioxidant and antitumor capabilities of flavonoids toward multiple cell lines. Moreover, they have a large variation in functional groups, rendering them useful for applications as templates toward future drug design. However, the molecular basis and mechanism of the antioxidation activity of the structurally diverse flavonoids and their capability of scavenging free radicals have not been conclusively revealed. In general, the phenoxy groups on the flavonoids have been associated with radical scavenging property. Thus, those with a higher number of hydroxyl substituents are considered better antioxidants.
Quercetin (Qr), 3,3',4',5,7-pentahydroxyflavone, is one of the extensively studied flavonoids with potent antioxidant activity (Figure 2.3 I).\textsuperscript{51,52} It is largely found in apple, onion, tea, red wine, blue berry, and grape.\textsuperscript{54} The structure of Qr contains a hydroxylated pyrone ring (C ring) conjugated to a \textit{meta}-diphenolic ring (A ring) and an \textit{ortho}-diphenolic ring (B ring). Like most flavonoids, Qr has the ability to scavenge free radicals and chelate metal ions. Studies have shown that Qr binds metal ion(s) in a bidentate manner.\textsuperscript{51b,d} There are three possible metal-binding sites on quercetin: β-keto-phenolate (4-carbonyl and 5-phenolate), α-keto-enolate (4-carbonyl and 3-enolate), and catecholate (3'- and 4'-phenolate) moiety (Figure 2.3 I). The metal-binding ability of Qr has been extensively studied with potentiometric and different spectroscopic techniques, using alkaline-earth (i.e. Ca\textsuperscript{II} and Mg\textsuperscript{II}), transition (i.e. Cu\textsuperscript{II} and Fe\textsuperscript{III}), and lanthanide (i.e. Yb\textsuperscript{III}) metal ions. Nevertheless, the coordination environment and metal binding modes have not reached consensus.\textsuperscript{51}

The redox-active metal dependent oxidative stress is proposed to be the culprit of neurodegeneration in the pathology of AD. We have confirmed this by showing the catechol- and phenol-oxidase-like chemistry of small Aβ fragments in the presence of redox-active Cu\textsuperscript{II} \textit{in vitro}.\textsuperscript{38,63,64} Thus Qr, with its radical-scavenging and metal-chelating properties, is one of many candidates as an inhibitor toward the metal-induced oxidative stress.\textsuperscript{55} Qr can potentially degrade the ROS generated by the oxygen-bound Cu\textsuperscript{II} center or can directly bind to the active Cu\textsuperscript{II} center to prevent further chemistry. The latter has been shown by the ability of Qr and related flavonoids in inhibiting the
Figure 2.3. Structure of quercetin (I), 5-hydroxyflavone (II), and catechin (III). There are three potential metal-binding sites on quercetin: β-keto-phenolate (4-carbonyl and 5-phenolate); α-keto-enolate (4-carbonyl and 3-enolate); and catecholate (3'- and 4'-phenolate), while 5-hydroxyflavone only has the β-keto-phenolate moiety and catechin only possesses the catecholate moiety for metal binding.
chemistry of tyrosinase, a dinuclear Cu\textsuperscript{II}-containing oxidase capable of converting phenol derivatives to o-quinone.\textsuperscript{56} Both Qr and kaempferol (a Qr analogue with a missing OH\textsuperscript{−} group at 3′ position) competitively inhibit tyrosinase activity, with an estimated IC\textsubscript{50} values were 0.13 and 0.23 mM, respectively.\textsuperscript{57,58} The mechanism of inhibition was suggested to be due to metal chelation at the active site.

Qr along with several other flavonoids have been suggested to have neuroprotective properties.\textsuperscript{59} To better understand the neuroprotective activity of Qr, the redox chemistry and coordination properties must be clearly revealed to provide a relationship between their structures and activities. In this study, the antioxidative mechanism of Qr toward the observed Cu\textsuperscript{II}-centered oxidation chemistry of A\textsubscript{β} was determined through optical, NMR, and reactivity studies. The A\textsubscript{β} peptide and aggregates have been proposed to cause a rise in the intracellular Ca\textsuperscript{II} concentration, leading to memory loss and cognitive impairments in AD patients. Thus, the metal binding properties of Qr and the influence of Ca\textsuperscript{II} on the antioxidative property of Qr was also determined.

2.2  \textit{Materials and Methods}

The N-terminal amyloid-β (A\textsubscript{β1-20}) was synthesized at the University of South Florida Peptide Synthesis and Mass Spectometry Center and the accuracy and purity checked with a MALDI-TOF mass spectrometer. Quercetin (Qr), Pyrocatechol, \textasciidewidth~99\% (CA), deuterated dimethyl sulfoxide d\textsubscript{6}-DMSO (99.9\%), and 3-methyl-benzyl
thiazolinone hydrochloride (MBTH), the lanthanide(III) chloride salts (99.99%) and CaCl₂ were purchased from Sigma-Aldrich Chemical Co. (Milwaukee, WI); CuSO₄ and 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer (HEPES) from Fisher Scientific Co. (Fair Lawn, NJ); and CoCl₂•6H₂O from Mallinckrodt (Paris, KY). All chemicals were used without further purification. All other solvents and reagents were the highest grade available from the commercial sources. The deionized water (18.2 MΩ) was obtained from a Millipore Milli-Q system. Plasticware and glassware were demetallized with EDTA and extensively rinsed prior to use. Due to the low solubility of Qr in aqueous solution, some experiments were performed in DMSO or d₆-DMSO. Both the metal and the ligand solutions were freshly prepared just prior to the experiments. Quartz cuvettes were used in all the kinetic and optical measurements.

2.2.1 **Kinetic Studies**

The 1:1 Cu²⁺Aβ₁–20 complex was prepared in deionized water. The oxidation of catechol by 1.0–3.0 μM Cu²⁺Aβ₁–20 in the presence or absence of different concentrations of Qr, Ca²⁺, and Yb³⁺ was monitored in 100.0 mM HEPES at pH 7.0 and 25 °C. Equal amounts of substrate and of the specific ω-quinone indicator, MBTH, were used in the assay.⁶⁰ All components were dissolved in DI water, and the final volume in the cuvette was fixed at 1.0 mL. The formation of the quinone product was followed photometrically (ε = 32,500 M⁻¹ cm⁻¹ for ω-quinone-MBTH complex)⁶⁰ at 500 nm with a Varian Cary50 spectrophotometer equipped with a water peltier PCB-150 thermostable cell.

53
(Varian, Palo Alto, CA), and the initial rate was determined from the slope of the change in the absorbance with respect to time (0–5 mins) in the linear region. Inhibition/activation of the reaction was performed with various amounts of an inhibitor/activator, and the rate determined and fitted to appropriate rate law to reveal the inhibition pattern. Sigma Plot 8.0 was used for plotting and fitting the data.

2.2.2 Optical Studies

All optical studies were performed in DMSO; triethylamine (TEA) was added as needed to enhance metal binding to Qr. The electronic spectra were acquired on the cary50 spectrophotometer. For metal-binding studies, 20.0 μM Qr was added with different concentrations of metal to a total volume of 1.0 mL, and the mixture was monitored from 200–800 nm. An optical Job plot can be utilized for revealing the metal-to-ligand stoichiometry, and has previously been demonstrated useful for the determination of complicated metal-drug binding stoichiometry. In the plot, a distinct absorption of the complex as a function of metal mole fraction (X_M) or ligand mole fraction (X_L) was obtained with a constant amount of the total concentration ([M] + [L]), wherein the ratio of X_M:X_L at which the absorption reaches the maximum in the plot reflects the stoichiometry of the M-L complex in solution. For all the Job plot studies, the metal and ligand concentrations were varied from 0–20.0 μM while keeping the overall concentration constant.
2.2.3 **NMR Studies**

1D and 2D $^1$H NMR spectra were acquired on a Varian INOVA500 spectrometer (at 500 MHz $^1$H resonance) with a 5-mm bio-TR (triple resonance) probe. A 90° pulse (~5 μs) was used for the acquisition of 1D $^1$H NMR spectra with 8 K data points, whereas 1024 × 512 data points were used for 2D EXSY (EXchanged SpectroscopY) experiment which reveals chemical exchange and the exchange pairs, with a presaturation pulse as well as superWEFT technique for solvent suppression. In 1D $^1$H saturation transfer experiments to reveal chemical exchange, a signal of the exchange partners is saturated with a presaturation pulse, and the different spectrum is obtained from the irradiated and the reference spectra (the presaturation pulse was applied at a signal-free region of the spectrum). A line-broadening of 40 Hz was applied to improve the signal-to-noise ratio of the paramagnetically shifted signals. The spin-lattice relaxation times ($T_1$) for the paramagnetically shifted signals were determined using the inversion-recovery method ($\tau_1$-180°-$\tau_2$-90°-FID). The peak intensities versus the $\tau$ values were fitted with a three-parameters fitting program on the spectrometer to afford the $T_1$ values.

2.3 **Results and Discussion**

The presence of Zn$^{II}$ along with redox-active Cu$^{II}$ and Fe$^{III}$ in amyloid plaques is evidence that AD is a metal-dependent pathology. Numerous studies have shown the possible involvement of Cu$^{II}$ and Fe$^{III}$ as the cause of oxidative stress, leading to damages that can potentially cause neurodegeneration in the brain. The ability of Cu$^{II}$Aβ$_{1-16}$ and
Cu\textsuperscript{II}A\textbeta\textsubscript{1–20} to perform catechol- and phenol-oxidase-like chemistry has been recently proposed.\textsuperscript{63, 64} The suggested metal-centered redox catalysis involves two electron transfers from the Cu\textsuperscript{II} complex to the substrate for the oxidation of catechol to ortho-quinone. In the presence of O\textsubscript{2} or H\textsubscript{2}O\textsubscript{2}, the continuation of oxidative chemistry is enhanced.\textsuperscript{63, 64} The ability of these Cu\textsuperscript{II}A\textbeta complexes to oxidize neurotransmitters, such as dopamine and serotonin, has been shown through \textit{in vitro} studies,\textsuperscript{38} suggesting a possible mechanism for the oxidative stress. Owing to the fact that oxidative stress is in this case metal-dependent chelation therapy has gained much interest in AD search.

Two well known attributes of Qr in terms of antioxidant activity are metal chelation and free radical scavenging. As a result, this natural metal chelator is a potential candidate for use as a therapeutic agent or a template for future drug design for the treatment of AD. In fact, several studies have indicated the neuroprotective role of this ubiquitous natural product.\textsuperscript{45} The structure of Qr has three potential metal-binding sites, rendering it a very effective metal chelator. In addition, the B ring of Qr has a catechol moiety, which may allow this flavonoid to potentially act as a suicide substrate. This, in turn, may prevent the depletion of catechol- and phenol-containing neurotransmitters, such as dopamine and serotonin, in the brain.

The hypothesis was tested by checking the influence of Qr toward the metal-centered oxidation of catechol by Cu\textsuperscript{II}A\textbeta\textsubscript{1–20}. The catechol oxidation was followed by monitoring the red adduct formed between the oxidized product, o-quinone, and MBTH, an o-quinone-specific indicator, at 500 nm. When different concentrations (0–153.6 \textmu M)
of Qr was incubated with 3.0 μM Cu^{II}Aβ_{1-20}, a sharp decrease in the oxidation activity toward 400.0 μM catechol was observed in a concentration dependent manner, and then followed by a less drastic decrease (Figure 2.4 inset). From this observation, Qr can effectively inhibit the oxidation chemistry of Cu^{II}Aβ_{1-20} at submicromolar concentrations, and at roughly 10.0 μM Qr, the loss of 50% activity is observed. A full inhibition study was performed in order to reveal the inhibition pattern (Figure 2.4).

Different concentrations of catechol were incubated with 3.0 μM Cu^{II}Aβ_{1-20} at several concentrations of Qr in 100.0 mM HEPES at pH 7.0 and 25 °C. At all concentrations of Qr, the data show an initial increase in catechol oxidation, followed by a saturation at higher substrate concentrations. This pattern suggests a possible pre-equilibrium kinetics, which is in agreement with previous studies. The pre-equilibrium kinetics (Eq. 1) can be described as follows:

\[
[Cu(II)-Amyloid] + [CA] \xrightleftharpoons[k_{-1}]{k_1} [Cu(II)-Amyloid-CA] \xrightarrow[k_{cat}]{\text{Kcat}} [Cu(II)-Amyloid] + o-quinone
\]

where \( v_0 = \frac{V_{Max}[CA]}{K_M + [CA]} \) (1) and \( V_{Max} \) are the initial and maximum velocity, respectively,

and \( K_M = \frac{k_{cat} + k_{-1}}{k_1} \) is the virtual dissociation constant of the Cu^{II}Aβ_{1-20}-CA complex.

At high concentration of the substrate, the activities of Qr are similar, suggesting similar maximum velocity values, \( V_{max} \). The dissociation constant, \( K_M \) when substrate
concentration is $\sim 0.5*V_{max}$, for the Cu$^{II}$Aβ$_{1-20}$-CA complex increases with increasing Qr concentration. These behaviors are typical to that of competitive inhibition.$^{65}$

In competitive inhibition, the substrate [CA] and the inhibitor [Qr] are considered mutually exclusive according to this relationship:

$$[\text{Cu(II)-Amyloid}] + [\text{CA}] \xrightarrow{K_{CA}} \text{[Cu(II)-Amyloid-CA]} \xrightarrow{k_{cat}} [\text{Cu(II)-Amyloid}] + o\text{-quinone}$$

$$[\text{[Qr]}]$$

The equation 2 below represents a competitive inhibition:

$$v = \frac{V_{max}[CA]}{K_{CA} + \frac{[Qr]}{K_{I}} + [CA]} (2),$$

where $K_{I}$ is the inhibition constant and $K_{CA}$ is the dissociation constant of the Cu$^{II}$Aβ$_{1-20}$-CA complex. The observed behaviors in the data can be described accordingly. The only difference between Eq. 1 and 2 is $K_M$ versus $K_{CA}\left(1 + \frac{[Qr]}{K_{I}}\right)$, and in the absence of inhibitor, $K_{CA} = K_M$. Thus, in competitive inhibition the $V_{max}$ values at all inhibitor concentrations are similar and changes are only seen in the apparent $K_M$ values.$^{65}$ The data at each Qr concentration was fitted to Eq. 2 to obtain
individual $K_I$ values. The fitted inhibition constant (the average of individual $K_I$ values) for the Qr inhibition of catechol oxidation by Cu$^{II}$Aβ$_{1-20}$ is 4.26 μM.

The data were also plotted and fitted linearly, as the Lineweaver-Burk plots ($1/v$ vs. $1/[\text{catechol}]$) (Eq. 3). The reciprocal form of the velocity equation for competitive inhibition is:

$$\frac{1}{v} = \frac{K_M}{V_{\text{max}}} \left(1 + \frac{[Qr]}{K_I}\right) + \frac{1}{V_{\text{max}}}$$

(Eq. 3)

where $K_I = -[Qr]$ when slope = 0 and $K_I$ is the inhibition constant which can be easily obtained from a secondary plot (Eq. 4). The point of convergence from the fitted lines can also suggest the type of inhibition present. The lines from the fits converge at the y-axis, suggesting a competitive inhibition (Figure 2.5 left).$^{65}$ This conclusion is in congruence with the above non-linear behaviors. From a secondary plot of the fitted slopes versus Qr (inhibitor) concentrations, an inhibition constant, $K_I$, of 4.24 μM, ($K_I = -x$-intercept) is determined, which is in agreement with the value from the non-linear fit (Figure 2.5 right). The observed competitive inhibition is in agreement with the inhibition of the di-Cu$^{II}$ center protein tyrosinase ($K_I = 38.6$ μM) by O$_2$. The inhibition of tyrosine by Qr was credited to the metal binding property of Qr.$^{56,57}$
**Figure 2.4.** Quercetin inhibition of catechol oxidation by Cu$^{II}$Aβ$_{1-20}$. Different concentrations of catechol (0–3.2 mM) were incubated with 3.0 μM Cu$^{II}$Aβ$_{1-20}$ with different concentrations of Qr (0–8.0 μM) in 100.0 mM HEPES pH 7.0 and 25°C. The inset is the Dixon plot or direct titration of Qr, monitored with the activity.
Figure 2.5. Lineweaver-Burk analysis of quercetin inhibition of catechol oxidation by Cu$^{II}$A$\beta_{1-20}$ (left) and replot of the slope versus inhibitor concentrations (right). The Lineweaver-Burk fitted lines intersect at $x = 0$, which is an indication of a competitive inhibition pattern. The inhibition constant, $K_i$, is equal to the negative of the $x$-intercept of the secondary plot of the slope from the plot on the right or a function of [Qr].
The mechanism of inhibition by Qr can be due to metal chelation or free radical scavenging. In our recent publication, we have shown the inability of mannitol and DMSO, hydroxyl and superoxide free radical scavengers, respectively, to stop the oxidation chemistry. Thus, the inhibitory action may be from the metal chelation property of Qr. Based on the observed competitive inhibition, it is possible that Qr binds to the Cu$^{II}$ center and prevents the substrate from binding. This may be proposed from the non-linear plots of the inhibition study, where the $K_M$ (approximately $0.5*V_{max}$), the binding or dissociation constant for the Cu$^{II}A\beta_{1-20}$-catechol complex, increases as the Qr concentration is increased (Figure 2.4). Qr may be competing against catechol in binding to the Cu$^{II}$ center. As a result, the binding between the catalyst and substrate is weakened as indicated by the increasing $K_M$ at higher Qr concentrations.

Metal-binding studies of Qr have been previously done, using multiple physical techniques. Qr has three potential metal-binding sites: β-keto-phenolate (4-carbonyl and 5-hydroxyl); α-keto-enolate (4-carbonyl and 3-hydroxyl); and catecholate (3’- and 4’-hydroxyl) (Figure 2.3). However, no consensus has been reached on the metal binding mode of Qr. Since the antioxidant activity of Qr may be due to metal-binding, it is important to determine which metal-binding site and how well this flavonoid can chelate the metal ion in solution. Thus, the metal binding by Qr was studied with UV-Vis and NMR spectroscopy in DMSO. Qr (20.0 μM) was slowly titrated with copper sulfate (CuSO$_4$) in DMSO and monitored from 200–800 nm. Triethylamine (TEA), was added as needed to ensure full deprotonation of all metal-binding sites.
The electronic spectrum of Qr in the absence of metal shows two absorptions at ~280 and ~380 nm (Figure 2.6); they have been previously assigned to the benzoyl (A ring including the β-keto-phenolate moiety) and cinnamoyl (C ring including the α-keto-enolate and catecholate moieties), respectively. Upon addition of CuII, a new absorption appears at 450 nm, accompanied by a decrease in the absorption of free ligand at 380 nm. This new absorption along with two isosbestic points at 325 and 410 nm indicates the formation of CuII-Qr complex (Figure 2.6). The CuII-to-Qr stoichiometry has been proposed from 1:1 up to 2:3, depending on the phase (i.e. solid vs. liquid) as well as solvent (i.e. EtOH vs. aqueous). In this study, the plot of the molar absorptivity value at 450 nm versus the equivalents of CuII added shows a saturation approximately at 1.2 equivalents of CuII in DMSO (Figure 2.6 inset). Then, the plot of the molar absorptivity value at 450 nm against CuII concentration can be fitted to a 1:1 metal-to-ligand binding quadratic equation to afford an affinity constant, $K_{Cu}$, of $1.06 \times 10^6$ M$^{-1}$ (Figure 2.6 inset).

The preferred stoichiometry between CuII and Qr was determined using the Job method. It is a continuous variation method that can suggest the optimum binding between a metal and a ligand or interaction between a catalyst and a substrate. In the optical Job study, the concentrations of CuII and Qr were arrayed while keeping the overall concentration constant. The molar absorptivity of the CuII-Qr complex at 450 nm
Figure 2.6. Optical titration of Cu$^{II}$ (0–2.0 equivalents) to Qr (20.0 μM) in DMSO. An isosbestic point at 410 nm along with an increase in the absorption at 450 nm indicates the formation of Cu$^{II}$-Qr complex. Fitting the absorbance values with respect to [Cu$^{II}$] affords an affinity constant, $K_{Cu}$, of $1.06 \times 10^6$ M$^{-1}$ (the inset).
was plotted against the mole fraction of Cu\textsuperscript{II} \((\frac{[\text{Cu}^{\text{II}}]}{[\text{Cu}^{\text{II}}]+[Qr]})\) (Figure 2.7 inset). The result shows an increase in the absorption of the complex with \(X_{\text{Cu(II)}}\) and reaches a maximum at \(X_{\text{Cu(II)}} = 0.5\), followed by the decrease (Figure 2.7 inset). The result can be fitted to a stoichiometry of 1:1 for the binding between Cu\textsuperscript{II} and Qr (Figure 2.7 inset). The slow electronic relaxation of Cu\textsuperscript{II} can broaden the NMR signals beyond detection, rendering it useless in the metal-binding study by means of NMR spectroscopy.

However, the fast relaxing Co\textsuperscript{II} has been effectively utilized as a paramagnetic NMR probe in studying the metal-coordination site of various systems.\textsuperscript{66} To ensure that Co\textsuperscript{II} binds Qr in a similar way to Cu\textsuperscript{II}, the optical studies were repeated with Co\textsuperscript{II}. A gradual addition of Co\textsuperscript{II} into 20.0 \(\mu\text{M}\) Qr was monitored with a spectrophotometer from 200–800 nm. Similar to the Cu\textsuperscript{II} binding study, a new absorption appears at 450 nm, accompanied by a corresponding decrease in the absorption at 380 nm. The absorption at 450 nm and the two isosbestic points at 305 and 410 nm suggest the formation of the Co\textsuperscript{II}-Qr complex in a single equilibrium (Figure 2.8). The affinity constant \(K_{\text{Co}}\) of \(5.58 \times 10^5 \text{ M}^{-1}\) was determined by fitting the plot of molar absorptivity value of the complex versus Co\textsuperscript{II} concentration (Figure 2.8 inset). The stoichiometry of the bound species was further determined with the Job method. Similar to the data from Cu\textsuperscript{II} binding study, a gradual increase in the absorption of the Co\textsuperscript{II}-Qr complex (450 nm) with \(X_{\text{Co(II)}}\) reaches a maximum at \(X_{\text{Co(II)}} = 0.5\), followed by a decrease (Figure 2.9 inset). This suggests that a 1:1 species is the preferred stoichiometry between Co\textsuperscript{II} and Qr under the experimental conditions.
Figure 2.7. Optical Job plot of Cu$^{II}$ (0–20.0 μM) binding by Qr (0–20.0 μM) in DMSO. The molar absorptivity values at 450 nm of the complex was plotted against the mole fraction with respect to the Cu$^{II}$ concentration (the inset). The data was fitted to a simple 1:1 metal-to-ligand binding with the r$^2$ value of 0.93.
Figure 2.8. Optical titration of Co$^{II}$ (0–12.8 equivalents) to Qr (20.0 μM) in DMSO. An isosbestic point at 405 nm along with an increase in the absorption at 450 nm indicates the formation of a Co$^{II}$-Qr complex. Fitting the absorbance values at 450 nm with respect to [Co$^{II}$] affords an affinity constant $K_{Co}$ of $5.58 \times 10^5$ M$^{-1}$ (the inset).
conditions herein. Overall, Qr binds Cu\textsuperscript{II} and Co\textsuperscript{II} similarly, and the decrease in the absorption at 380 nm for both studies suggests the possible involvement of \(\alpha\)-keto-enolate or catecholate moiety (the cinnamoyl group) in chelating metal ion.\textsuperscript{51b, c}

To further elucidate the preferred binding site among the three possible moieties on Qr in solution, the optical Co\textsuperscript{II} binding to 5-hydroxyflavone (5HF) and catechin (CN) were performed. While the structure of 5HF only has the \(\beta\)-keto-phenolate moiety for metal binding, the only functional group available for chelating metal on CN is the catecholate moiety (Figure 2.3). The electronic spectrum of free CN shows an absorption at approximately 285 nm. Upon Co\textsuperscript{II} addition to CN, a new absorption appears at 313 nm (an isosbestic point at 298 nm) with a corresponding minor decrease in the absorption at 285 nm, indicating the complexation between Co\textsuperscript{II} and CN (Figure 2.10 left). Similarly, the increase in a new absorption at 410 nm (with an isosbestic point at 368 nm) coupled with the decrease in the absorption of free 5HF at 335 nm upon gradual addition of Co\textsuperscript{II} marks the formation of a Co\textsuperscript{II}-5HF complex (Figure 2.10 right). The absorptions of the Co\textsuperscript{II}-catecholate (313 nm) and -\(\beta\)-keto-phenolate (410 nm) complexes can be clearly distinguished from that of the Co\textsuperscript{II}-Qr complex, which appears at 450 nm, indicating the Co\textsuperscript{II} binding site cannot be the \(\alpha\)-keto-phenolate and the catecholate site. By default, the Co\textsuperscript{II} binding site in Qr is \(\alpha\)-keto-enolate. However, the possibility of Co\textsuperscript{II}-\(\beta\)-keto-phenolate binding cannot be completely ruled out since the absorptions of the complexes are close. The metal-binding site was further confirmed by means of NMR spectroscopy.
Figure 2.9. Optical Job plot of Co$^{II}$ (0–20.0 μM) binding to Qr (0–20.0 μM) in DMSO. The plot of the molar absorptivity values at 450 nm versus the mole fraction with respect to the mole fraction of Co$^{II}$ fits well to a 1:1 species with the $r^2 = 0.95$ (the inset).
Figure 2.10. Optical Co$^{II}$ titration of catechin (left) and 5-hydroxyflavone (right). The new absorption at 313 nm for catechin and 410 nm for 5-hydroxyflavone along with their corresponding isosbestic points at 298 and 368 nm, respectively, indicates the formation of Co$^{II}$-ligand complexes.
NMR is a powerful spectroscopic technique for studying the coordination environment of paramagnetic metal ions, such as Co\textsuperscript{II}, Fe\textsuperscript{II}, and Ni\textsuperscript{II} as well as some lanthanide\textsuperscript{III} ions, in both simple complexes and metallo-proteins.\textsuperscript{66} Because of the overwhelming electron magnetic moment of the unpaired electron(s) in the paramagnetic systems (658 times that of the proton), the coordination environment is very sensitive to changes due to electron-nuclear hyperfine interaction. As a result, metal ions such as Co\textsuperscript{II} and Yb\textsuperscript{III} have been commonly utilized as paramagnetic NMR probes for studying the metal coordination environments in Zn\textsuperscript{II}- and Ca\textsuperscript{II}-containing metalloproteins, respectively, upon replacing the native metal ions in the proteins with Co\textsuperscript{II} and Yb\textsuperscript{III} ions.\textsuperscript{66} The same electron-nuclear hyperfine interaction is responsible for shifting the signals of the coordinated ligand and outside the typical diamagnetic spectral region (0–15 ppm). In addition, the fast electronic relaxation of these paramagnetic agents can enhance nuclear relaxation; thus, the relaxation time of the signals close to the metal ion is considerably shortened. The slow electronic relaxation of Cu\textsuperscript{II} (10\textsuperscript{-8}–10\textsuperscript{-11}s) can reduce the nuclear relaxation time and can lead to broadening of the signals beyond detection. The fast relaxing Co\textsuperscript{II} (10\textsuperscript{-11}–10\textsuperscript{-13}s), however, can afford relatively sharp shifted signals.\textsuperscript{66} Thus, Co\textsuperscript{II} was used in place of Cu\textsuperscript{II} for the determination of the metal binding site on Qr. A complete assignment of the \textsuperscript{1}H NMR spectrum of the Co\textsuperscript{II}-Qr complex is accomplished by the use of 1D and 2D saturation transfer NMR techniques,\textsuperscript{62,67} owing to the presence of exchange between the metal-bound and metal-free Qr according to the equilibrium, M + Qr \iff M–Qr.
The $^1$H NMR spectrum of Co$^{II}$-Qr acquired in $d_6$-DMSO displays 8 paramagnetically shifted signals (out of 10 protons) within the spectral region of 60 to –80 ppm (Figure 2.11). TEA was added as needed to ensure full deprotonation of all three metal-binding sites on Qr. The proton spectrum in Fig. 2.11 was acquired with the superWEFT (modified Water Eliminated Fourier Transform) ($\tau_1$-180°-$\tau_2$-90°-FID), a solvent suppression technique for paramagnetic systems that selectively suppresses the slowly relaxing diamagnetic signals. As a result, the diamagnetic signals appeared distorted.

An exchanging system allows the use of 2D saturation transfer experiment, EXSY (EXchanged SpectroscopY), for the assignment of paramagnetic signals once the diamagnetic free ligand signals are fully assigned. From the EXSY spectrum, 5 out of 8 shifted paramagnetic signals can be connected to their corresponding diamagnetic counterparts. The shifted signals at 17.0 and 16.3 ppm of the Co$^{II}$-Qr complex are in exchange with the signals of the 3’-OH and 4’-OH protons, respectively, of the catechol moiety of free Qr (3’ and 4’ cross peaks Figure 2.12). This assignment clearly shows that the catecholate moiety on Qr does not involve in metal binding, otherwise the 3 and 4 protons should not be detected since they deprotonate upon metal binding. This conclusion is in agreement with the finding from optical studies. The remaining three signals are correlated with 6-H, 8-H, and 5’-H protons (Fig. 2.12 and Table 2.1).
Figure 2.11. 1D NMR spectrum of Co(II)-(25.0 mM) Qr-TEA in a 1:5:1.5 ratio in $d_6$-DMSO. The spectrum was acquired with the spectral width of 110k (–100 to 100 ppm) on the Varian INOVA500 using superWEFT (Water Eliminated Fourier Transform) technique to selectively observe the fast-relaxing paramagnetically shifted signals over the diamagnetic ones. The signal intensities are quite different owing to their very different relaxation times which result in signal saturation to different extents. The inverted signals are due to “diamagnetic protons” far away from the paramagnetic metal center, which do not relax to gain positive intensity after the 180° pulse and the short delay.
The broadness of the three furthest shifted signals (56.4, 49.8, and –75.1 ppm) did not allow the assignment by 2D EXSY experiment. However, they can be assigned with 1D saturation transfer technique. The irradiation of the most upfield shifted signal at –75.1 ppm reveals saturation transfer to the signal at 12.5 ppm corresponding to 5-H of the hydroxyl proton of the β-keto-phenolate moiety on the structure of Qr. Furthermore, the protons of the two most downfield shifted signals at 56.4 and 49.8 ppm are in exchange with 2'-H and 6'-H protons, respectively (Figure 2.13). The solvent exchangeable hydroxyl OH signals can be easily identified with the addition of a few drops of D2O to the d6-DMSO solution. The OH signals are identified as the signals at 17.0, 16.3, and –75.1 ppm, correlating to 3'-H, 4'-H, and 5-H. They are in agreement with the assignments by 2D EXSY and 1D saturation transfer experiments.

After all the paramagnetic signals have been assigned, the two proton signals left unaccounted for are the hydroxyl protons at 7-H and 3-H. The former is a free-standing hydroxyl group and unlikely to be the preferred metal binding site. The latter, however, is the hydroxyl proton for the α-keto-enolate moiety, and the absence of a paramagnetic counterpart may indicate the deprotonation of the enol group and binding of the metal ion. On the other hand, the shifted signal for 5-H suggests that the β-keto-phenolate site is still protonated. The protons closest to the metal ion will be most affected by the electron-nuclear interaction, and they are expected to have larger paramagnetic shifts as well as shorter relaxation times. With the conclusion above that Qr binds CoII at the α-keto-enolate moiety, the protons in the closest proximity are the 5-H on the β-keto-
Figure 2.12. The 2D $^1$H EXSY spectrum of the 1:1:0.1 Co$^{II}$-(31.0 mM) Qr-TEA complex in $d_6$-DMSO. The data was acquired with the mixing time of 90 ms. The paramagnetic signals (marked with asterisks) of the complex are correlated to their diamagnetic counterparts, and the numbers next to the crosspeaks represent the protons according to Figure 2.3.
Figure 2.13. 1D saturation-transfer results for the Co$^{11}$-Qr complex. The difference spectra from the 1D $^1$H saturation transfer experiments for the three farthest shifted signals, $-75.1$, $49.8$, and $56.4$ ppm show connections with their corresponding diamagnetic protons ($7.5$, $7.6$, and $12.5$ ppm, bottom to top) of the 1:1 Co$^{11}$-Qr complex.
phenolate site and both 6'-H and 2'-H, since the single bond allows the rotation of the B ring (Figure 2.3). Thus, the 5-H, 2'-H, and 6'-H protons are expected to have the largest chemical shifts, –75.1, 49.8, and 56.4 ppm, respectively, in comparison to the remaining shifted signals. In addition, $T_1$ relaxation times for all shifted signals were determined with the standard inversion recovery method ($\tau_1–180–\tau_2–90$) and are reported in Table 1. As expected, 5-H, the closest proton to the Co$^{II}$ ion, has the lowest $T_1$ value (4.0 ms) among all, followed by the 6'-H (5.4 ms) and 2'-H (6.2 ms) signals. From the combined results, the metal binding on Qr can be unambiguously assigned to the $\alpha$-keto-enolate moiety.

Physiological studies have shown that soluble Aβ peptides of the sequence 1–40, 1–42, and 25–35 can increase the intracellular Ca$^{II}$ concentration in hippocampus, deteriorating learning ability and memory.33 Both the soluble and aggregated forms of the amyloid-β peptides can span lipid membrane which creates channels with poor ion selectivity35 and can cause membrane depolarization and impair Na$^+$/K$^+$-ATPase which leads to an increase in Ca$^{II}$ influx.36,37 The rise in the Ca$^{II}$ level can defect neurons and synapses, deter long-term potentiation in certain brain regions, and lead to impairments in learning and memory.32–37

As shown earlier, Qr is a potent inhibitor ($K_I = 4.24 \mu M$) against the catechol oxidation chemistry of Cu$^{II}$Aβ$_{1–20}$. A possible mechanism of inhibition is chelation of the Cu$^{II}$ center by Qr; this prevents the substrate from binding to the Cu$^{II}$ center.
<table>
<thead>
<tr>
<th>$^1$H Position</th>
<th>$\sigma_{\text{dia}}$ (ppm)</th>
<th>$\sigma_{\text{para}}$ (ppm)</th>
<th>$T_1$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6'-H</td>
<td>7.5</td>
<td>56.4</td>
<td>5.4</td>
</tr>
<tr>
<td>2'-H</td>
<td>7.6</td>
<td>49.8</td>
<td>6.2</td>
</tr>
<tr>
<td>5'-H</td>
<td>6.9</td>
<td>19.1</td>
<td>89</td>
</tr>
<tr>
<td>3'-OH</td>
<td>9.3</td>
<td>17.0</td>
<td>213</td>
</tr>
<tr>
<td>4'-OH</td>
<td>9.6</td>
<td>16.3</td>
<td>408</td>
</tr>
<tr>
<td>8-H</td>
<td>6.4</td>
<td>13.0</td>
<td>158</td>
</tr>
<tr>
<td>6-H</td>
<td>6.2</td>
<td>-4.3</td>
<td>134</td>
</tr>
<tr>
<td>5-OH</td>
<td>12.5</td>
<td>-75.1</td>
<td>4.0</td>
</tr>
</tbody>
</table>

**Table 2.1.** Full assignment of the $^1$H NMR spectrum of the 1:1:0.1 Co$^{II}$-(31.0 mM) Qr-TEA complex from 1D saturation transfer and 2D EXSY experiments and their corresponding $T_1$ relaxation times.
Alternatively, Qr can act as a suicide substrate and can be oxidized by the Cu$^{II}$Aβ$_{1-20}$ complex, competing with other physiologically important substrates. There are three possible sites on Qr for interacting with the metal ion, and the mode of binding can suggest the possible mechanism of inhibition. For example, if the catecholate moiety is the binding site, Qr is potentially oxidized since Cu$^{II}$Aβ$_{1-20}$ has been shown to have both catechol- and phenol-oxidase activity.$^{63, 64}$ The metal binding site of Qr has been determined to be at the α-keto-enolate moiety by means of optical and NMR spectroscopy discussed above. Thus, the antioxidative mechanism of Qr is possibly through chelation of the metal center. This finding is important since Qr is a naturally occurring antioxidant which may be applicable as a therapeutic agent against the redox metal-dependent oxidative stress in the pathology of AD.

Since Qr is a good metal chelator,$^{51}$ it may indiscriminately bind Ca$^{II}$ at the same site and blocks its binding to the metal in Cu$^{II}$Aβ$_{1-20}$. As a result, the increased intracellular Ca$^{II}$ concentration may reduce the antioxidative effect of Qr in the brain. To gain further information about the metal-binding versatility of Qr, the binding of the biologically significant Ca$^{II}$ to Qr was investigated. A new absorption at 450 nm and an isosbestic point at 405 nm (Fig. 2.14) are analogous to the transition metal complexes of Qr (Figs. 2.6 and 2.8), confirming the formation of Ca$^{II}$-Qr complex. Fitting of the molar absorptivity value (at 405 nm) as a function of Ca$^{II}$ concentration gives a smaller affinity constant $K_{Ca}$ of 530 M$^{-1}$. 

79
Figure 2.14. Optical titration of Ca$^{II}$ (0–819.2 equivalents) to Qr (20.0 μM) in DMSO. An isosbestic point at 405 nm along with an increase in the absorption at 450 nm indicates the formation of Ca$^{II}$-Qr complex. Fitting the absorbance values versus [Ca$^{II}$] affords an affinity constant, $K_{Ca}$, of $5.35 \times 10^2$ M$^{-1}$ (the inset).
The influence of Ca\textsuperscript{II} on the antioxidative activity of Qr was probed with respect to catechol oxidation. Several concentrations of Ca\textsuperscript{II} (0–160 mM) were incubated with 3.0 μM Cu\textsuperscript{II}Aβ\textsubscript{1–20} and a fixed concentration of catechol in the presence of 4.0 μM Qr in 100 mM HEPES at pH 7.0 and 25 °C. The catechol oxidation gradually increases with increasing Ca\textsuperscript{II} concentrations and eventually reaches a plateau at high millimolar (mM) concentrations of Ca\textsuperscript{II} (Figure 2.15 inset). The saturation profile potentially indicates that the presence of Ca\textsuperscript{II} is somehow promoting the substrate’s ability to interact with the redox Cu\textsuperscript{II} center, since Ca\textsuperscript{II} is a redox-inert metal. It is possible that Ca\textsuperscript{II} is interfering with the Cu\textsuperscript{II} center chelated by Qr. Even though the affinity of Qr toward Ca\textsuperscript{II} is not as strong as that of Cu\textsuperscript{II}, the binding and removing of Qr from the Cu\textsuperscript{II} center may occur in the presence of high concentrations of Ca\textsuperscript{II}. This is evident in the requirement of mM concentration in order to clearly see the effect.

The effect of Ca\textsuperscript{II} was further investigated with kinetics. Different concentrations of catechol (0–3.2 mM) were incubated with 4.0 μM Qr (K\textsubscript{i} concentration) and 3.0 μM Cu\textsuperscript{II}Aβ\textsubscript{1–20} with different concentrations of Ca\textsuperscript{II} (0–80.0 mM) in 100.0 mM HEPES pH 7.0 and 25 °C. At all concentrations of Ca\textsuperscript{II}, the data show an initial increase in catechol oxidation, followed by a saturation at higher substrate concentrations (Figure 2.15). This pattern suggests a possible pre-equilibrium kinetics, which indicates Ca\textsuperscript{II} binding to Qr. A rate law (Eq. 5) is derived according to the following relationship:
\[ \text{[Cu(II)-Amyloid] + [CA]} \xrightleftharpoons[k_{\text{Catechol}}]{K_{\text{Catechol}}} \text{[Cu(II)-Amyloid-CA]} \xrightarrow{k_{\text{cat}}} \text{[Cu(II)-Amyloid] + o-quinone} \]

\[ [\text{Qr}] + [\text{Cal}] \xrightleftharpoons[K_{\text{Cal}}]{K_{1}} [\text{Cal-Qr}] \]

\[ [\text{Cu(II)-Amyloid-Qr}] \]

\[ \nu = \frac{V_{\text{max}}[\text{CA}]}{K_{\text{Catechol}} \left( 1 + \frac{K_{\text{Cal}} [I]_0}{[\text{Ca}^\text{II}]_0 + K_{\text{Cal}}} \right) + [\text{CA}]} \]  \hspace{1cm} (5)

where \([I]_0 =\) experimental \([\text{Qr}], [\text{Ca}]_0 =\) experimental \([\text{Ca}^\text{II}], K_{1} = \text{Qr inhibition constant}, K_{\text{Catechol}} = \text{intrinsic (in the absence of Qr and Ca}^\text{II}) \text{dissociation constant of catechol, and } K_{\text{Cal}} = \text{dissociation constant for Ca}^\text{II}. \] The results are fitted to Eq. 5 to afford \(K_{\text{Cal}} = 0.263 \text{ M.}\) This large dissociation constant for \(\text{Ca}^\text{II}\) is consistent with the small affinity constant observed from the optical titration, which also suggests the influence of \(\text{Ca}^\text{II}\) on Qr inhibition of catechol oxidation chemistry by \(\text{Cu}^\text{II}A\beta\) is the result of binding to Qr.

Since the effect of \(\text{Ca}^\text{II}\) toward Qr inhibition of catechol oxidation by \(\text{Cu}^\text{II}A\beta_{1-20}\) is potentially due to binding to Qr and removing of Qr from the \(\text{Cu}^\text{II}\) center for oxidation, it is essential to know how Qr binds \(\text{Ca}^\text{II}\) in order to make a correlation between the structure and observed activity. Thus, the \(\text{Ca}^\text{II}\) binding mode of Qr was elucidated by means of NMR spectroscopy. Since \(\text{Ca}^\text{II}\) is spectroscopically inert, the lanthanide Yb\textsuperscript{III}
Figure 2.15. Calcium influence on quercetin inhibition of catechol oxidation by

Cu^{II}Aβ_{1–20}. Different concentrations of catechol (0–3.2 mM) were incubated with 4.0 μM

Qr at K_i concentration and 3 μM Cu^{II}Aβ_{1–20} with different concentrations of Ca^{II} (0–80.0

mM) in 100.0 mM HEPES pH 7.0 and 25 °C. The inset is the direct titration of Ca^{II} in

the presence of the inhibitor, Qr, monitored with the activity.
was used as a paramagnetic NMR probe. The lanthanides have similar radii and ligand binding preferences as Ca$\text{II}$ and thus are good Ca$\text{II}$ substitutes in biological systems.$^{69}$ Similar to the Co$\text{II}$ binding study, the $^1$HNMR spectrum of the Yb$\text{III}$-Qr complex shows the exchange between the free and metal-bound form of Qr. As a result, 1D and 2D saturation transfer experiments, such as 1D NOE difference and 2D EXSY, were used in the assignment of paramagnetically shifted signals. The $^1$H NMR spectrum displays 9 shifted signals within the range of 20 to –40 ppm (marked, Figure 2.16). Unlike the Co$\text{II}$-Qr spectrum, some signals of the Yb$\text{III}$-Qr complex appear within the diamagnetic spectral region (0–15 ppm). This is due to the difference between the transition and lanthanide metal ions in their electron-nuclear interaction. While the through-bond interaction mainly occurs in the transition metal complexes, only the through-space or dipolar interaction can take place in the lanthanide complexes, since the $f$ orbitals in the lanthanides are shielded and do not overlap with the ligand orbitals. Furthermore, the dipolar interaction only occurs in the presence of magnetic anisotropy, and the direction and magnitude of magnetic anisotropy determine the direction and magnitude of the dipolar shift.$^{66}$

From the EXSY spectra, 8 shifted signals of the Yb$\text{III}$-Qr complex can be assigned to their diamagnetic counterparts of metal-free Qr (Figures 2.17 and 2.18). Half of these signals are determined to be solvent-exchangeable OH protons based on the disappearance of signals upon D$_2$O addition. Among them, the signals at 19.2, 4.1, and
Figure 2.16. 1D NMR spectrum of Yb(III)-Qr-TEA in a 1:1:0.7 ratio in $d_6$-DMSO. The spectrum was acquired with the spectral width of –100 to 100 ppm on the Varian INOVA500 using superWEFT technique.
2.1 ppm are exchanging with the diamagnetic signals at 12.5, 9.6, and 9.3 ppm, corresponding to 5-H, 4'-H, and 3'-H on the structure of Qr (Figure 2.3). The one remaining signal at 6 ppm was assigned to 6-H with the 1D saturation transfer experiment. Similar to the results from the Co$^{II}$ binding study, the protons at the β-keto-phenolate (5-H) and catecholate (4'-H and 3'-H) sites are present, which indicate their noninvolvement in metal binding. On the opposite note, the absence of 3-H signal along with short T$_1$ relaxation times of the nearby protons (5-H, 6'-H, and 2'-H) clearly indicate the α-keto-enolate site to be the Yb$^{III}$ binding site. The exchange correlations along with the corresponding T$_1$ values are summarized in Table 2.2.

Based on the Yb$^{III}$ binding study, Qr may potentially bind Ca$^{II}$ at the same site as Cu$^{II}$ and Co$^{II}$, which is at the α-keto-enolate moiety. This may explain the effect of Ca$^{II}$ toward the Qr inhibition of catechol oxidation by Cu$^{II}$Aβ1–20. At sufficiently high concentration of Ca$^{II}$, it can compete with Cu$^{II}$ in binding to Qr, since Qr binds both Ca$^{II}$ and Cu$^{II}$ at the same site. As a result, the Cu$^{II}$ center of the Cu$^{II}$Aβ1–20 complex is made available for binding and oxidation of catechol. In order to confirm this hypothesis, the influence of Yb$^{III}$ on the Qr inhibition was determined with optical and kinetic studies. While the optical study ensures the similarity between Ca$^{II}$ and Yb$^{III}$ binding to Qr, the kinetic study will help to determine if the Ca$^{II}$ interference in the Qr inhibition is the result of its binding to Qr.

Consistent with the transition metals and Ca$^{II}$ binding studies, a new absorption appears at approximately 440 nm upon addition of Yb$^{III}$ to Qr (Figure 2.19). A
**Figure 2.17.** The 2D $^1$H EXSY spectrum of the 1:1:0.7 Yb$^{III}$-Qr-TEA complex in $d_6$-DMSO. The data was acquired with mixing time of 8.3 ms. The protons shifted the farthest with respect to the diamagnetic envelope, indicating their close proximity to the metal center.
Figure 2.18. The 2D $^1$H EXSY spectrum of the expanded region of ~12 to –4 ppm for Fig. 2.17 of the 1:1:0.7 Yb$^{III}$-Qr-TEA complex in d$_6$-DMSO. The data was acquired with a mixing time of 8.3 ms. The presence of a crosspeak for 3'-OH eliminates the catechol moiety from being a possible metal-binding site.
<table>
<thead>
<tr>
<th>$^1$H Position</th>
<th>$\sigma_{\text{dia}}$ (ppm)</th>
<th>$\sigma_{\text{para}}$ (ppm)</th>
<th>$T_1$ (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-OH</td>
<td>12.5</td>
<td>19.2</td>
<td>8.3</td>
</tr>
<tr>
<td>7-H</td>
<td>10.9</td>
<td>8.8</td>
<td>832</td>
</tr>
<tr>
<td>6-H</td>
<td>6.2</td>
<td>6.0</td>
<td>420</td>
</tr>
<tr>
<td>3'-OH</td>
<td>9.3</td>
<td>2.1</td>
<td>638</td>
</tr>
<tr>
<td>8-H</td>
<td>6.4</td>
<td>–1.1</td>
<td>554</td>
</tr>
<tr>
<td>5'-H</td>
<td>6.9</td>
<td>–3.5</td>
<td>339</td>
</tr>
<tr>
<td>2'-H</td>
<td>7.8</td>
<td>–24.6</td>
<td>32.1</td>
</tr>
<tr>
<td>6'-H</td>
<td>7.7</td>
<td>–32.2</td>
<td>24.3</td>
</tr>
</tbody>
</table>

Table 2.2. Full assignment of the $^1$H NMR spectrum of the 1:1:0.7 Yb$^{III}$-Qr-TEA complex from 1D saturation transfer and 2D EXSY experiments and their corresponding $T_1$ relaxation times.
corresponding decrease in the absorption of the free Qr in 380 nm is observed along with the isosbestic point at roughly 400 nm, which indicates the formation of Yb$^{III}$-Qr complex. Fitting the molar absorptivity at 440 nm versus Yb$^{III}$ concentration to a simple 1:1 binding pattern affords an affinity constant $K_{Yb} = 5.60 \times 10^5$ M$^{-1}$ (Figure 2.19, inset). The stoichiometry of the binding between Yb$^{III}$ and Qr was further determined with the Job method as described above. The plot of the molar absorptivity values at 440 nm versus the mole fraction of Yb$^{III}$ shows a maximum absorption intensity at $X_{Yb(III)} = 0.5$, which indicates the preferred stoichiometry to be 1:1 (Figure 2.20, inset).

The influence of Yb$^{III}$ on the antioxidative activity of Qr was further investigated with respect to catechol oxidation. Several concentrations of Yb$^{III}$ (0–600.0 μM) were incubated with 3.0 μM Cu$^{II}$Aβ1–20 and a fixed concentration of catechol in the presence of 4.0 μM Qr in 100.0 mM HEPES at pH 7.0 and 25 °C. An increase in catechol oxidation was observed with increasing Yb$^{III}$ concentration and reaches a plateau at high micromolar (μM) concentration.

The effect of Yb$^{III}$ was further investigated with kinetics. Different concentrations of catechol (0–3.2 mM) were incubated with 4.0 μM Qr ($K_f$ concentration) and 3.0 μM Cu$^{II}$Aβ1–20 with different concentrations of Yb$^{III}$ (0–600.0 μM) in 100.0 mM HEPES pH 7.0 and 25 °C. At all concentrations of Yb$^{III}$, the data show an initial increase in catechol oxidation, followed by a saturation at higher substrate concentrations (Figure 2.21). This pattern suggests a possible pre-equilibrium kinetics, which indicates Yb$^{III}$ binding to Qr. A rate law (Eq. 6) is derived according to the following relationship:
Figure 2.19. Optical titration of Yb\textsuperscript{III} (0–6.4 equivalents) to Qr (20.0 μM) in DMSO. An isosbestic point at ~400 nm along with an increase in the absorption at 440 nm indicates the formation of Yb\textsuperscript{III}-Qr complex. Fitting the molar absorptivity values with respect to the added Yb\textsuperscript{III} equivalent affords an affinity constant, $K_{Yb}$, of $5.60 \times 10^5$ M\textsuperscript{-1} (the inset).
\[
\begin{align*}
\text{[Cu(II)-Amyloid] + [CA]} & \xrightarrow{K_{\text{Catechol}}} [\text{Cu(II)-Amyloid-CA}] \xrightarrow{k_{\text{cat}}} [\text{Cu(II)-Amyloid} + o-quinone] \\
+ [\text{Qr}] + [\text{Yb}] & \xrightarrow{K_Y} [\text{Yb-Qr}] \\
\text{[Cu(II)-Amyloid-Qr]} & \xrightarrow{K_1} \text{[Cu(II)-Amyloid-Qr]} \\
\end{align*}
\]

\[
V = \frac{V_{\text{max}} [CA]}{K_{\text{Catechol}} + \left( \frac{K_Y [I]_0}{[Yb^{III}]_0 + K_Y} \right) + [CA]} \quad (6)
\]

where \([I]_0 = \text{experimental [Qr]}, [Yb^{III}]_0 = \text{experimental [Yb^{III}]}, K_I = \text{Qr inhibition constant, } K_{\text{Catechol}} = \text{intrinsic (in the absence of Qr and Yb^{III}) dissociation constant of catechol, and } K_Y = \text{dissociation constant for Yb^{III}}. \text{ The results are fitted to Eq. 6 to afford } K_Y = 4.85 \times 10^{-4} \text{ M. Compared to Ca^{II}, a smaller dissociation constant for Yb^{III} is expected since its affinity toward Qr is approximately 1000 times higher than that of Ca^{II} based on the affinity constants obtained from the optical titration experiments.}
Figure 2.20. Optical Job plot of Yb$^{III}$ (0–20.0 μM) binding to Qr (0–20.0 μM) in DMSO.

The plot of the absorbance at 440 nm versus the mole fraction of Yb$^{III}$ shows a maximum absorption intensity at $X_{Yb(III)} = 0.5$, which indicates the preferred stoichiometry to be 1:1 (the inset).
Figure 2.21. Ytterbium influence on quercetin inhibition of catechol oxidation by Cu$^{II}\text{A}_{1-20}$. Different concentrations of catechol (0–5.0 mM) were incubated with 4.0 µM Qr ($K_I$ concentration) and 3.0 µM Cu$^{II}\text{A}_{1-20}$ with different concentrations of Yb$^{III}$ (0–600.0 µM) in 100.0 mM HEPES pH 7.0 and 25°C. The inset is the direct titration of Yb$^{III}$ in the presence of the inhibitor, Qr, monitored with the activity.
2.4 **Conclusions**

It is originally thought that the aggregation of Aβ peptide is the cause of neurodegeneration in the pathology of Alzheimer’s disease.\textsuperscript{15} However, there is inconsistency in the amount of plaque present and the severity of neurodegeneration.\textsuperscript{16} Furthermore, the presence of high concentration of redox-active metal ions such as Cu\textsuperscript{II} and Fe\textsuperscript{III} in the plaques\textsuperscript{11} along with oxidized biological molecules\textsuperscript{17} indicates that the metal dependent oxidative stress may be a culprit of neuronal damages in the brain. As a result, the metal chelation therapy has become a center of attention. A good metal chelator such as clioquinol has been proposed.\textsuperscript{39,41} However, the testing of this drug has been stopped at the Phase II clinical trials due to its intrinsic toxicities.\textsuperscript{40–44}

Recently, naturally occurring flavonoids have gained the interests of researchers due to their biological properties.\textsuperscript{47} One of the most studied among them is Qr. Qr is well known for its free radical scavenging and metal chelating abilities.\textsuperscript{50,51} We have recently shown the ability of short N-terminal Aβ fragments (Aβ\textsubscript{1–16} and Aβ\textsubscript{1–20}, which contains the metal binding domain) to perform catechol- and phenol-oxidase-like chemistry in the presence of Cu\textsuperscript{II}.\textsuperscript{63,64} In this study, the antioxidant ability of Qr toward the observed oxidation chemistry is determined. Qr can potentially serve two purposes. It can be a metal chelator (block the Cu\textsuperscript{II} center and prevent the substrate from binding) or a suicide substrate (the C ring contains a catechol moiety, which can be oxidized). Based on kinetic studies, Qr has been determined to competitively inhibit catechol oxidation by Cu\textsuperscript{II}Aβ with a $K_i$ of 4.24 μM. Qr has three possible metal binding sites: β-
keto-phenolate, \( \alpha \)-keto-enolate, and catecholate moiety. The site of binding may suggest the mechanism of inhibition. For example, if the metal is bound through the catecholate moiety, Qr is potentially oxidized by Cu\( ^{II} \)A\( \beta \), acting as a suicide substrate. Thus, the metal binding studies were performed by means of optical and NMR spectroscopy. According to NMR results, the metal binding site can be unambiguously assigned to the \( \alpha \)-keto-enolate moiety, suggesting that the mechanism of inhibition by Qr may be due to Cu\( ^{II} \) center chelation. A recent study showing tyrosinase inhibition by Qr supports this conclusion.\(^{57}\)

The A\( \beta \) peptide has been shown to cause a disruption in Ca\( ^{II} \) homeostasis in AD, leading to the rise in intracellular Ca\( ^{II} \) concentration. Memory loss and cognitive impairments have been associated with an increase in Ca\( ^{II} \) level.\(^{32-37}\) Since Qr can bind metal indiscriminately, the presence of Ca\( ^{II} \) may interfere with its antioxidant ability, or on the other hand, the free Ca\( ^{II} \) can be chelated by Qr which may decrease the damage caused by the extra free Ca\( ^{II} \). As expected, the recovery of catechol oxidation by Cu\( ^{II} \)A\( \beta \) was observed in the presence of high concentration of Ca\( ^{II} \). The fact that the interference is due to Ca\( ^{II} \) binding was shown by using Yb\( ^{III} \) as a spectroscopic probe (for spectroscopically inert Ca\( ^{II} \)) in optical and NMR studies. Once again, the NMR results showed that Qr binds Yb\( ^{III} \) at the \( \alpha \)-keto-enolate site. This indicates that Ca\( ^{II} \) binds Qr at the same site as Cu\( ^{II} \) and thus releasing the Cu\( ^{II} \) center for catechol oxidation. These findings suggest that Qr may have dual protective roles in AD, preventing both the metal-
centered oxidative stress by binding to the metal in Cu\textsuperscript{II}Aβ as well as Ca\textsuperscript{II}-induced memory and cognitive impairments by chelating Ca\textsuperscript{II}.

Since Qr-containing fruits and vegetables make up a large part of our daily diets, this compound is relatively safe.\textsuperscript{47} Thus, Qr may be potentially used as a therapeutic agent toward AD. However, one main concern for the flavonoids is their bioavailability, especially for Qr. The hydrophobic nature of the polyphenolic structures greatly reduces its absorbability into the body. Furthermore, metabolic processes can potentially change or modify the chemical structure(s), leading to the reduction or loss of function.\textsuperscript{70} Another possibility that may contribute to this discrepancy is the inability of flavonoids to be at the places when they are needed, which includes being presence when they are not needed (e.g. when ROS homeostasis is well maintained) and possible interaction with biomolecules (e.g. hydrogen bonding through the hydroxyl group).\textsuperscript{71} As a result, the efficacies of naturally occurring antioxidants \textit{in vitro} cannot be directly translated to the situations \textit{in vivo}. Thus, further \textit{in vivo} studies in animal models are needed. Nonetheless, Qr may be a good starting point, serving as a template for future drug design.
References

1 National Institute on Aging, a division of U.S. National Institutes of Health.  


Heber, S.; Ring, S.; Fuhrmann, M.; Kretzschmar, H.; Sisodia, S. S.; Muller, U. Cortical
dysplasia resembling human type 2 lissencephaly in mice lacking all three APP family

6 Suh, Y. H.; Checler, F. Amyloid Precursor Protein, Presenilins, and α-Synuclein:
Molecular Pathogenesis and Pharmacological Applications in Alzheimer's Disease.

7 Evin, G.; Weidemann, A. Biogenesis and metabolism of Alzheimer's disease Aβ

8 Evin, G.; Zhu, A.; Holsinger, D.; Masters, C. L.; Li, Q. Proteolytic Processing of the
Res. 2003, 74, 386–392.

9 (a) Glenner, G. G.; Wong, C. W. Alzheimer's disease: Initial report of the purification
disease and Down's syndrome: Sharing of a unique cerebrovascular amyloid fibril

10 (a) Hou, L.; Shao, H.; Zhang, Y.; Li, H.; Menon, N. K.; Neuhaus, E. B.; Brewer, J.
M.; Byeon, I. J.; Ray, D. G.; Vittek, M. P.; Iwashita, T. Makula, R. A.; Brzybyla, A. B.;
Zagorski, M. G. Solution NMR Studies of the Aβ(1-40) and Aβ(1-42) Peptides
Establish that the Met35 Oxidation State Affects the Mechanism of Amyloid


56 Parvez, S.; Kang, M.; Chung, H.-S.; Bae, H. Naturally occurring tyrosinase inhibitors: mechanism and applications in skin health, cosmetics and agriculture industries. 


61 Polster, J.; Lachmann, H. *Spectrometric Titrations*; VCH: New York, **1989**.


CHAPTER 3

ELUCIDATION OF THE *IN VITRO* OXIDATION CHEMISTRY OF COPPER(II)–BACITRACIN COMPLEX
3.1 **Bacitracin**

Bacitracin (Bc) is an antibiotic peptide, isolated from *Bacillus subtilis* and *B. licheniformis*.[1, 2] It was first isolated from an open wound of a young girl named Margaret Tracey.[1] This antibiotic has come to be termed *bacitracin*. It is active primarily against Gram-positive bacteria, including *Staphylococcus*, *Streptococcus*, and *Clostridium difficile*.[3] In addition to its worldwide use in animal feed as a preventive drug for livestock,[4] Bc makes up one of the ingredients in the commonly used topical triple antibiotic ointments, Neosporin® and Polysporin®, along with polymyxin B and neomycin since 1956.[5] The three antibiotics have been suggested to work synergistically. This may be one of the reasons for the rare occurrences of bacterial resistance, despite more than 50 years of over-the-counter usage.[6] In addition, bacitracin has other well known functions, such as an inhibitor of disulfide isomerase[7] as well as platelet and multiple agonist aggregation.[8]

Although it is relatively safe for oral intake owing to the low absorption by the gastrointestinal tract, Bc can cause nephrotoxicity when given systemically.[3, 5] The minute intestinal absorption has been previously shown in animal studies.[9] It is generally safe for use in topical applications, but a growing concern in the topical usage of bacitracin is the increasing reports of allergic contact dermatitis.[10] A recent publication suggested this to be the result of improper or over usage of the drug (i.e. application on all surgical wounds in hospitals and on fresh tattoo wounds).[10b] The same study proposed white petrolatum to be used as an alternative for cleaning surgical wounds.[10b]
Furthermore, bacitracin is one of the world’s massively produced and used food additives for farm animals it is until recently banned in European countries, which is an act of precaution against bacterial resistance.\textsuperscript{11} Up to 60\% resistance by bacteria has been proposed,\textsuperscript{12} but a full resistance to the drug has not been reported over 5 decades of application. Thus, relatively speaking bacitracin resistance still is a rare occurrence, and this is possibly due to its unique structure.\textsuperscript{2}

3.1.1 \textit{Structure of Bacitracin}

Bacitracin is synthesized non-ribosomally by bacitracin synthetase ABC, a multi-enzyme complex consisted of BacA (598 kDa), BacB (297 kDa), and BacC (723 kDa). Each synthetase is responsible for the incorporation as well as chemical modification of different parts of the full-length peptide.\textsuperscript{13,14} This peptide is produced as a mixture of closely related peptides, possibly as many as 50 variants.\textsuperscript{15} Among the congeners, bacitracin A\textsubscript{1} (BcA) is the major component with the most potent antibiotic activity.\textsuperscript{16,17} Combined bacitracin A and B components are accountable for >90\% of the biological activity although BcA\textsubscript{2} has low activity.\textsuperscript{18} Bacitracin F is biologically inactive and has been proposed to be the cause of nephrotoxicity. It is the oxidized product of BcA\textsuperscript{19} that can act as a vasoconstrictor toward kidneys.\textsuperscript{20}

Bacitracin was initially isolated using counter-current distribution technique,\textsuperscript{21} and the structure was determined by NMR.\textsuperscript{22} Several structures were proposed, but the structure was not fully characterized until the early 1990’s by means of modern 2D NMR
techniques.\textsuperscript{23} The congeners can also be purified with reverse-phase HPLC and characterized with mass spectrometry and NMR spectroscopy.\textsuperscript{24} The structure of BcA consists of a thiazoline ring formed by the condensation reaction between the carboxylate of Ile-1 and the –NH\textsubscript{2} and the –SH groups of Cys-2. It is a dodecapeptide, containing four D-amino acids, with a unique cyclic heptapeptide structure formed via an amide linkage between the side chain of Lys-6 and the C-terminus of Asn-12.\textsuperscript{23} In the characterized structure of Bc, the N-terminal end folds over the cyclic heptapeptide, placing the thiazoline ring, Glu-4, and His-10 in proximity.\textsuperscript{23} It has been suggested that these unusual structural features may prevent this peptide from being degraded by proteases.\textsuperscript{25} Structures of bacitracin congeners are displayed in Figure 3.1.

3.1.2 \textit{Metal-Binding and Antibacterial Mechanism}

Bacitracin requires Zn\textsuperscript{II} for its antibacterial activity\textsuperscript{26} and has been shown to bind several divalent metal ions, including Co\textsuperscript{II}, Ni\textsuperscript{II}, and Cu\textsuperscript{II}, in a 1:1 stoichiometry.\textsuperscript{27, 28} A previously proposed antibacterial mechanism of bacitracin involves the inhibition of peptidoglycan cell wall synthesis. At the final step of the synthesis, the uridine diphosphate sugar molecule is transported to the cell wall by a lipid carrier known as C\textsubscript{55}-isoprenyl pyrophosphate.\textsuperscript{29} After the incorporation of the disaccharide into the peptidoglycan layer, the lipid carrier is dephosphorylated to its monophosphate form in order to bind another sugar molecule. The metal\textsuperscript{III}-Bc complex inhibits the cell wall
Figure 3.1. Structures of bacitracin congeners (adopted from ref. 2). Bacitracin is a dodecapeptide, containing four D-amino acids, with a unique cyclic heptapeptide structure formed via an amide linkage between the side chain of Lys-6 and the C-terminus of Asn-12. In BcA, the two N-terminal amino acids form a thiazoline ring by the condensation reaction between the carboxylate of Ile-1 and the –NH$_2$ and the –SH groups of Cys-2.
synthesis by binding to the lipid carrier with high affinity \( (K_f = 1.05 \times 10^6 \text{ M}^{-1}) \). Thus, metal coordination is an essential part of its biological activity.

Metal binding of Bc have been studied by means of multiple techniques, including proton release titration, ORD, EPR, EXAFS, \(^1\)H NMR, and UV-Vis spectroscopy.\(^{28, 30, 31}\) One of the earlier studies proposed that the metal binding site is consisted of His-10 nitrogen, thiazoline ring (nitrogen or sulfur), and the amino group of Ile-1, based on a proton release titration.\(^{31b}\) Nonetheless, later studies did not suggest the involvement of Ile-1 group. Another metal binding study with Cu\(^{II}\) and Mn\(^{II}\) suggested the binding through the imidazole ring of His-10, and the carbonyl group of acidic amino acid residues such as Asp and Glu according to \(^{13}\)C NMR results.\(^{31d}\) EPR\(^{28}\) and EXAFS\(^{31f}\) studies showed metal binding through thiazoline ring, Glu-4, and His-10 but disagreed on the fourth ligand, Asp-11 and Ile-1-NH\(_2\) for EPR and EXAFS study, respectively.

Finally, the metal binding site of Bc was unambiguously assigned by the use of Co\(^{II}\) as a paramagnetic NMR probe.\(^{24}\) The suggested metal-binding ligands are the thiazoline ring nitrogen, the N\(\epsilon\) nitrogen of His-10, and the carboxylate side chain of D-Glu-4.\(^{24}\) Furthermore, the study suggested a correlation between the structure and function of bacitracin. According to the authors, both BcA\(_2\) and BcF cannot effectively bind the metal ion to create a proper binding site for the lipid carrier. Although Ile-1 is not involved in metal binding, it is important in creating a correct environment as seen in the inability of BcA\(_2\) (D-\(allo\)-Ile-1 versus L-Ile-1 of BacA\(_1\) at the N-terminus) to bind the
metal properly. Likewise, BcF, having an oxidized thiazole ring instead of a thiazoline ring at the N-terminus, is only able to bind the metal ion weakly through the imidazole group of His-10. In addition, no metal binding by D-Glu-4 was observed in both congeners.

The structure of a metal-bound bacitracin was generated based on the $T_1$ relaxation results with a molecular modeling program.\textsuperscript{24} According to the proposed structure, the four N-terminal amino acid residues fold back onto the His-10-bound metal ion to complete the tridentate coordination sphere. The structure also suggested the presence of a hydrophobic pocket (Ile-5, Phe-9, and Ile-10 are in close proximity). This hydrophobic binding site was proposed to play an important role in lipid pyrophosphate binding, aided by the stabilization effect of Ile-1-NH$_3^+$ through H-bonding. As a result, inability of binding metal correlates to the biological activity of Bac congeners.\textsuperscript{24, 2}

Pyrophosphate and its several derivatives were found to bind to Co$^{II}$-bacitracin to form kinetically inert ternary complexes by means of NMR spectroscopy.\textsuperscript{2b} The study suggested the pyrophosphate moiety to be the minimum necessity for binding to the metallo-bacitracin complex, since sodium phosphate was shown only to bind very weakly.\textsuperscript{2b} The ability of the metal$^{II}$-Bc complex (not free Bc) to bind pyrophosphate may suggest a potential interaction with the phospho-group on DNA. Thus, in the presence of a redox-active metal such as Cu$^{II}$, bacitracin may bind and perform an oxidative damage of DNA. This hypothesis is supported by the Cu$^{II}$-dependent oxidation chemistry by metal-binding short peptides (e.g. the amyloid-β peptides in the brain of the patients with
AD, Aβ_{1-16} and Aβ_{1-20}) has been previously proposed.\textsuperscript{32, 33} In this study described herein, the catechol oxidation chemistry of Cu\textsuperscript{II}-Bc was explored using optical and kinetic methods.

### 3.2 Materials and Methods

Bacitracin and pyrocatechol (~99\%) were purchased from Sigma–Aldrich Inc. (St. Louis, MO), the plasmid pQE30Xa was purchased from Qiagen (Valencia, CA), and 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate, 98\%, was acquired from and Acros Organics (NJ). Copper Sulfate (anhydrous) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer (HEPES) used for the oxidation study was purchased from Fisher Scientific Co. (Fair Lawn, NJ). All chemicals were used without further purification. All other solvents and reagents were the highest grade available from commercial sources. Deionized (DI) water (18.2 MΩ) was obtained from a Millipore Milli-Q system. Plastic ware and glassware were demetallized with EDTA and extensively rinsed. All components were freshly prepared just prior to the experiments. Quartz cuvettes were used in all the kinetic and optical studies.

#### 3.2.1 Kinetic Studies

The catechol oxidation assay was carried out as previously described.\textsuperscript{32, 33} Briefly, different concentrations of the substrate catechol ranging from 0.1 to 19.2 mM (along with each corresponding concentrations of MBTH as an ortho-quinone trapper)
were incubated with 20.0 μM (or 2.0 μM in H2O2 related experiments) CuII-bacitracin (1:1 metal-to-peptide stoichiometric ratio) in 100.0 mM HEPES buffer at pH 7.0 and 25 °C in the presence and absence of different H2O2 concentrations (0 to 64.0 mM). All components were dissolved in DI water, and the final volume in the cuvette was fixed at 1.0 mL. The binding of oxidized o–quinone to MBTH formed a red adduct, which was monitored at 500 nm (ε = 32, 500 M⁻¹cm⁻¹) on a Varian Cary50 Bio UV-Vis spectrophotometer, and the initial rate was determined from the slope of the change in the absorbance with respect to time.

The optimal ratio between the catalyst and substrate during catalysis was determined using the Job method with respect to catalytic activity. The Job method is a continuous variation technique, where the concentrations of catalyst [CuII-Bc] and substrate [CA] were varied while keeping the total concentration constant ([CuII-Bc]+[CA] = 50.0 μM). The result, with respect to activity, is plotted versus the mole fraction of catalyst (XCu(II)-Bc) or substrate (XCA). The ratio of XCu(II)-Bc:XCA at which the activity reaches the maximum in the plot reflects the preferred stoichiometry of the (CuII-Bc)-CA complex for the given experimental conditions. Since the oxidation reaction follows the enzyme-like kinetics (discussed in the result section), the kinetic constants, such as K_B and V_{Max}, were determined by fitting the data to the Michaelis-Menten equation with the Sigma Plot 8.0.
3.2.2 Optical Studies

The slow substrate 4,5-di-chloro-catechol (DCC) was used as a ligand in the binding study to show the interaction between the Cu$^{II}$-Bc complex and the catechol substrate. Due to low solubility in water, DCC was dissolved in DMF. The stock solution of the Cu$^{II}$-Bc complex was prepared in DI water, since only the peptide and not the copper salt would dissolve in DMF. The experiment was run in DMF, and all components were completely miscible. Briefly, DCC ranging from ~60.0 to 600.0 µM was gradually titrated into a 1.0 mL solution of Cu$^{II}$-Bc (300.0 µM) with the sample cuvette left in the spectrometer to keep the baseline intact. The background was zeroed with only the solvent, and the mixture was scanned from 200 to 900 nm on the Varian Cary50 Bio UV-Vis spectrophotometer. The dilution factor from each addition was corrected in the final concentrations. The molar absorptivity value of the (Cu$^{II}$-Bc)-DCC complex was plotted with respect to the titrated DCC concentration, and the data fitted to simple 1:1 binding quadratic equation in Sigma Plot 8.0 for the determination of the affinity constant, $K_{DCC}$.

The stoichiometry of (Cu$^{II}$-Bc)-to-DCC binding was determined with the Job method. As described in section 3.2.1, the metal complex [Cu$^{II}$-Bc] and ligand [DCC] were continuously varied while keeping a constant overall concentration ([Cu$^{II}$-Bc]+[DCC] = 500.0 µM). The maximum ratio ($X_{Cu^{II}-Bc}:X_{DCC}$) on the plot of the molar absorptivity of the (Cu$^{II}$-Bc)-DCC complex versus the mole fraction of DCC ($X_{DCC}$) or
Cu$^{II}$-Bc ($X_{Cu(II)-Bc}$) indicates the preferred binding stoichiometry between the Cu$^{II}$-Bc complex and DCC.

3.2.3 **Anaerobic Studies**

For anaerobic experiments, the dissolved oxygen from all components was removed through freeze-thawing on a vacuum-argon line. Briefly, the oxygenated solutions were first frozen in liquid nitrogen and then evacuated. Next, the frozen sample was thawed in warm water, followed by application of pure Ar gas. The process was repeated 5–8 times to ensure the removal of oxygen and replacement by Ar. All components, except the catalyst, were transferred to the evacuated quartz cuvette with a gas-tight syringe. The reaction was immediately monitored following the addition of the catalyst (Cu$^{II}$-Bc).

3.2.4 **DNA Cleavage Assay**

The 1:1 Cu$^{II}$-Bc stock solution was prepared by dissolving the peptide in ultrapurified water. The metal-complexes were freshly prepared in all experiments. The DNA cleavage assay contained 225 ng of plasmid DNA, 0.05% H$_2$O$_2$, and 25.0 μM of Cu$^{II}$-Bc in 100.0 mM HEPES at pH 7.00 and 37 °C in a volume of 25.0 μL. A time-course (0–60 mins) experiment was performed and analyzed in a 1.0 % agarose gel stained with ethyldium bromide, and then photographed on a transilluminator.
3.3 Results and Discussion

A divalent metal ion is an essential cofactor for the biological activity of bacitracin.\textsuperscript{26} The metal-bound complex can bind the sugar carrier, $C_{55}$-isoprenyl pyrophosphate, with high affinity.\textsuperscript{30} Furthermore, the Co\textsuperscript{II}-Bc complex has been shown to interact with various phosphate derivatives through NMR studies.\textsuperscript{2b} Thus, in the presence of redox-active metal ion such as Cu\textsuperscript{II}, Bc can potentially interact and cause oxidative damage of biomolecules, such as DNA.

The ability of (1:1) Cu\textsuperscript{II}-Bc to perform oxidative DNA cleavage was studied in the presence of $H_2O_2$ following the previously proposed protocol with a slight modification.\textsuperscript{32} The plasmid DNA (225 ng) was incubated with 25.0 $\mu$M Cu\textsuperscript{II}-Bc and 0.05\% $H_2O_2$ in 100.0 mM HEPES at pH 7.0 and 37 °C over a time course (0–60 mins). On the gel (Figure 3.2), the first lane is the 1 kb DNA molecular weight marker (M on gel), and the second lane is the reference (R on gel), which only contains the plasmid DNA (pQE30Xa) incubated over the same time course. Lanes 1–5 represent different incubation times (i.e. 5, 10, 20, 40, 60 mins) for the Cu\textsuperscript{II}-Bc complex. For the reference lane, both supercoiled (lower) and nicked circular (top) bands can be clearly seen. In lanes 1–3 corresponding to 5, 10, and 20 mins incubation, a middle band of approximately 3.5 kbp based on the marker corresponds to a linearized plasmid from the manufacturer. This indicates a double-stranded DNA cleavage has occurred. In lanes 4 and 5, corresponding to 40 and 60 mins incubation, the plasmid DNA is completely cleaved. Short peptides with Cu\textsuperscript{II} binding ability have been shown to perform oxidative
Figure 3.2. Oxidative cleavage of 225 ng of plasmid DNA by 25.0 μM Cu$^{II}$-Bc with 0.05% H$_2$O$_2$ in 100.0 mM HEPES buffer at pH 7.0 and 25 °C. The first lane is the 1 kb DNA molecular weight marker (M on gel), and the second lane is the reference (R on gel). Lanes 1–5 represent different incubation times (i.e. 5, 10, 20, 40, 60 mins) for the Cu$^{II}$-Bc complex.
cleavage of plasmid DNA. Thus, the above results suggest the ability of Cu\(^{II}\)-Bc to perform oxidation chemistry.

The oxidation chemistry catalyzed by the Cu\(^{II}\)-Bc complex was further studied with catechol (CA) as a substrate. Different concentrations of CA were incubated with 20.0 µM Cu\(^{II}\)-Bc in 100.0 mM HEPES at pH 7.0 and 25°C. The formation of the oxidized o-quinone product was followed optically, by monitoring the red adduct formed between the product and MBTH (an o-quinone specific indicator). The catechol oxidation by the Cu\(^{II}\)-Bc complex increases with increasing amount of substrate and eventually reaches a plateau. The observed saturation at higher concentrations of substrate suggests a possibility of enzyme-like pre-equilibrium kinetics shown below.

The rate law for this reaction can be described accordingly to Eq. (1), with the assumption that [CA]>>[Cu(II)-Bc-CA]:

\[
[Cu(II)-Bc] + [CA] \stackrel{k_1}{\rightleftharpoons} [Cu(II)-Bc-CA] \stackrel{k_{cat}}{\rightarrow} [Cu(II)-Bc] + o-quinone
\]

where \(v_0\) and \(V_{Max}\) are the measured and maximum velocity, respectively, and

\[
K_B = \frac{k_{cat} + k_{-1}}{k_1}
\]

is the virtual dissociation constant of the (Cu\(^{II}\)-Bc)-CA complex. The data fits well to Eq. (1) to afford \(K_B = 3.31 \times 10^{-3}\) M, a first-order rate constant or the turnover number \(k_{cat}\) (\(V_{Max}/[Cu(II)-Bc]\)) = 6.99 \times 10^{-3}s^{-1}, and a second-order rate...
constant or the catalytic efficiency $k_{cat}/K_B = 2.11 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 3.3). Compared to the auto-oxidation rate constant of catechol ($4.74 \times 10^{-7}\text{ s}^{-1}$) in the absence of Cu$^{II}$-Bc, there is a $1.47 \times 10^4$-time increase with respect to the first-order rate constant.

The interaction between the Cu$^{II}$-Bc complex and the substrate catechol is confirmed by an optical binding study with a slow substrate, 4,5-dichlorocatechol (DCC). The activity of DCC is roughly 200 times slower than catechol with respect to $k_{cat}$ as a ligand. A 1.0-mL solution of 300-μM Cu$^{II}$-Bc was slowly titrated with DCC in DMF at 25 °C. The formation of complex was monitored from 200–800 nm on the Varian Cary50 Bio UV-Vis spectrophotometer. Upon addition of DCC, a new absorption corresponding to a complex between Cu$^{II}$-Bc and DCC appears at approximately 304 nm (Figure 3.4). The catecholate moiety has been previously proposed to bind the metal ion in a bidentate manner. A recent Co$^{II}$ binding study of bacitracin by means of NMR spectroscopy and molecular modeling suggested the presence of a possible hydrophobic binding pocket and the metal coordination sphere to be a distorted 5 or 6 instead of 4 coordination. Thus, it may be possible for the catecholate moiety on DCC (or catechol) to bind the metal$^{II}$ center in a 1:1 stoichiometry to give a penta-coordinated sphere. The plot of molar absorptivity ($\epsilon$) value of the Cu$^{II}$-Bc-DCC complex at 304 nm versus the added equivalence of DCC can be fitted well to a 1:1 binding quadratic formula to afford the affinity constant, $K_{DCC}$, of $2.40 \times 10^4 \text{ M}^{-1}$ (Figure 3.4 inset). Even though the affinity constant can be determined from the fitting, the stoichiometry of the binding cannot be clearly abstracted.
Figure 3.3. Catechol oxidation by Cu\textsuperscript{II}-Bc in 100.0 mM HEPES at pH 7.0 and 25 °C. The activity increases with increasing substrate concentration and reaches saturation at higher concentration, which may suggest an enzyme-like kinetics. The data fits well to Eq. (1), affording $K_B$, a first-order rate constant or the turnover number $k_{cat} (V_{Max}/[\text{Cu(II)}$-Bc]), and a second-order rate constant or the catalytic efficiency $k_{cat}/K_B$ of $3.31 \times 10^{-3}$ M, $6.99 \times 10^{-3}$ s\textsuperscript{-1}, and 2.11 M\textsuperscript{-1} s\textsuperscript{-1}, respectively.
Figure 3.4. 4,5-dichlorocatechol (DCC) binding study of Cu\textsuperscript{II}-Bc in DMF. DCC is added in 60.0 μM (0.2 equivalent) increment. The Cu\textsuperscript{II}-Bc-DCC complex gives a strong absorption at 304 nm. The changes in the absorbance at 304 nm with respect to the added DCC equivalence afford an affinity constant of 2.40 \times 10^4 \text{ M}^{-1} (the inset). The spectra are baselined with the Cu\textsuperscript{II}-Bc complex prior to the addition of DCC.
Thus, the Job method was used to determine the stoichiometry of the (Cu\text{II}-Bc)-DCC complex. The method calls for continuously varying the concentrations of the Cu\text{II}-Bc complex and DCC while keeping the overall concentration constant.\textsuperscript{35} For the optical study, the concentrations of the two components were arrayed to a total concentration of 500.0 μM in 100.0 mM HEPES buffer at pH 7.0 and 25 °C (i.e. [Cu\text{II}-Bc]:[DCC] = 500:0 μM, 450:50 μM, ..., 0:500 μM). The absorption of the complex (310 nm) in buffer slightly shifted toward the higher wavelength (Figure 3.5). As a result, the ratio that corresponds to the maximum intensity represents the preferred binding between Cu\text{II}-Bc and DCC. The plot of the molar absorptivity value at 310 nm versus the mole fraction of DCC (X_{DCC}) shows the maximum around 0.5 (0.5:0.5) which suggests that the stoichiometry of the preferred species is $X_{\text{Cu(II)-Bc}}:X_{\text{DCC}} = 1:1$ (Figure 3.5 inset).

Similarly, since an equilibrium is present between the Cu\text{II}-Bc complex and substrate the interaction between the catalytic center (Cu\text{II}-Bc) and substrate (CA) was further investigated with the Job method, monitored with the activity toward catechol oxidation. The total concentration of [Cu\text{II}-Bc]+[CA] was kept constant at 50 μM, and the experiment was conducted in 100 mM HEPES buffer at pH 7.0 and 25 °C. The result shows a gradual increase in activity with $X_{\text{Cu(II)-Bc}}$ and reaches a maximum at $X_{\text{Cu(II)-Bc}} = 0.5$, followed by a decrease in the activity of subsequent mole fractions which can be fitted to a 1:1 stoichiometry (Figure 3.6). From the result, the most active species has a ratio of (Cu\text{II}-Bc):CA = 1:1 (i.e. 0.5:0.5), establishing the equilibrium between reactants and the (Cu\text{II}-Bc)-CA complex in the catalysis.
**Figure 3.5.** Optical Job plot of DCC binding to the Cu\textsuperscript{II}-Bc complex. The absorbance at 310 nm versus the molar ratios between the Cu\textsuperscript{II}-Bc complex and DCC suggests that the stoichiometry of the preferred species is 1:1.
Figure 3.6. Interaction between the Cu$^{II}$-Bc and catechol using the Job method. The total concentration of the molar ratio is 50.0 μM. The assays were performed in 100.0 mM HEPES pH 7.0 buffer at 25 °C, and the interaction was monitored with the oxidation activity. The plot of the initial rates versus the molar ratios indicates the preferred stoichiometry to be 1:1.
The results from the two binding studies (Cu\textsuperscript{II}-Bc:DCC = 1:1 and Cu\textsuperscript{II}-Bc:CA = 1:1) using the Job method clearly indicate that the observed catechol oxidation chemistry occurs through a mononuclear Cu\textsuperscript{II} center. The oxidation chemistry of mononuclear Cu\textsuperscript{II} systems has been thoroughly studied with synthetic ligand complexes, several spectroscopic techniques, and density functional theory (DFT) calculations.\textsuperscript{37} These studies have proven the important role of dioxygen in mononuclear Cu\textsuperscript{II}-center catalysis. Since the oxidation of catechol to o-quinone is a 2e\textsuperscript{−} transfer process, the involvement of dioxygen in recycling of the metal center (Cu\textsuperscript{I}/Cu\textsuperscript{II}) is suspected. Thus, the ability of Cu\textsuperscript{II}-Bc to perform catechol oxidation was determined in the absence of dioxygen (under anaerobic conditions).

The dissolved oxygen in all components was removed by the method described in the experimental section. The reaction (100.0 \(\mu\)M Cu\textsuperscript{II}-Bc and 2.5 mM CA) was performed in methanol, and the formation of o-quinone was monitored from 400–600 nm for 5 mins. (Figure 3.7). The o-quinone-MBTH adduct immediately appears upon Cu\textsuperscript{II}-Bc addition approximately at 500 nm. A gradual and minor increase in the absorption from the first scan (Abs = 0.015) can be seen over 5-minute period and then stop increasing after 2–3 mins. The difference in the intensity is estimated to be 0.010 (\(\Delta\text{Abs} = 0.025 – 0.015\)), and the overall concentration of o-quinone is roughly estimated to be 770 nM (based on \(\varepsilon_{\text{MBTH}} = 32, 500 \text{ M}^{-1}\text{cm}^{-1}\)), which indicates a very inefficient catalysis. This is to be expected since a single Cu\textsuperscript{II} can only oxidize one of the two hydroxyl groups on catechol at a time.
Figure 3.7. The oxidation of 2.5mM catechol by 100.0 μM Cu^{II}-Bc under anaerobic condition. The reaction was immediately monitored upon Cu^{II}-Bc addition up to 5 minutes. A small, gradual increase was observed up to 2–3 mins. The change in absorbance is approximately 0.01.
The involvement of oxygen was further shown by introducing some air into the same sample and scanning over the same region. An air-tight syringe was used to apply the air from the surrounding, and the listed air volume is based on the volume of the syringe. As a result, this is a qualitative experiment. From 5 to 40 mL of air was added into the mixture over 4 different occasions. The increase in the absorbance was observed after each addition (Figure 3.8). Quantification of the product based on the volume of air added is not feasible since factors such as the amount of oxygen in a given volume of syringe and the dissolvability of oxygen (which may be time-dependent) are not known. Qualitatively, however, this experiment clearly shows the importance of dioxygen in the observed mononuclear Cu$^{II}$-centered catechol oxidation.

The binding between copper and dioxygen depends on the oxidation state of metal (Cu$^{I}$ vs. Cu$^{II}$) and the binding mode of dioxygen (end-on vs. side-on). Two possible binding modes of dioxygen are end-on ($\eta^1$) and side-on ($\eta^2$) binding, where the dioxygen binds the metal ion in a monodentate and bidentate fashion, respectively.$^{37}$ In the presence of a bidentate catechol, dioxygen may only be able bind the Cu$^{II}$-Bc center (a 3-coordinated complex) through the end-on mode due to steric hindrance. The steric effect is another important factor in the selection of dioxygen binding mode. A classic example is the very first synthetic Cu$^{II}$-ligand complex, showing the mononuclear Cu$^{II}$-superoxo complex with a side-on binding mode, by Kitajima et. al. in 1994.$^{38}$ The side-on binding by dioxygen in this complex was induced by steric effect of the ligand.
Figure 3.8. Addition of air into anaerobic sample containing 100.0 μM Cu^{II}-Bc and 2.5 mM CA. The same air-tight syringe was used to suck up and bubble in the air from surrounding, and the listed air volume is based on the volume of the syringe. From 5 to 40 mL of air were added into the mixture over 4 different additions.
Upon catechol binding, the Cu$^{II}$ center may be reduced to Cu$^{I}$ while the catechol is oxidized to a semiquinone. Under aerobic conditions, the Cu$^{I}$ can interact with dioxygen to give the following three potential copper-oxygen intermediates: Cu$^{II}$-superoxo (-O$_2^-$), Cu$^{II}$-hydroperoxo (-OOH) and Cu$^{III}$-peroxo (-O$_2^{2-}$).$^{37}$ The Cu$^{III}$-O$_2^{2-}$ species may not be the intermediate since side-on binding to the Cu center may not be possible (this binding mode will result in 7-coordination of the Cu). On the other hand, both Cu$^{II}$-O$_2^-$ and Cu$^{II}$-OOH species can have both end-on and side-on binding mode. In its protonated form, the Cu$^{II}$-OOH species forms an end-on intermediate. However, it can potentially form a side-on intermediate once deprotonated.$^{37}$ The side-on binding mode may be too crowded in the metal coordination sphere as with the Cu$^{III}$-O$_2^{2-}$.

Furthermore, a recently solved crystal structure of a mononuclear Cu$^{II}$ enzyme, peptidylglycine $\alpha$-hydroxylating monooxygenase (PHM), shows the end-on O$_2$ binding of the Cu center.$^{39}$ Thus, the end-on Cu$^{II}$-O$_2^-$ or Cu$^{II}$-OOH species is very likely to be the intermediate.

Unlike oxygen, hydrogen peroxide (H$_2$O$_2$) prefers to bind the Cu$^{II}$ center through a side-on mode upon full deprotonation, possibly forming the Cu$^{II}$-Bc-O$_2^{2-}$ intermediate. Hence, H$_2$O$_2$ was included in the reaction in order to determine its influence on the observed chemistry. Different concentrations (0–64.0 mM) of H$_2$O$_2$ were incubated with 20.0 $\mu$M Cu$^{II}$-Bc and 0.7 mM catechol in 100.0 mM HEPES buffer at pH 7.0 and 25 ºC. The increase in catechol oxidation was observed with increasing H$_2$O$_2$ and reaches saturation at higher concentrations of H$_2$O$_2$ (Figure 3.9 left panel). As discussed above,
the saturation profile indicates the possibility of an enzyme-like kinetics, thus suggesting H$_2$O$_2$ as another substrate capable of binding to the metal center. The data can be fitted to Eq. 2 to afford a $k_{cat}$ of $9.37 \times 10^{-3}$ s$^{-1}$ and an apparent binding or dissociation constant $K_{H_2O_2}$ of $7.49 \times 10^{-3}$ M for H$_2$O$_2$. This value compared to $K_B = 3.31 \times 10^{-3}$ M, the binding constant for catechol in the absence of H$_2$O$_2$, is approximately twice as higher, suggesting this substrate to bind ~2× lower than catechol toward the Cu$^{II}$-Bc complex.

$$V_0 = V_{Background} + \frac{V_{Max}[H_2O_2]}{K_{H_2O_2} + [H_2O_2]}$$

(2)

Since both substrates (i.e. both catechol and H$_2$O$_2$ show saturation) can independently bind to the Cu$^{2+}$ center, it is essential to determine how the binding of one affects the other. Different concentrations (0–9.6 mM) of catechol were incubated with the Cu$^{II}$-Bc (2.0 μM) complex in the presence of different H$_2$O$_2$ concentrations (0–64.0 mM). The enhancements in activity of catechol oxidation in the presence of H$_2$O$_2$ were clearly observed (Figure 3.9 right). While $K_B$ is increased by ~7 folds, the addition of 32.0 mM H$_2$O$_2$ in the assay increased the $k_{cat}$ and $k_{cat}/K_B$ by 55 and 7 folds, respectively. Because the two substrates may bind to the Cu$^{II}$-Bc complex independently of each other, the Hanes plot can be used to determine the influence or relationship between the two substrates.

From the Hanes analysis, the apparent binding equilibrium constants for both substrates and an intrinsic binding constant for one substrate can be calculated. The
Figure 3.9. Hydrogen peroxide influence on the catechol oxidation by 2.0 μM Cu$^{II}$-Bc at pH 7.0 in 100.0 mM HEPES buffer at 25°C (right). The concentrations of H$_2$O$_2$ were varied from 0 to 64.0 mM (right). The titration of H$_2$O$_2$ at a constant catechol concentration of 0.7 mM displays saturation, which indicates the direct interaction between the Cu$^{II}$-Bc center and H$_2$O$_2$ (left). 20.0 μM Cu$^{II}$-Bc was used in the latter experiment (left).
Hanes analysis requires an initial linear treatment of the data in Figure 3.6 according to the following rate law (Eq. 3),

\[
\frac{[\text{Catechol}]}{V_0} = \left(1 + \frac{K_{\text{H}_2\text{O}_2}^{\text{App}}}{[\text{H}_2\text{O}_2]}\right)\frac{[\text{Catechol}]}{V_{\text{max}}} + \frac{K_{\text{Catechol}}^{\text{App}}}{V_{\text{max}}} \left(1 + \frac{K_{\text{H}_2\text{O}_2}^{\text{Int}}}{[\text{H}_2\text{O}_2]}\right) \tag{3}
\]

where \(K_{\text{H}_2\text{O}_2}^{\text{Int}}\) is the intrinsic binding constant for \(\text{H}_2\text{O}_2\), \(K_{\text{H}_2\text{O}_2}^{\text{App}}\) and \(K_{\text{Catechol}}^{\text{App}}\) are the apparent binding constants for \(\text{H}_2\text{O}_2\) and catechol, and \(V_0\) and \(V_{\text{max}}\) are the experimental and maximum velocity, respectively (Figure 3.10 A). The ratio between the corresponding apparent and intrinsic equilibrium constants for each substrate can suggest how the binding of one substrate affects the binding of another. Then, the secondary plots of the fitted y-intercept (Eq. 4) and slope (Eq. 5) values versus \(1/[\text{H}_2\text{O}_2]\) afford the two apparent equilibrium constants \(K_{\text{H}_2\text{O}_2}^{\text{App}} = 1.91 \times 10^{-2} \text{M}\) and \(K_{\text{Catechol}}^{\text{App}} = 8.38 \times 10^{-3} \text{M}\) and the intrinsic binding constant \(K_{\text{H}_2\text{O}_2}^{\text{Int}} = 9.03 \times 10^{-3} \text{M}\) (Figure 3.10 B and C).

\[
y - \text{int ercept} = \frac{K_{\text{Catechol}}^{\text{App}}}{V_{\text{max}}} + \left(\frac{K_{\text{Catechol}}^{\text{App}} K_{\text{H}_2\text{O}_2}^{\text{Int}}}{V_{\text{max}}}\right) \frac{1}{[\text{H}_2\text{O}_2]} \tag{4}
\]

\[
slope = \frac{1}{V_{\text{max}}} + \left(\frac{K_{\text{H}_2\text{O}_2}^{\text{App}}}{V_{\text{max}}}\right) \frac{1}{[\text{H}_2\text{O}_2]} \tag{5}
\]

The ratio between the apparent and intrinsic binding constants is 2.53 for catechol \((K_{\text{Catechol}}^{\text{App}}/K_B)\) and 2.11 for \(\text{H}_2\text{O}_2\) \((K_{\text{H}_2\text{O}_2}^{\text{App}}/K_{\text{H}_2\text{O}_2}^{\text{Int}})\), which suggests that both substrates are equally affected by the presence of the other. Since \(\text{O}_2^{2–}\) has been shown to bind Cu in a
Figure 3.10. Hanes analysis of the oxidation of catechol by Cu$^{II}$-Bc at different concentrations of H$_2$O$_2$ (kinetic data from Figure 3.9 right). Plot B and C are the replots of the slope and y-intercept values from plot A with respect to [H$_2$O$_2$]$^{-1}$. The apparent binding equilibrium constants for catechol ($K_{App}^{Catechol}$) and H$_2$O$_2$ ($K_{App}^{H_2O_2}$) as well as the intrinsic binding constant for H$_2$O$_2$ ($K_{int}^{H_2O_2}$) can be determined from the secondary plots.
side-on (bidentate) mode, the reduction in the effectiveness of binding by catechol (also a bidentate binding) is expected due to inability of the Cu$^{II}$ center to support a 7-coordination sphere. Thus, the observation described herein is more consistent with the end-on binding mode of HO$_2^-$ when the catechol is bound to the metal center.

In the attempt to differentiate between a mononuclear and dinuclear Cu$^{II}$-centered catalysis, “redox-dilution” experiments were performed as previously proposed. Even though the studies above strongly suggest a mononuclear Cu$^{II}$-based catalysis, these interesting results, which may be of mechanistic relevance, are included. Similar to the Job method, the concentrations of redox-inert Zn$^{II}$ to Cu$^{II}$ were continuously arrayed with the total concentration kept constant ([Zn$^{II}$]+[Cu$^{II}$] = 20.0 μM). Two additional experiments with Co$^{II}$ and Ni$^{II}$ in place of Zn$^{II}$ were performed. The interaction between the two metal ions and the peptide was monitored with the catechol oxidation activity. The catechol (1.6 mM) oxidation by 20.0 μM metal-Bc complex was performed in 100.0 mM HEPES buffer at pH 7.0 and 25 ºC. Since Bc can bind both metals, the presence of Zn$^{II}$-Bc complex can essentially “dilute” the redox activity of the Cu$^{II}$-Bc complex.

A mononuclear versus dinuclear mechanism may be suggested based on the resulted pattern. In a mononuclear mechanism, the Zn$^{II}$ complex has no influence on the redox activity of the Cu$^{II}$ complex; thus, a linear trend is expected for the activity with respect to the Cu$^{II}$ concentration. However, the redox-inactive Zn$^{II}$ species can essentially interfere with the redox chemistry in the dinuclear mechanism by competing for the formation of the dinuclear center. Thus, in the dinuclear mechanism, only a slow
and steady increase in the activity is expected up to 50% Zn$^{II}$ and 50% Cu$^{II}$ ratio. Then, a sharper increase in the activity is expected due to the presence of more Cu$^{II}$ species for the formation of dinuclear center when the Cu$^{II}$ to Zn$^{II}$ ratio surpasses 1:1, affording a sigmoidal-like pattern.

The data can be fitted to the Hill equation (Eq. 6) which suggests the presence of cooperativity during the catalysis. In the Hill equation,

$$V = V_{\text{Background}} + \frac{V_{\text{max}} [\text{Cu(II)} - \text{Bac}]^\theta}{K_A + [\text{Cu(II)} - \text{Bac}]^\theta} \quad (6)$$

$v$ and $V_{\text{max}}$ are the usual experimental and maximum velocity, respectively; $K_A$ is equivalent to the Michaelis constant accounting for all interactions; and $\theta$ is the fitted Hill coefficient that indicates the presence of cooperativity when greater than 1.\textsuperscript{33} The results for Zn$^{II}$ and Co$^{II}$ show an initial small and gradual increase in activity up to $X_{\text{Cu(II)}} = 0.5$, then followed by larger increase in activity (Figure 3.11 A and B). A similar trend is observed for Ni$^{II}$; however, the sigmoidal-like nature is not as apparent (Figure 3.11 C).

The sigmoidal-like patterns in the case of catechol oxidation by Cu$^{II}$-Bc in the presence of Zn$^{II}$ or Co$^{II}$ can be fitted to the Hill equation to afford $\theta = 1.93$ ($r^2 = 0.99$) and 2.37 ($r^2 = 0.99$), respectively (Figure 3.11 A and B), suggesting the presence of cooperativity. For comparison, the results were also fitted with a simple 1:1 quadratic binding equation. As shown in Figure 3.11, the 1:1 binding pattern for Cu$^{II}$ binding to Bc may not be appropriate for fitting the data (the points deviate from the fitted dotted line), showing the $r^2$ values of 0.92 and 0.89, respectively, for Zn$^{II}$ and Co$^{II}$ dilution.
difference in $r^2$ values for the Hill ($r^2 = 0.991$) and quadratic ($r^2 = 0.986$) equation, unlike for Zn$^{II}$ and Co$^{II}$, is less apparent for Ni$^{II}$. This is also reflected in the Hill coefficient of 1.38.

At a first glance, the presence of cooperativity can suggest a di-Cu$^{II}$ center catalysis. Nevertheless, the optical (DCC binding) and mechanistic (catechol oxidation) Job data strongly indicate a mononuclear Cu$^{II}$ center oxidation chemistry. The results from the latter two experiments also suggest the binding of catechol to the Cu$^{II}$ center in a bidentate manner (i.e. $X_{Cu(II)} = 0.5$). A possible explanation for the presence of cooperativity is the electron transfer between the nearby Cu$^{II}$-Bc complexes. Thus, when the metal$^{II}$:Cu$^{II}$ ratio is greater than 1:1 ($X_{Cu(II)}>0.5$), the increase in Cu$^{II}$-Bc (higher probability to be in close proximity) species corresponds to increase in activity.

Structural change upon metal binding was observed, which might account for the cooperativity. Although not common, the presence of cooperativity between two Cu$^{II}$ centers in close proximity has been described in PHM and D$\beta$M for a mononuclear Cu$^{II}$ center oxidation chemistry. PHM is responsible for C-H bond hydroxylation of C-terminal glycine, and D$\beta$M hydroxylates the benzylic C-H bond of dopamine to generate norepinephrine. Even though they are di-Cu enzymes, their Cu centers (Cu$_M$ and Cu$_H$) are ~11 Å apart, and only Cu$_M$, the active site, directly interacts with the substrate and oxygen. As a result, these proteins are still categorized as mononuclear Cu monooxygenases. However, Cu$_H$ has been proposed to perform a long-range transfer of an electron to the Cu$_M$ site for regeneration of Cu$^{I}$ from Cu$^{II}$.43
Figure 3.11. Zn$^{II}$ (A), Co$^{II}$ (B), and Ni$^{II}$ (C) dilution of Cu$^{II}$ for the analysis of mononuclear versus dinuclear metal center in the catalysis of catechol oxidation by Cu$^{II}$-Bc. The experiments were followed by the oxidation of catechol in pH 7.0 100.0 mM HEPES buffer at 25 °C. The two metal ions were arrayed in different ratios while the overall concentration of all the metal ions was fixed. The data can be better fitted to the Hill equation (solid trace) than the quadratic equation for a simple 1:1 binding (dashed trace).
Based on the experimental results, the following mechanism is proposed for the oxidation of catechol by Cu\textsuperscript{II}-Be through a mononuclear Cu center (Figure 3.12). First, catechol binds to the Cu\textsuperscript{II} center in a bidentate manner, followed by 1e\textsuperscript{−} transfer to Cu\textsuperscript{II} to afford a Cu\textsuperscript{I}-semiquinone complex (step A and B). The Cu\textsuperscript{I}-semiquinone complex binds O\textsubscript{2} and transfers 1e\textsuperscript{−} to O\textsubscript{2} give a Cu\textsuperscript{II}-superoxo-semiquinone complex (step C). The semiquinone is further oxidized to give $o$-quinone and a Cu\textsuperscript{II}-peroxo complex (step D). The Cu\textsuperscript{II}-peroxo complex can go through another cycle since O\textsubscript{2}\textsuperscript{2−} can take 2 more e\textsuperscript{−} before released as 2H\textsubscript{2}O, or H\textsubscript{2}O\textsubscript{2} can be released with a regenerated Cu\textsuperscript{II} center (step E).
Figure 3.12. Proposed mechanism for catechol oxidation by Cu$^{II}$-Bc through a mononuclear Cu$^{II}$ center catalysis in the presence of O$_2$. First, catechol binds to the Cu$^{II}$ center in a bidentate manner, followed by 1e$^-$ transfer to Cu$^{II}$ to afford a Cu$^{I}$-semiquinone complex (step A and B). The Cu$^{I}$-semiquinone complex binds O$_2$ and transfers 1e$^-$ to O$_2$ to give a Cu$^{II}$-superoxo-semiquinone complex (step C). Then, the semiquinone is further oxidized to give o-quinone and a Cu$^{II}$-peroxo complex (step D). The Cu$^{II}$-peroxo complex can go through another cycle since O$_2$$^{2-}$ can take 2 more e$^-$ before released as 2H$_2$O, or H$_2$O$_2$ can be released with a regenerated Cu$^{II}$ center (step E). In the absence of O$_2$, the oxidation is not catalytic.
3.4 Conclusions

Bacitracin, a dodecapeptide with a cyclic structure, is a naturally occurring potent antibiotic specifically against Gram positive bacteria.\textsuperscript{2} It is an effective component of probably the most used topical antibiotic ointment in North America. While topical application of this antibiotic is considered safe, a systemic administration has been proposed to cause nephrotoxicity.\textsuperscript{2} The proposed antibacterial mechanism involves the inhibition of bacterial cell wall synthesis by the tight binding of Zn\textsuperscript{II}-Bc complex to the pyrophosphate group of lipid sugar carrier.\textsuperscript{26, 29, 30}

In this study, I have shown the ability of bacitracin to perform oxidation chemistry in the presence of redox-active Cu\textsuperscript{II}. A high affinity binding of metallo-Bc to C\textsubscript{55}-isoprenyl pyrophosphate\textsuperscript{29} as well as the NMR study of binding between Co\textsuperscript{II}-Bc and several phosphate derivatives\textsuperscript{2b} led to the hypothesis that the Cu\textsuperscript{II}-Bc complex may bind DNA through the phosphate moiety and perform the oxidative cleavage of DNA. Indeed, oxidative cleavage of plasmid DNA by Cu\textsuperscript{II}-Bc was observed (Figure 3.2). The oxidation chemistry of Cu\textsuperscript{II}-Bc was studied with catechol as a substrate. The substrate was indeed observed to be oxidized effectively by Cu\textsuperscript{II}-Bc in the absence and presence of 32.0 mM H\textsubscript{2}O\textsubscript{2}, showing first-order rate constant of $6.99 \times 10^{-3} \text{ s}^{-1}$ and $9.37 \times 10^{-3} \text{ s}^{-1}$, respectively, accounting for a rate enhancement of $1.47 \times 10^{4}$ and $1.98 \times 10^{4}$ fold, respectively with respect to the auto-oxidation under the same condition.

Studies using the Job method, an optical binding of a slow substrate, 4,5-dichlorocatechol, (1:1 Cu\textsuperscript{II}-Bc-DCC) and a mechanistic binding of catechol (1:1 Cu\textsuperscript{II}-Bc-
CA) with the Cu\textsuperscript{II}-Bc complex nicely suggest a mononuclear Cu\textsuperscript{II} center oxidation chemistry. Since catechol oxidation is a 2e\textsuperscript{−} transfer process, oxygen must be involved in the redox cycling of the Cu (Cu\textsuperscript{II}/Cu\textsuperscript{I}) center. The 1:1 binding indicates a bidentate binding of CA to the Cu\textsuperscript{II} center, which results in a 5-coordination sphere. This further leads to the proposal of a monodentate end-on oxygen binding. Furthermore, “dilution” experiments indicate the possible presence of cooperativity. A mechanism is postulated based on the experimental results (Figure 3.12).

Upon catechol binding (in a bidentate manner) to the Cu\textsuperscript{II} center, an electron is readily transferred to the Cu center to form a Cu\textsuperscript{I}-bound semiquinone (Figure 3.12 steps A and B). Next, dioxygen binds to the Cu\textsuperscript{I} center in an end-on (η\textsubscript{1}) mode. This is immediately followed by transfer of an electron from the Cu center to the oxygen, affording a Cu\textsuperscript{II}-superoxo intermediate (Figure 3.12 step C). The semiquinone is then fully oxidized to \textit{o}-quinone and released after another electron transfer to the Cu center (Figure 3.12 step D). The bound oxygen may be able to accept up to four electrons before released as two water molecules, or H\textsubscript{2}O\textsubscript{2} and a regenerated Cu\textsuperscript{II} center may be released without further electron transfer (Figure 3.12 step E).\textsuperscript{44} A possible presence of cooperativity between nearby Cu\textsuperscript{II}-Bc complexes may also be suggested. Furthermore, the cooperativity may be explained by the bridging of dioxygen to two different Cu\textsuperscript{II}-Bc complexes, which may help stabilize the overall structure.

The chemistry of di-Cu\textsuperscript{II} centers with oxygen has been extensively studied and well characterized with a combination of synthetic organic models, multiple
spectroscopic techniques, and DFT calculations.\textsuperscript{45} Although our understanding of mononuclear Cu\textsuperscript{II} centers has increased recently, it is still a work in progress. The above proposed mechanism is deduced based on limited information from the experimental results and our understanding of mononuclear Cu systems. Thus, further detailed kinetic experiments combined with spectroscopic techniques, such as EPR and resonance Raman spectroscopy, are essential to validate the oxidation mechanism of Cu\textsuperscript{II}-Bc.

In general, bacitracin is a relatively safe antibiotic. Because of its low intestinal absorption, bacitracin has been globally utilized as animal feeds to prevent bacterial infections in farm animals.\textsuperscript{4} In addition, it has been largely used as wound prophylaxis in hospitals and on tattoo wounds.\textsuperscript{10} However, there is an increase in reported cases of allergic reactions from this drug recently. An earlier study has proposed these allergic reactions to be due to polymerization of bacitracin into macromolecules with high molecular weight.\textsuperscript{46} Metal-induced conformational changes and aggregations are commonly observed among small peptides and proteins especially by redox-active Cu\textsuperscript{II} and Fe\textsuperscript{III}.\textsuperscript{47} Thus, the oxidation chemistry of Cu\textsuperscript{II}-Bc reported in this chapter may potentially contribute toward the allergic reactions caused by bacitracin.
References


44 (a) Tabner, B. J.; Turnbull, S.; Fullwood, N. J.; German, M.; Allsop, D. The production of hydrogen peroxide during early-stage protein aggregation: a common pathological mechanism in different neurodegenerative disease? *Biochem. Soc. Trans.*


CHAPTER 4

ELUCIDATION OF THE IN VITRO OXIDATION CHEMISTRY OF COPPER(II)–HISTATIN 5 COMPLEX BY MEANS OF REACTIVITY STUDIES
4.1 **Traditional Antibiotics**

The serendipitous discovery of *penicillin* (isolated from *Penicillium notatum*) by the bacteriologist and immunologist Sir Alexander Fleming of Ayrshire, Scotland in 1928 marked the beginning of the use of naturally occurring antibiotics.\(^1,2\) It is inarguably one of the most important discoveries in the field of medicine in the 20\(^{th}\) century. Although Fleming published his findings in 1929, the clinical application of penicillin was not fully established until 1940 by chemist Ernst Boris Chain (Berlin, Germany) and pathologist Howard Walter Florey (Adelaide, South Australia). The three shared the Nobel Prize for their findings in 1945.\(^1,2\) Since then, antibiotics have been irreplaceable weapons against disease-causing microbes.

By definition, antibiotics are chemical substances produced by bacteria and fungi to selectively inhibit the growth or survival of other organisms. In general, they deter the growth of microorganisms by interacting with DNA/RNA (e.g. \(\text{Fe}^{II}\)-bleomycin can bind and cleave DNA and RNA molecules through \(O_2\) activation\(^3\)) and the cell membrane (e.g. gramicidin can penetrate and span the lipid bilayer, leading to eventual bacterial cell death\(^4\)), preventing cell wall synthesis (e.g. penicillin prevents bacterial cell wall synthesis by binding irreversibly to the DD-transpeptidase or the penicillin binding protein, which is responsible for cross-linking polysaccharide chains\(^5\)), or inhibiting protein synthesis (e.g. tetracycline inhibits protein synthesis by binding to the ribosome\(^6\)). They are effective against a broad spectrum of infectious microbes, ranging from Gram positive and negative bacteria to fungi. In addition, some have antitumor and
anticancer properties. Thus, naturally-occurring antibiotics such as aminoglycosides, cephalosporins, macrolides, and vancomycin were considered “wonder drugs” and were extensively used. As a result of such extensive as well as improper usages (such as incorrect dosing and treatment duration) over the years, bacterial resistance has become an alarming issue. For example, streptococci became resistant to penicillin only after one year of use, while tetracycline is obsolete against many bacteria and vancomycin is gradually losing its potency. Furthermore, there are different levels of toxicity associated with many antibiotics, ranging from minor (i.e. headache, diarrhea, and nausea) to serious (i.e. cardiotoxicity, ototoxicity, and nephrotoxicity) side effects, reducing effectiveness in their applications.

4.1.1 **Antimicrobial Peptides (AMPs)**

In the early 1970s, a new way of combating infectious microorganisms came with the discovery of peptides having antimicrobial activities. They are commonly called antimicrobial peptides (AMPs) and are found in both plants and animals. In human, AMPs are an essential part of the innate immunity, where they form the first line of defense against the invading pathogens in various locations of the body. These natural antibiotics are typically small cationic peptides of 3–6 kDa. Although their amino acid compositions vary, these peptides have been suggested to have similar structural motifs, a random coil in an aqueous environment and an α-helical or a β-sheet conformation in a hydrophobic environment.
AMPs display a wide range of antimicrobial activities against Gram-positive and -negative bacteria, fungi, parasites, protozoa, and some viruses. A generally accepted antibacterial mechanism of these peptides is their ability to penetrate the bacterial membrane and create pores through charge–charge (positively charged peptide and negatively charged cell membrane) interactions, which lead to cell death as a result of cytoplasmic leakage.\(^\text{10}\) Nevertheless, AMPs have also been suggested to interact with DNA, RNA, and specific proteins or enzymes as well as inhibit DNA, protein, or cell wall synthesis. Buforin II, a 21-amino acid antimicrobial peptide, is isolated from the stomach tissue of the Asian toad, \textit{Bufo bufo garagriozans}, and causes cell death by binding to DNA and RNA.\(^\text{13}\)

In addition to their antimicrobial functions, these antibiotic peptides are also associated with the processes in immunomodulation and detoxification. They can act as a flag in directing immune or inflammatory cells to the infectious sites, can enhance angiogenesis and wound healing, and can bind and neutralize lipopolysaccharides (LPSs). LPSs are released from the lysed bacterial cell wall as a result of antibiotic treatment. These molecules can over-activate the host’s immune system, which can ultimately lead to lethal endotoxicity.\(^\text{14}\) In addition, a salivary peptide, histatin 5, prevents the toxic tannin from intestinal absorption by binding and precipitating out the plant toxin.\(^\text{15}\)

Moreover, some of these peptide antibiotics have antiviral or anticancer properties other than their normal antimicrobial activities (antibacterial and antifungal). The three well-studied AMPs in humans are defensins, cathelicidins, and histatins.\(^\text{10c}\) Other well known
AMPs are the amphibian peptides, magainins and dermaseptins, and the insect peptides, cecropins and melittins.\textsuperscript{10b}

4.1.2 \textit{Candida albicans}

Human oral mucosa, a highly permeable tissue that lines the oral cavity, houses over 200 bacterial and fungal species.\textsuperscript{16} The oral cavity in which these microbes reside is consisted of the saliva, the tongue, and the tooth-associated supra-gingival and sub-gingival plaques. While the \textit{Streptococcus} species make up the majority of the bacterial flora, the \textit{Candida} species are the most predominant among the fungal families, including \textit{Candida albicans}, \textit{C. dubliniensis}, \textit{C. glabrata}, \textit{C. krusei}, and \textit{C. tropicalis}.\textsuperscript{16} Up to 80\% of healthy individuals have been suggested to retain these fungal microbes at various regions of the body (i.e. the oral cavity, lungs, the gastrointestinal tract, the vaginal tract, blood, and skin) but mostly in the oral cavity. Furthermore, these fungi are prominent (65–80\%) among healthy children, the elderly, and HIV patients.\textsuperscript{16}

Under normal circumstances, these fungal microorganisms are kept under control by the host’s innate defense, the \textit{whole saliva}. Nevertheless, this delicate equilibrium is disrupted in immunosuppressed or immunocompromised individuals, and conditions such as diabetes mellitus, immunosuppressive therapy, Sjogren’s syndrome, radiation therapy for head and neck tumour, and HIV infection can lead to \textit{candidiasis}.\textsuperscript{16} Moreover, \textit{C. albicans} makes up the largest percentile among the above-listed fungi and mainly implicated in HIV-related oral candidiasis (up to 90\%).\textsuperscript{16} Thus, the severity of its
infections can be used as an indicator for the disease progression in AIDS, owing to the prominent nature of oral candidiasis among immunocompromised patients.\(^\text{17}\) Although oral fungal infections are transient for the healthy individuals, they can be progressively infectious and may even be lethal for the immunodeficient HIV-infected adult and pediatric patients. In fact, the rise in the antifungal drug resistance has increased the mortality rate among AIDS patients over the years.\(^\text{18}\)

Whole saliva contains arrays of antimicrobial peptides and proteins that help control infections caused by these fungal microbes; some of the well-studied ones are mucins, histatins, defensins, calprotectin, myeloperoxidase, lysozyme, lactoferrin, secretory leukoprotease inhibitor, and proteolytic peptide fragments.\(^\text{16}\) In the case of fungal infections among the immunosuppressed individuals such as HIV patients, a number of drugs have been used: 5-fluorocytosine, the polyenes (i.e. amphotericin B and nystatin), and the azole-based antifungal drugs (i.e. triazole fluconazole). However, the fungal drug resistance has become an alarming issue over the years similar to the antibiotic resistance in bacteria.\(^\text{19}\) As a result, scientists are intensely studying the naturally-occurring antimicrobial peptides in hopes of finding a new way to combat those opportunistic microorganisms.

4.1.3 **Histatin 5 (Hn5)**

The search for a salivary component that enhances the glycolysis of oral microorganisms led to the discovery of a small basic peptide,\(^\text{20}\) which was later
determined to have multiple congeners and termed histatins (Hn) owing to their histidine-rich primary structures.\textsuperscript{21, 22} The ability of these peptides to bind hydroxyapatite with high affinity\textsuperscript{23} and the difficulty in the separation of the congeners initially interested researchers.\textsuperscript{24} Histatins are a family of histidine-rich cationic peptides secreted from the parotid and submandibular glands.\textsuperscript{22} Along with other salivary proteins and peptides, such as proline-rich proteins and defensins, histatins possess antimicrobial (against \textit{Streptococcus mutans})\textsuperscript{25} and antifungal (against \textit{Candida albicans}) properties and are active especially toward one of the most prevalent and opportunistic pathogenic yeasts, \textit{Candida albicans}.\textsuperscript{26} In addition, Hn5 show antifungal activity against \textit{Cryptococcus neoformans} and \textit{Saccharomyces cerevisiae}\textsuperscript{27} as well as azole-resistant strains of \textit{Candida}.\textsuperscript{28}

4.1.3.1 \textit{Structure of Histatins}

Histatins are present only in higher primates. Two genes, HTN1 and HTN2 localized on chromosome 4q13, are responsible for the synthesis of histatin 1 (DpSHEKRHHGY\textsuperscript{10} RRKFHEKHHS\textsuperscript{20} HREFPFYGDY\textsuperscript{30} GSNYLYDN) and histatin 3 (DSHAKRHGHY\textsuperscript{10} KRKFHEKHHS\textsuperscript{20} HRYGYSNYLY\textsuperscript{30} DN), respectively.\textsuperscript{29, 30} Histatin 1 is composed of 38 amino acids, with Ser-2 phosphorylated; whereas, histatin 3 is made up of 32 amino acids, having similar sequence as histatin 1.\textsuperscript{22} Shorter variants of these two peptides have been isolated and identified, which have also been shown to be
active toward *C. albicans*. Histatin 5 (Hn5), consists of the first 24 amino acids of histatin 3, is the most active fragment at physiological concentrations (15–30 μM). Hn5 is expressed at the highest concentration among the variants in human saliva. Hn1, 3, and 5 make up 85–90% of all histatin peptides in the whole saliva,\(^2^2\) and the variants are the proteolytic products with different antifungal activities toward *C. albicans*.\(^3^1\)

Based on a circular dichroism study, the full length along with several shorter length peptides (the shortest fragment is made up of 10 C-terminal amino acid residues) of Hn5 have different tendencies to form α-helices in hydrophobic environments; whereas, the random coil conformation is favored in aqueous environments. The same study suggests that the C-terminal (a minimum of 14 amino acids) but not the N-terminal residues of Hn5 are essential for appreciable candidacidal activity. The α-helical conformation is not required for its function, since less active fragments can also assume such secondary structure.\(^3^2\)

### 4.1.3.2 Antimicrobial Mechanisms of Histatin 5

Even though the antibacterial and antifungal activities of histatins have been confirmed,\(^2^5,\,2^6\) the antibiotic mechanism is still unclear. Two mechanisms have been proposed in the explanation of antimicrobial activity of Hn5. Initially, Hn5 was proposed to damage cell membranes, which result the release of K\(^+\) ions that are associated with the loss of cell viability.\(^2^5,\,2^6\) However, unlike other antimicrobial peptides, Hn5 has a weak amphipathic nature and lack the ability to form pores in the bacterial cell
membranes.\textsuperscript{33} The calcein dye permeability study by Edgerton et. al. supports this hypothesis by proposing that the release of intracellular calcein from \textit{C. albicans} is the aftermath of cell death and is not associated with cytotoxicity.\textsuperscript{34} In this study, it was proposed that the peptide destabilizes the cell membrane, followed by the interaction with specific cellular components leading to cell death and cytoplasmic leakage (which takes place after cell death).\textsuperscript{34} The same group later proposed that Hn5 induces cell death through non-lytic release of ATP.\textsuperscript{35} In this mechanism, Hn5 initially interacts and binds to Ssa1/2, the heat shock protein on the cell wall of \textit{C. albicans} and internalized.\textsuperscript{36} After entering the cell wall, Hn5 interacts with TRK1, the potassium transporter, leading to the loss of cell integrity.\textsuperscript{37} Extracellular ATP, in turn, binds and activates purinergic-like receptors, leading to apoptosis.\textsuperscript{35}

Oppenheim and co-workers proposed a different mechanism that involves the disruption of the yeast’s respiratory machinery.\textsuperscript{38} According to this group, the Hn5 peptide is first internalized, possibly by translocation down the membrane-potential gradient. This is followed by further internalization of the peptide into the mitochondrion through a similar mechanism. The internalization of Hn5 has been shown by the aggregated intensity of the fluorescent agents, \textit{tetramethylrhodamine isothiocyanate} and \textit{fluorescein isothiocyanate}, coupled to Hn5 in mitochondria.\textsuperscript{38b} Once inside, Hn5 interferes with the electron transfer processes, which leads to the generation of reactive oxygen species (ROS). The presence of ROS was confirmed by the accumulation of the oxygen radical sensitive fluorescent probe, \textit{dihydroethidium}, in mitochondria.\textsuperscript{38a}
Furthermore, they suggested that cellular respiration is necessary for the candidacidal activity since respiratory inhibitors such as cyanide and azide as well as anaerobic conditions reduce or prevent the antifungal activity of Hn5. The disruption of cellular respiration and the oxidative damages to biological molecules caused by ROS lead to eventual cell death.38

4.1.3.3 Metal-Binding of Histatin 5

Having multiple histidine residues allows Hn5 to have great flexibility in binding metal ions. Hn5 has a high-affinity Cu\textsuperscript{II} and Ni\textsuperscript{II} binding site (Asp-1-Ser-His known as ATCUN— Amino Terminal Cu\textsuperscript{II}- and Ni\textsuperscript{II}-binding)\textsuperscript{39} on the N-terminus and a preferential Zn\textsuperscript{II} binding site (HEXXH motif)\textsuperscript{40} on the C-terminus.\textsuperscript{41} The affinity constants determined from an isothermal calorimetry study are $2.6 \times 10^7$ M\textsuperscript{-1} and $1.2 \times 10^5$ M\textsuperscript{-1} for the Cu\textsuperscript{II}- and Zn\textsuperscript{II}-binding site, respectively.\textsuperscript{42} In addition to Cu\textsuperscript{II} and Zn\textsuperscript{II}, Hn5 can bind Ni\textsuperscript{II}, Ca\textsuperscript{II}, and Fe\textsuperscript{II}, although the binding affinities for the latter two are very weak. The order of metal binding to Hn5 is Cu\textsuperscript{II} > Ni\textsuperscript{II} > Zn\textsuperscript{II} in aqueous environments. Both Hn3 and Hn5 have been shown to be able to bind more than one (and up to 5) equivalent of metal ions.\textsuperscript{41}

A different study shows the binding of Hn5 to divalent transition metals, such as Cu\textsuperscript{II}, Ni\textsuperscript{II}, and Zn\textsuperscript{II}, and displays DNA nuclease activity.\textsuperscript{43} It was shown that the Cu\textsuperscript{II} complexes of synthetic variants of Hn5, containing both the ATCUN and HEXXH regions, can damage plasmid DNA through redox chemistry. Based on NMR results, it
was suggested that the peptide structure is stabilized by metal binding. In a recent study by the same group, another short ATCUN containing synthetic variant of Hn5 was proposed to have a pro-oxidant activity. They proposed a possible generation of ROS by Hn5 in the presence of Cu$^{II}$, supporting the ROS-induced antimicrobial mechanism. ROS generation was proposed to follow oxygen activation by the Cu$^{II}$-Hn5 complex.

It is apparent that there is a trend in the ability of Cu$^{II}$-binding peptides to perform oxidation chemistry. This has been demonstrated in recent studies, where the short soluble and metal-binding fragments of amyloid peptides have been shown to perform catechol- and phenol-oxidase-like chemistry in the presence of Cu$^{II}$. The oxidation mechanism has been proposed to involve oxygen binding and activation through a transient di-Cu$^{II}$ center. Another example is the antibiotic peptide, bacitracin, discussed in the previous chapter. Bacitracin, however, appears to perform a mononuclear Cu-centered catalysis, based on experimental results. In both cases, only one Cu$^{II}$ ion can bind to the peptide. With histatin 5, at least two Cu$^{II}$ ions can bind to each peptide, owing to the presence of 7 histidine residues. Thus, it will be interesting to study the potential oxidation chemistry with a peptide containing more than one binding site in the sequence which is the theme of this chapter.
4.2 **Materials and Methods**

Histatin 5 (Hn5) was synthesized and the purity determined using MALDI-TOF mass spectrometer at the University of South Florida Peptide Synthesis and Mass Spectrometry Center. Copper Sulfate (anhydrous) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonate buffer (HEPES) used for the reactivity experiments was purchased from Fisher Scientific Co. (Fair Lawn, NJ). Pyrocatechol (~99%) and 3-Methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (98%), MBTH, were acquired from Sigma–Aldrich Inc. (St. Louis, MO) and Acros Organics (NJ), respectively. All chemicals were used without further purification. All other solvents and reagents were of the highest grade available from commercial sources. Deionized water (18.2 MΩ) was obtained from a Millipore Milli-Q system. Plastic ware and glassware were demetallized with EDTA and extensively rinsed. All components were freshly prepared prior to the experiments.

4.2.1 **Kinetic Studies**

Catechol oxidation assays were carried out as previously described.\(^{46}\) Different concentrations (0–6.4 mM) of catechol (with equal concentrations of o-quinone specific indicator, MBTH) were incubated with 5.0 μM Cu\(^{II}\)–Hn5 (4:1 metal-to-peptide) in the presence and absence of H\(_2\)O\(_2\) in 100.0 mM HEPES buffer at pH 7.0 and 25 °C. The final volume in the cuvette is 1.0 mL. The oxidized o–quinone can bind to MBTH to form a red adduct, which was monitored at 500 nm (\(ε = 32, 500 \text{ M}^{-1}\text{cm}^{-1}\))\(^{47}\) on a Varian Cary50
Bio UV-Vis spectrophotometer equipped with a water peltier PCB-150 thermostable cell (Varian, Palo Alto, CA). The initial rate was determined from the slope of the change in the absorbance with respect to time (0–5 mins) in the linear region. The kinetic constants, $K_M$ and $k_{cat}$, were determined by fitting the data to the Michaelis-Menten equation in the Sigma Plot 8.0.

### 4.2.2 Optical Studies

The slow substrate, 4,5-di-chloro-catechol (DCC), was used as a ligand in binding studies to show the interaction between the Cu$^{II}$-Hn5 complex and the catechol substrate. Due to low solubility in water, DCC was dissolved in DMF. The stock solution of the Cu$^{II}$-Hn5 complex was prepared in DI water, since only the peptide and not the copper salt would dissolve in DMF. The experiment was performed in DMF, and all components were completely miscible. Briefly, DCC ranging from 0 to 1.0 mM was gradually titrated into a 1.0mL solution of Cu$^{II}$-Hn5 (200.0 μM) with the sample cuvette left in the spectrometer to keep the baseline intact. The background was zeroed with only the solvent, and the mixture was scanned from 200 to 900 nm on the Varian Cary50 Bio UV-Vis spectrophotometer. The dilution factor from each addition was corrected in the final concentrations. The molar absorptivity value of the Cu$^{II}$-Hn5-DCC complex was plotted with respect to the titrated DCC concentration, and the data was fitted to simple 1:1 binding quadratic equation in Sigma Plot 8.0 for the determination of the affinity constant, $K_{DCC}$. 

178
4.2.3 **NMR Studies**

$^1$H NMR spectra were acquired on Varian INOVA500 spectrometer (at 500 MHz $^1$H resonance) with a 5-mm bio-TR (triple resonance) probe. A 90° pulse (~9 μs) was used for the acquisition of 1D $^1$H NMR spectra with 8 K data points. The superWEFT technique\(^4\) was used for the suppression of slowly relaxing signals. Both peptide and cobalt stock solutions were prepared in d$_6$-DMSO. Hn5 peptide sample (0.5 mM) was titrated with Co$^{II}$, and the complex formation was monitored over 300 ppm spectral width (~150 to 150 ppm). Triethylamine was added as needed. A line-broadening of 40 Hz was applied to improve the signal-to-noise ratio of the paramagnetically shifted signals.

4.3 **Results and Discussion**

There are 7 histidine residues on Hn5, which afford more than one metal-binding site. Metal-binding studies have been previously done for Hn5, and two possible sites were proposed: a high-affinity Cu$^{II}$ and Ni$^{II}$ binding site on the N-terminus (known as ATCUN—Amino Terminal Cu$^{II}$- and Ni$^{II}$-binding)\(^3\) and a preferential Zn$^{II}$ binding site on the C-terminus (HEXXH motif)\(^4\). However, up to 4 equivalents of Cu$^{II}$ have been suggested to bind 1 equivalent of Hn5 peptide.\(^4\) Thus, a 4:1 Cu$^{II}$-to-Hn5 complex was used in the reactivity studies.

Only two metal binding motifs have been proposed for Hn5 although this peptide of 24 amino acids has 11 possible metal binding residues (i.e. 1 Asp, 1 Glu and 2 Tyr in...
addition to 7 His residues). A recent study on Zn$^{II}$ and Cu$^{II}$ binding by means of isothermal calorimetry suggests that Hn5 has three possible metal binding sites for both metals.$^{42}$ Since the presence of 2 versus 3 Cu$^{II}$ ions may react with catechol differently, it is essential to know how many Cu$^{II}$ ions are bound per Hn5 peptide. Thus, Cu$^{II}$ binding by Hn5 was studied by means of optical, EPR, and NMR spectroscopy to determine the stoichiometry.

The optical Cu$^{II}$ titration was performed in 100.0 mM HEPES buffer at pH 7.0 and 25 ºC on Cary50 spectrophotometer. Hn5 (200.0 μM) was gradually titrated with Cu$^{II}$ and the electronic spectrum collected from 200–800 nm. Upon Cu$^{II}$ addition, two strong absorptions around 250 and 300 nm and a d–d transition absorption at ~520 nm were observed (Figure 4.1). As more Cu$^{II}$ is added, a gradual red shift by all three signals was observed. The sample was titrated with up to ~6.0 equivalents of metal, resulting in ~100 nm shift in the d–d transition. The resulted electronic spectra suggest the presence of more than one Cu$^{II}$-bound Hn5 species. Since the number of species present cannot be clearly distinguished from the electronic spectra, the Cu$^{II}$ binding was further investigated with EPR spectroscopy.

The continuous-wave (CW) EPR spectra of Cu$^{II}$-Hn5 complexes were acquired on a Bruker Elexsys E580 cw X-band spectrometer assisted by Dr. Angerhofer at the University of Florida. The spectra were acquired on the following complexes of Cu$^{II}$
Figure 4.1. Optical Cu$^{II}$ binding study of Hn5 (200.0 μM) in 100.0 mM HEPES buffer at pH 7.0 and 25 °C. Approximately 100.0 μM Cu$^{II}$ (0.5 equivalent) is added for each spectrum. The inset is the expansion of the wavelength from 400–800 nm. A gradual shift in all three observed signals was observed upon addition of metal. The $d$–$d$ transition at ~520 nm shifted approximately 100 nm after up to ~6.0 equivalents of Cu$^{II}$ has been added.
-(250.0 μM) Hn5 in DMF: 1:1, 2:1, and 4:1 (Figure 4.2 and 4.3). The spectra display characteristics typical of CuII complexes, having 4 hyperfine splittings in an axial orientation (i.e. \( g_x = g_y < g_z \)), with “overshoot” signals. The magnetic field is converted into g values, and the spectra are plotted with respect to g values (on x-axis). The g-parallel values for 1:1 and 2:1 species are approximately 2.198 and 2.269, respectively, while the g-perpendicular value is estimated to be 2.062 for both species (Figure 4.2). In the spectrum for 1:1 CuII-Hn5, only one set of hyperfine splittings can be clearly seen; whereas, in the 2:1 spectrum, another set of hyperfine signals in addition to the former is apparent. The difference in the g values also indicates the presence of two different CuII species in 2:1 spectrum, and these values fall within the normal range for CuII species bound to nitrogen-donor ligand such as histidine. At 4:1 species, the hyperfine splittings of 1:1 and 2:1 species are replaced by a single set of hyperfine signals with g-parallel and g-perpendicular values of 2.313 and 2.082, respectively (Figure 4.3). Based on the clear difference between 1:1 and 2:1 versus 4:1 species indicates there may be two or more types of CuII centers present at 4 equivalents of CuII.

Next, the metal coordination by Hn5 was further defined by means of NMR spectroscopy. The slow electronic relaxation of CuII can broaden the NMR signals beyond detection, rendering it useless in metal-binding studies by means of NMR spectroscopy. However, fast relaxing CoII has been effectively utilized as a paramagnetic NMR probe in studying the metal-coordination site of various systems. A 0.5-mM apo-Hn5 peptide sample was gradually titrated with CoII in d6-DMSO, and the
Figure 4.2. EPR spectra of 2:1 (dotted line) and 1:1 (solid line) Cu$^{II}$-(250.0 μM) Hn5 in DMF. The g-parallel values for 1:1 and 2:1 species are 2.198 and 2.269, respectively, while the g-perpendicular value is estimated to be 2.062 for both species. The 2:1 spectrum clearly shows the presence of two different species of Cu$^{II}$, which can be distinguished based on their g-parallel values.
Figure 4.3. EPR spectrum of 4:1 Cu$^{II}$-(250.0 μM) Hn5 in DMF. The estimated g values are 2.313 and 2.082 for g-parallel and g-perpendicular, respectively.
data was acquired on the INOVA500 Varian spectrometer with a bio-triple resonance 5 mm probe at 25 °C. Up to 6.0 equivalents of CoII were added to the peptide in small increments. No signal was observed upon addition of 0.5 equivalent of CoII within the monitoring spectral region (–150 to 150 ppm). At 1.5 equivalents, three very weak signals can be observed at ~50, ~60, and ~70 ppm, which can be clearly seen at 2.0 equivalents of CoII (Figure 4.4). The two signals at 58 and 67 ppm are broader compared to a relatively sharp signal at 50 ppm. A new signal appears approximately at 48 ppm after 2.5 equivalents of CoII, and continued addition of CoII up to 6.0 equivalents only enhances the intensity of the shifted signals. The typical chemical shift range for CoII-bound imidazole group of histidine residue is approximately from 40–80 ppm. Addition of 30.0 uL D2O to the sample significantly reduces the signal intensity, which clearly shows these signals to be the solvent-exchangeable imidazole NH protons. Thus, the signals observed may be assigned to the CoII-bound histidine residues on Hn5.

An important observation from this experiment is the indication that there are only two types of metal binding modes. From 0.5 to 2.5 equivalents of CoII, one binding mode of CoII-Hn5 can be seen from a simultaneous increase of signal intensity at 50, 58, and 67 ppm. A second mode of metal binding becomes observable starting at 2.5 equivalents of CoII, showing a new signal at 47 ppm and no additional signals are observed up to 6.0 equivalents of CoII. The three signals from the first binding mode may be assigned to His-15, His-18, and His-19 in the HEXXH motif, since there is only one histidine in the ATCUN motif. In the ATCUN motif, the metal is bound through the
Figure 4.4. $^1$H NMR spectra of Co$^{II}$-Hn5 at 2:1 (bottom) and 6:1 (top) ratios with 8k scans. Up to 2.5 equivalents of Co$^{II}$, only three signals at 50, 58, and 67 ppm are observed, and from 2.5 to 6.0 equivalents of Co$^{II}$, a new signal at 47 ppm appears. All signals are solvent-exchangeable (i.e. determined by signal reductions after D$_2$O addition). The three signals may represent one metal binding mode by 3 histidine residues in HEXXH motif, and the latter signal at 47 ppm may be assigned to a single histidine residue in ATCUN motif.
amide backbones of the first three amino acids and the imidazole group of \textsuperscript{3}His. Thus, a single signal from the second binding mode may be assigned to the ATCUN site.

Different concentrations of catechol were incubated with 5.0 μM Cu\textsuperscript{II}-Hn5 (4:1) in 100.0 mM HEPES buffer at pH 7.0 and 25 °C. An increase in the rate of catechol oxidation was observed with increasing substrate, which reaches saturation at higher concentrations of catechol (Figure 4.5). The saturation profile suggests the presence of enzyme-like kinetics, and the rate law can be described according to the following equations, with the assumption that [CA] >> [Cu(II)-Hn5-CA]:

\[
[Cu(II)-Hn5] + [CA] \overset{k_1}{\underset{k_{-1}}{\rightleftharpoons}} [Cu(II)-Hn5-CA] \overset{k_{cat}}{\rightarrow} [Cu(II)-Hn5] + o-quinone
\]

\[v_0 = \frac{V_{Max}[CA]}{K_{Hn5} + [CA]} \quad (1)\]

where \(v_0\) and \(V_{Max}\) are the measured and maximum velocity, respectively, and

\[K_{Hn5} = \frac{k_{cat} + k_{-1}}{k_1}\]

is the virtual dissociation constant of the Cu\textsuperscript{II}-Hn5-CA complex. The data can be well fitted to Eq. 1 to afford \(K_{Hn5}\), the first-order rate constant or the turnover number \(k_{cat}\) (\(k_{cat} = V_{Max}/[Cu\textsuperscript{II}-Hn5]\)), and the second-order rate constant or the catalytic efficiency \(k_{cat}/K_{Hn5}\) of 3.06 × 10\textsuperscript{-3} M, 4.53 × 10\textsuperscript{-2} s\textsuperscript{-1}, and 14.83 M\textsuperscript{-1} s\textsuperscript{-1}, respectively (Figure 4.5). Compared to the auto-oxidation rate of catechol (4.74 × 10\textsuperscript{-7} s\textsuperscript{-1}) in the absence of Cu\textsuperscript{II}-Hn5,\textsuperscript{45} there is a 9.56 × 10\textsuperscript{4}-times rate acceleration with respect to the first-order rate constant.
Figure 4.5. Catechol oxidation by Cu^{II}-Hn5 (4:1) in 100.0 mM HEPES at pH 7.0 and 25 °C. The saturation profile suggests a possible pre-equilibrium kinetics. The data are fitted to Eq. 1, an analogue of Michaelis-Menten equation, to afford $K_{Hn5}$, $k_{cat}$, and $k_{cat}/K_{Hn5}$.
Since Cu$^{II}$-Hn5 can oxidize catechol, the Cu$^{II}$ binding to Hn5 was monitored with catechol oxidation. Different amounts of Cu$^{II}$ (0–16.0 equivalents) were incubated with 5.0 μM Hn5 and 5.0 mM catechol/MBTH in 100 mM HEPES buffer at pH 7.0 and 25 °C. From 0–1.0 equivalent of Cu$^{II}$, no significant activation was observed (Figure 4.6). However, a sudden increase in the activity was observed after the first equivalent of Cu$^{II}$ and does not reach saturation until 16.0 equivalents of metal. Since Hn5 has multiple metal binding residues and two proposed metal binding sites on the sequence, the peptide may bind the Cu$^{II}$ ion in such a way that no binding site is left open for substrate interaction. In the presence of more Cu$^{II}$, however, more substrate binding sites can become available. As a result, the 1:1 Cu$^{II}$-Hn5 species appears inactive, and an abrupt increase in the activity is observed at higher Cu$^{II}$ concentrations.

The interaction between the Cu$^{II}$-Hn5 complex and the catechol was confirmed by an optical binding study with a slow substrate, 4,5-dichlorocatechol (DCC). The activity of DCC is roughly 200 times slower than catechol with respect to $k_{cat}$ as a ligand.$^{45}$ A 1.0-mL solution of 200-μM Cu$^{II}$-Hn5 was slowly titrated with DCC in DMF at 25 °C. The formation of complex was monitored from 200–800 nm on the Varian Cary50 Bio UV-Vis spectrophotometer. Upon addition of DCC, a new absorption corresponding to a complex between Cu$^{II}$-Hn5 and DCC appears at 298 nm (Figure 4.7). The plot of the (Cu$^{II}$-Hn5)-DCC complex against equivalents of DCC added shows the binding to reach a saturation at approximately 3.0 equivalents of DCC. The results suggest a possible presence of three metal binding sites for substrate binding.
Figure 4.6. Cu$^{II}$ binding of Hn5 monitored with catechol oxidation activity. No significant activity was observed approximately up to 1.0 equivalent of Cu$^{II}$, which is followed by a sudden increase in the activity for subsequent assays.
In the presence of H\textsubscript{2}O\textsubscript{2}, a significant rate enhancement in catechol oxidation was observed for the Cu\textsuperscript{II}-Bc system in the previous chapter. Similar experiments were performed to determine the effect of H\textsubscript{2}O\textsubscript{2} on catechol oxidation by Cu\textsuperscript{II}-Hn5. Different concentrations (0–64.0 mM) of H\textsubscript{2}O\textsubscript{2} were incubated with 0.5 \, \mu\text{M} Cu\textsuperscript{II}-Hn5 and 3.2 mM catechol in 100.0 mM HEPES buffer at pH 7.0 and 25 °C. The increase in catechol oxidation was observed with increasing H\textsubscript{2}O\textsubscript{2} and reaches saturation at higher concentrations of H\textsubscript{2}O\textsubscript{2} (Figure 4.8 inset). The saturation profile indicates a possible presence of a pre-equilibrium kinetics, suggesting H\textsubscript{2}O\textsubscript{2} as another substrate capable of interaction with the metal center(s). The data can be fitted to Eq. 2 to afford an apparent binding or dissociation constant $K_{H2O2}$ of $8.63 \times 10^{-3}$ M for H\textsubscript{2}O\textsubscript{2}.

$$V_0 = V_{Background} + \frac{V_{Max}[H_2O_2]}{K_{H_2O_2} + [H_2O_2]}$$  \hspace{1cm} (2)

This compared to $K_{Hn5} = 3.06 \times 10^{-3}$ M, the binding constant for catechol in the absence of H\textsubscript{2}O\textsubscript{2}, is approximately 2.8 times higher, suggesting that this substrate binds ~3× lower than catechol toward the Cu\textsuperscript{II}-Hn5 complex.

The fact that both substrates can independently bind to the Cu\textsuperscript{II} center (i.e. both catechol and H\textsubscript{2}O\textsubscript{2} show saturation), it is essential to determine how the binding of one affects the other. Different concentrations (0–6.4 mM) of catechol were incubated with the Cu\textsuperscript{II}-Hn5 (0.2 \, \mu\text{M}) in the presence of different H\textsubscript{2}O\textsubscript{2} concentrations (0–64.0 mM). The enhancements in activity of catechol oxidation in the presence of H\textsubscript{2}O\textsubscript{2} were clearly observed (Figure 4.8). While $K_{Hn5}$ is increased by ~2 folds, the addition of 32.0 mM...
Figure 4.7. 4,5-dichlorocatechol binding of Cu$^{II}$-Hn5 in DMF. All the spectra have been zeroed with that of Cu$^{II}$-Hn5 complex alone. The (Cu$^{II}$-Hn5)-DCC complex plotted versus DCC added shows saturation at 3.0 equivalents of DCC.
H$_2$O$_2$ in the assay increased both the $k_{cat}$ and $k_{cat}/K_{Hn5}$ by 7 and 4 folds, respectively.

Because the two substrates may bind to Cu$^{II}$-Hn5 independently of each other, the Hanes plot analysis can be used to determine the interaction between the two substrates.

From the Hanes analysis, the apparent binding equilibrium constants for both substrates and an intrinsic binding constant for one substrate can be calculated. The Hanes analysis requires an initial linear treatment of the data in Figure 4.8 according to the following rate law (Eq. 3),

$$
\frac{[\text{Catechol}]}{v_0} = \frac{1 + \frac{K_{App}^{H_2O_2}}{[H_2O_2]}}{V_{max}}[\text{Catechol}] + \frac{K_{Catechol}^{App}}{V_{max}} \left(1 + \frac{K_{Int}^{H_2O_2}}{[H_2O_2]}\right)
$$

where $K_{Int}^{H_2O_2}$ is the intrinsic binding constant for H$_2$O$_2$, $K_{App}^{H_2O_2}$ and $K_{Catechol}^{App}$ are the apparent binding constants for H$_2$O$_2$ and catechol, and $v_0$ and $V_{max}$ are the experimental and maximum velocity, respectively (Figure 4.9 A). The ratio between the corresponding apparent and intrinsic equilibrium constants for each substrate can suggest how the binding of one substrate affects the binding of another. The secondary plots of the fitted y-intercept (Eq. 4) and slope (Eq. 5) values versus 1/[H$_2$O$_2$] afford the two apparent equilibrium constants $K_{App}^{H_2O_2} = 5.23 \times 10^{-3}$ M and $K_{Catechol}^{App} = 3.63 \times 10^{-3}$ M and the intrinsic binding constant $K_{Int}^{H_2O_2} = 2.26 \times 10^{-3}$ M (Figure 4.9 B and C).
Figure 4.8. Hydrogen peroxide influence on catechol oxidation by 0.2 μM Cu$^{II}$-Hn5 at pH 7.0 in 100.0 mM HEPES buffer at 25 °C. The concentrations of H$_2$O$_2$ were varied from 0 to 64.0 mM. The titration of H$_2$O$_2$ at a constant catechol concentration of 3.2 mM displays saturation, which indicates the direct interaction between the Cu$^{II}$-Hn5 center and H$_2$O$_2$ (the inset).
The ratio between the apparent and intrinsic binding constants is 1.19 for catechol

\( \frac{K_{\text{App}}^{\text{Catechol}}}{K_{\text{App}}^{\text{Hn5}}} \) and 2.31 for \( \frac{K_{\text{App}}^{\text{H2O2}}}{K_{\text{Int}}^{\text{H2O2}}} \), suggesting that the binding of \( \text{H}_2\text{O}_2 \) to the \( \text{Cu}^{II}-\text{Hn5} \) complex does not affect the catechol binding; whereas, the binding of \( \text{H}_2\text{O}_2 \) is reduced by half in the presence of catechol.
**Figure 4.9.** Hanes analysis of the oxidation of catechol by Cu$^{II}$-Hn5 at different concentrations of H$_2$O$_2$ (kinetic data from Figure 4.8). Plot B and C are the replots of the slope and y-intercept values from plot A with respect to [H$_2$O$_2$]$^{-1}$. The apparent binding equilibrium constants for catechol ($K_{Catechol}^{app}$) and H$_2$O$_2$ ($K^{app}_{H_2O_2}$) as well as the intrinsic binding constant for H$_2$O$_2$ ($K_{H_2O_2}^{int}$) can be determined from the secondary plots.
4.4 Conclusions

In this study, the oxidative activity of Hn5 peptide in the presence of Cu\textsuperscript{II} has been studied. Recent results have shown the ability of short N-terminal metal binding fragments A\beta (i.e. A peptide of 1–40 or 1–42 amino acids isolated from the plaques of Alzheimer’s disease patients.), A\beta\textsubscript{1–16} and A\beta\textsubscript{1–20}, can perform catechol- and phenol-oxidase-like chemistry in the presence of Cu\textsuperscript{II}.\textsuperscript{45, 46} Furthermore, in the previous chapter I have shown catechol oxidation chemistry of the Cu\textsuperscript{II} complex of bacitracin, a dodecapeptide antibiotic. While the Cu\textsuperscript{II}-A\beta complexes oxidize catechol to o-quinone  through a di-Cu\textsuperscript{II} center catalysis, the Cu\textsuperscript{II}-Bc complex only requires a mononuclear Cu\textsuperscript{II} center. Both systems bind to Cu\textsuperscript{II} in a 1:1 ratio. Nevertheless, two Cu\textsuperscript{II}-A\beta complexes have been proposed to assemble into a di-Cu\textsuperscript{II} center either by the substrate or oxygen during catalysis.\textsuperscript{45, 46} Thus, it will be interesting to determine how will the Hn5 peptide, having two or more potential metal binding sites within per peptide sequence, perform catechol oxidation in the presence of redox-active Cu\textsuperscript{II}.

The Cu\textsuperscript{II} complex of Hn5 has been shown to effectively oxidize catechol with a rate enhancement of $9.56 \times 10^4$ times with respect to the first-order rate constant, compared to the auto-oxidation rate of catechol ($4.74 \times 10^{-7}$ s\textsuperscript{-1}).\textsuperscript{45} For Cu\textsuperscript{II}A\beta\textsuperscript{45} and Cu\textsuperscript{II}-Bc, the presence of H\textsubscript{2}O\textsubscript{2} significantly enhances the catechol oxidation. H\textsubscript{2}O\textsubscript{2} also significantly enhances the catechol oxidation by Cu\textsuperscript{II}-Hn5.

The preliminary results from the above studies only suggest the ability of Cu\textsuperscript{II}-Hn5 to perform catechol oxidation. According to EPR and NMR studies, there are two
potential metal binding sites on Hn5. Because the metal binding sites are on the same peptide, they may be in close enough proximity to perform a di-Cu$^{II}$ oxygen chemistry. However, more studies are necessary to differentiate between the mononuclear and dinuclear Cu$^{II}$ center. Thus, experiments such as optical DCC binding and catechol binding (i.e. with respect to activity) using the Job method can provide further evidence. In addition, similar studies with EPR and NMR spectroscopic techniques may provide more details on substrate binding; whereas, all the above studies under anaerobic conditions can provide a clear picture for the role played by dioxygen.

In conclusion, redox-active metals such as Cu$^{II}$ and Fe$^{III}$ are essential for biological processes but are heavily regulated due to oxidative damages they can impose on the biological molecules.$^{53}$ Dysregulation of metal ions have been associated with many human pathologies, such as amyotrophic lateral sclerosis, Wilson’s, Alzheimer’s, and Parkinson’s disease, just to name a few.$^{54}$ There seems to be a trend among some biologically available peptides, where they can effectively bind a wide range of metal ions.$^{55}$ It is possible that these metal binding peptides play an important role in the regulation of these redox metals. Thus, understanding of the oxidation chemistry of these metal-peptide complexes may be essential to better understanding of related pathologies. Even though the findings in this dissertation may not be directly extended to *in vivo* situations, they are intended for providing fundamental understandings in hopes of serving as a building block for future innovations.
References


23 Hay, D. I. Fractionation of human parotid salivary proteins and the isolation of an histidine-rich acidic peptide which shows high affinity for hydroxyapatite surfaces. 


ABOUT THE AUTHOR

William Maung Tay was born in 1979 in Rangoon, Burma (currently known as Yangon, Myanmar). He immigrated to the United States of America at the age of 12. After overcoming the language barrier, he graduated with high honor with distinction from Lakeland Senior High School. He attended Florida Southern College from 1998 to 2002 and graduated with a B.Sc. in both chemistry and mathematics. Then, he pursued his graduate studies in chemistry at the University of South Florida under the supervision of Dr. Li-June Ming. From 2002 to 2004, he served as a teaching assistant for General and Organic Chemistry Labs. Then, he took on the NMR teaching assistant position from 2004 to 2007.