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Metallopeptides From Design to Catalysis: Structure, Oxidative Activities, And Inhibition Studies Of Designed And Naturally Occurring Metallopeptides

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Metallopeptides From Design To Catalysis: Structure, Oxidative Activities, And Inhibition Studies Of Designed And Naturally Occurring Metallopeptides.

by

Alaa Hassan Hashim

A dissertation submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy
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Keywords: bioinorganic chemistry, metallopeptide models, structure elucidation, molecular dynamics, paramagnetic NMR, enzyme-like kinetics, beta amyloid, DNA cleavage

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DEDICATION

I would like to dedicate my work to my family for their endless love and support, and to my beloved grandmother who is currently suffering from advanced stages of Alzheimer's disease.
ACKNOWLEDGMENTS

Foremost, I would like to extend my deepest gratitude to my advisor and mentor, Professor Dr. Li-June Ming, for his immense knowledge, enthusiasm, motivation, research ingenuity, endless patience, and persistent in inspiring us to prosper. The experience acquired under his supervision has flourished within me as a chemist in research and in teaching. Endless thanks. I would also like to acknowledge the committee members, Dr. David Merkler, Dr. Jainfeng Cia, and Dr. Xiao Li, for their advice, and continuous support. I would also like to extend my acknowledgement to Dr. Abdul Malik for initially serving on my committee and for all his support. I would like to acknowledge Dr. William Tay for accepting to chair the defense, and for all his support along this journey. I must also acknowledge Dr. Edwin Rivera for his help, support, and encouragement, and for making the NMR facility at USF a great place to work at. I would also like to acknowledge everyone I have worked with or for as a teaching assistant here at USF: Dr. Rebecca O’Malley, Dr. Solomon Weldegirma, Dr. Vasiliki Lykourinou, and Dr. Kerrianne Greenhalgh. I must also acknowledge Dr. Nicole West, from the academy of teaching and learning excellence at USF, for her very insightful course on college teaching and for all her advice.

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I have enjoyed this journey, and thanks to all my friends and to everyone who had a positive impact in my life.
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<th>Description</th>
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<tr>
<td>1D:</td>
<td>One-dimensional</td>
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<tr>
<td>2D:</td>
<td>Two-dimensional</td>
</tr>
<tr>
<td>A:</td>
<td>Alanine</td>
</tr>
<tr>
<td>AD:</td>
<td>Alzheimer’s disease</td>
</tr>
<tr>
<td>Ala:</td>
<td>Alanine</td>
</tr>
<tr>
<td>APP:</td>
<td>Amyloid precursor protein</td>
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</tr>
<tr>
<td>EDTA:</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>EXSY:</td>
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gDQCOSY: Gradient double–quantum filtered correlation spectroscopy
H: Histidine
HEPES: N-(2-hydroxyethyl)-piperazine-N′-2-ethanesulfonic acid
His: Histidine
Hz: Hertz
$k_{cat}$: Catalytic turnover number
$K_i$: Inhibition Constant
$K_m$: Michaelis-Menten Constant
LMCT: Ligand to metal charge transfer transition
M: Molar
MBTH: 3-methyl-2-benzothiazolinone hydrazine
MePY2: Methylbis[2-(2-pyridyl)ethyl]amine
MES: 2-Morpholinoethanesulfonic acid
mM: Millimolar
N4PY2: Bis[2-(2-pyridyl)ethyl]amine with (CH$_2$)$_4$ linker
NADP$: Nicotinamide adenine dinucleotide phosphate, oxidized form
NADPH: Nicotinamide adenine dinucleotide phosphate, reduced form
NMR: Nuclear Magnetic Resonance
NOE: Nuclear Overhauser effect
NOSEY: Nuclear Overhauser effect spectroscopy
P: Proline
ppm: Parts per million
Pro: Proline
R: Arginine
ROS: Reactive Oxygen Species
$T_1$: Spin-lattice relaxation time
TEA: Triethylamine
THB: 1,2,3-trihydroxybenzene
TOCSY: Total correlation spectroscopy
UV-Vis: Ultraviolet-visible electronic spectroscopy
Zn: Zinc
Zn\(^{+2}\): Zinc (II)
δ: Chemical shift
ε: Molar absorptivity
ABSTRACT

Structural and mechanistic complexities of copper-dioxygen systems have attracted much attention in the field of bioinorganic chemistry, both in model systems and trapped protein intermediates. The research presented herein is focused on model and naturally occurring metallopeptide systems, from its design to catalysis. Copper is used as the coordinating metal ion, with cobalt and zinc as probes for metal binding. The bioinorganic chemistry of copper proteins and its coordination and spectroscopic properties are briefly discussed in chapter 1. The next two chapters are centered on the de novo design of a minimalistic metallopeptide system with an amino acid sequence of RHHPPHHE. Structural characterization of the peptide by means of CD and NMR spectroscopy techniques are presented in chapter 2, suggesting a characteristic beta-turn structure in its apo and di-metal bound form. The designed metallopeptide exhibits catecholase activity, which is presented in chapter 3. The data suggest the presence of two mononuclear copper active sites, exhibiting specificity towards the oxidation of catecholamine substrates. Similarly, the catecholase activity has been previously observed in copper complexes of Alzheimer’s disease related peptide β-amyloid, exhibiting metal-centered redox chemistry. The metallo-Αβ complexes are the hallmark Alzheimer’s disease and have been attributed to the generation of reactive oxygen species causing oxidative stress. Thus, inhibition of the observed oxidative activities was investigated. Probing the role of phosphate moieties in various
compounds as potential inhibitors against the induced oxidative stress is presented in chapter 4. The phosphate analogs of the studied compounds exhibit more pronounced potency, where mutation of the Aβ20 peptide at Arg-5 and Lys-16 give insight into the interactions of the side chains of Arg and Lys with the phosphate moiety. $^{31}$P NMR relaxation studies further support the binding/interaction of phosphate with the Cu$^{2+}$–Aβ complexes. The correlation of phosphate moiety binding/activity will allow for the design of more potent inhibitors toward the Cu$^{2+}$–Aβ induced oxidative stress.
CHAPTER 1: INTRODUCTION TO THE BIONINORGANIC CHEMISTRY OF COPPER CENTERS

1.1 Background

Structural and mechanistic complexities of copper-dioxygen$^1,^2$ systems have attracted much attention in the field of bioinorganic chemistry, both in model systems and trapped protein intermediates.$^3$ Reactions involving copper-dioxygen species have potential relevance in biochemical systems, synthetic catalysis, and industrial applications.$^4,^5$ In biological systems, the reaction of dioxygen with organic substances is thermodynamically favorable, but kinetically unfavorable and must be catalyzed by metalloenzymes. This is attributed to the valence configuration of dioxygen, $(1\pi_g^*)^2$, which gives a triplet ground state, high spin $^3\Sigma_g^-$, as compared to a singlet state for the majority of organic molecules, except organic radicals. Thus, the reaction between dioxygen and organic molecules is spin forbidden implying that it is slow. Biological molecules overcome this barrier in dioxygen binding and activation; by the use of copper and iron containing metalloproteins that function as efficient oxidation catalysts.$^6,^7,^8,^9$

Copper, heme, and non-heme iron containing metalloproteins catalyze a large number of biologically important reactions and play key roles in dioxygen binding and activation.$^{10}$ The active site environment and the redox properties of
the metal ion at the active site influence the mechanistic pathway of dioxygen binding and activation. For instance, Heme (cytochrome p450), non-heme iron (catechol dioxygenase), and copper (dopamine β-monooxygenase) containing enzymes catalyze the hydroxylation/oxygenation of alkanes/arenes. The first step involves C-H bond cleavage through H-atom abstraction. Heme enzymes stabilize the reactive iron(IV)-oxo porphyrin-radical intermediate. In non-heme iron enzymes, the low-spin ferric-hydroperoxo and iron(IV)-oxo are used as the reactive oxidants, while copper enzymes form superoxo/peroxo intermediates.

In biological molecules copper centers exist in mononuclear and multinuclear configurations and function in dioxygen binding, activation, and subsequent substrate oxidation. Copper-proteins have various functions as dioxygenases, monooxygenases, and oxidases (Figure 1.1). The immense variation in the coordination environment of copper centers in proteins and enzymes attributes to their diverse functions, which is correlated with its characteristic structural motifs. The redox properties, lability, and coordination flexibility of Cu(I) and Cu(II) ions is greatly affected by the coordinating ligands at the active site. Copper ions coordinate to the protein through amino acid residues and backbone amide, in addition to water molecules. Side chain ligands include the imidazole group of histidine, the sulfur in cysteine and methionine, and the carboxylate in glutamic acid and aspartic acid.

The design of model systems mimicking the active sites in proteins is an essential tool for characterizing the active site environment, and catalytic activity. This can further lead to the design of novel peptides and proteins with predictable
A) Mononuclear copper proteins:

B) Nonecoupled dinuclear copper proteins:

C) Coupled dinuclear copper proteins:

deoxyHc [CuI/CuI] + O2 \xrightarrow{\text{Hemocyanin}} \text{oxyHc [CuII}_2^{2-}/\text{CuII]} + O2

D) Trinuclear copper proteins:

Figure 1.1: Select catalytic reactions of mononuclear (A), dinuclear (B, C), and trinuclear (C) copper proteins involved in dioxygen binding and activation
structures and functions. Herein, a brief overview of copper proteins from a bioinorganic perspective will be presented. Classification of copper proteins as type-1 (T1Cu), type-2 (T2Cu), and type-3 (T3Cu) on the basis of spectroscopic features will be briefly discussed. Coordination of copper in biomolecules, and examples on the formation of Cu–O₂ species in metalloproteins and model systems will be presented. The chapter concludes with a brief discussion of Michaelis-Menten steady state enzyme kinetics.

1.2 Copper Coordination in Biomolecules

Copper coordination in biomolecules has attracted much attention in the field of bioinorganic chemistry. Copper exists in three oxidation states Cu(I), Cu(II), and the less common Cu(III). Cu(I) and Cu(II) play important roles in numerous biological systems due to their redox properties, lability, and coordination flexibility. Cu(I) has a d¹⁰ electronic configuration and can adopt different geometries controlled mainly by steric and structural constrains for polydentate ligands; tetrahedral and trigonal-monopyramidal four-coordinate are most common geometries. Cu(II) has a d⁹ electronic configuration in an octahedral field which leads to a significant Jahn-Teller distortion, an axial elongation. Hence, preferred coordination geometries of Cu(II) complexes are tetragonally distorted octahedral, square planar or square-pyramidal where the axial ligands are weakly coordinated at distances of 2.3-2.6 Å. The unpaired electron is localized in the dₓ²−ᵧ² orbital. Cu(II) complexes can also adopt a
trigonal-pyramidal coordination geometry, where the unpaired electron in the ground state is localized in the $d_{z^2}$ orbital. Different extent of elongations of the octahedral structure gives rise to square-pyramidal, square-bipyramidal, or square planar geometries. The less common, Cu(III) has a $d^8$ electronic configuration and generally adopt a square-planar geometry stabilized by strong basic anionic ligands. Cu(III) complexes are low-spin and diamagnetic in the presence of an oxygen ligand, but may exist as a high-spin centers.\textsuperscript{19}

In copper proteins, mononuclear\textsuperscript{20}, binuclear, and trinuclear Cu(II) sites are found. The copper proteins are classified into three types, T1Cu, T2Cu, and T3Cu, each with distinct geometries and ligand environment (Figure 1.2). The copper ions coordinate to the peptide chain through amino acid residues, backbone amide, and or water molecules. Soft sulfur ligands and other unsaturated ligands, such as 2,2'-bipyridine, are preferred by the soft Cu(I) ions. Hard nitrogen ligands are preferred by the relatively hard Cu(II) ions. The strong preference to electron donating ligand, nitrogen, pertains to its stability forming inert N–Cu(II) coordination bonds. Cu(II) centers may also coordinate to oxygen donor ligands or sulfur and phosphorus centers, forming a weaker more labile coordination bond as compared to N-Cu\textsuperscript{II}. The imidazole, $N_e$ and to a less extent $N_8$, group of histidine is the most common ligand in copper proteins. Redox active copper proteins feature a ligand environment to allow copper ions to exist in either oxidation state.

Cu–proteins play key roles in dioxygen binding and activation (Figure 1.1). Observed Cu–O\textsubscript{2} species and their corresponding O–O and Cu–O distances are summarized in (Figure 1.3). The reaction of substances with dioxygen is kinetically
**Figure 1.2:** Coordination geometries of type-1, type-2, and type-3 copper centers.
Figure 1.3: Cu–O$_2$ species formed in mononuclear and dinuclear Cu sites, and their O–O and Cu–O distances in Å.
unfavorable due to the triplet state of dioxygen, with two unpaired electrons in the doubly degenerate orbital, $\pi^*$. Let us first consider Cu(II)-O$_2$ coordination in mononuclear copper centers. The reduction of O$_2$ to peroxide, results in a fully occupied $\pi^*$ HOMO orbital, forming an end-on peroxo Cu(II) species (Figure 1.4A). One of the $\pi^*$ electrons is stabilized as a result of the $\sigma$ bonding with the half occupied d orbital of Cu(II). The unpaired electron is localized in the $d_{x^2-y^2}$ orbital and has a characteristic EPR spectrum with four small hyperfine splitting in the parallel region (Figure 1.4A). In addition to its EPR spectra, mononuclear copper-peroxo species exhibit characteristic UV-Vis and rR spectroscopic features. An intense transition $\sim$500 nm, $\varepsilon = 5000$ M$^{-1}$cm$^{-1}$, characteristic of an O$_2^{2-}$ $\pi^*$ to Cu(II) charge transfer transition, and an O–O stretch at 803 cm$^{-1}$ (Figure 1.4A).

In binuclear Cu(II) centers, coupled and uncoupled systems have been observed based on the magnetic interactions between the two Cu(II) sites. In coupled binuclear sites, strong magnetic interactions are present between the two Cu(II) sites as a result of direct bridging ligands, such as oxide or hydroxide, providing a mechanism for O$_2$ to bridge as side-on peroxo-dicopper (II) species. Antiferromagnetic and ferromagnetic coupling between dinuclear Cu(II) centers have been observed. Antiferromagnetic coupling is a result of spin paring of the two copper ion. In the MO energy diagram, the O$_2^{2-}$ $\pi^*_\sigma$ HOMO is involved in $\sigma$ bonding with d orbitals resulting in large splitting of energy, (Figure 1.4B). This large split in energy excludes electron-electron repulsion, thus resulting in spin paring of the two Cu(II) ions with a singlet ground state, hence, EPR silent. It is similar to mononuclear Cu(II) sites, but twice as intense charge transfer transition is
Figure 1.4: MO energy diagrams of (A) mononuclear copper-peroxo end-on species, and (B) dinuclear copper peroxo end-on species. In inset, the corresponding EPR, UV-Vis, and rR spectra.21
observed, $\varepsilon = 10,000 \text{ M}^{-1}\text{cm}^{-1}$, (Figure 1.4B). The increase of $\varepsilon$ is attributed to the bridging peroxo coordinating to two Cu(II) ions. A slight increase in its rR O-O stretch is also observed, 830 cm$^{-1}$ (Figure 1.4B).

1.3 Classifications of Copper Proteins

Copper and iron containing metalloproteins play an important role in catalysis and dominate the field of biological oxygen chemistry. Copper proteins are involved in numerous reactions (Figure 1.5) including the reversible dioxygen binding in hemocyanin, the two-electron oxidation of substrates in catechol oxidase and galactose oxidase, the hydroxylation in tyrosinase and dopamine beta-hydroxylase, the four-electron reduction of O$_2$ to water and substrate oxidation in laccase and ascorbate oxidase, and the proton pumping and electron transfer in cytochrome c oxidase. The structure and chemical reactivity of metalloproteins are controlled or tuned by the active site environment of the metal center (Figure 1.6).

The spectroscopic characteristics, UV-Vis and EPR, of copper proteins enabled their classification into three distinct types, type-1, type-2, and type-3 copper centers, summarized in Table 1.1. A combination of a type-1 and a type-3 center is found in some copper proteins containing a trinuclear copper unit, such as, laccase and ascorbate oxidase.

Type-1 copper centers, also known as blue copper centers, are found in electron transfer proteins such as amicyanin, plastocyanin, and azurin. A single Cu$^{II}$ ion is coordinated to two histidine residues, a cysteine, and a variable axial ligand, typically methionine, in a distorted tetrahedral geometry, i.e., a trigonal
Figure 1.5: Selected examples of different types of copper proteins. Type 1: Azurin, and plastocyanin. Type 2: Amine oxidase (AO), galactose oxidase (GO), and dopamine β-monooxygenase (DβM). Type 3: Catechol oxidase (CO), tyrosinase (Tyr), and hemocyanin (Hc).
Figure 1.6: Active site species of select Cu proteins in the reduced and oxygenated state (a-e). Species with an * have been crystallographically characterized.²
Table 1.1: Spectroscopic characteristics of select copper proteins.

<table>
<thead>
<tr>
<th>Name of Enzyme</th>
<th>Type/M&lt;sub&gt;e&lt;/sub&gt; (daltons, Da)</th>
<th>Absorption Bands, nm (ε, mM&lt;sup&gt;-1&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Reduction Potential (mV)</th>
<th>Details of EPR spectrum (A × 10&lt;sup&gt;-4&lt;/sup&gt; cm&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Azurin</td>
<td>I (blue)/14 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>625 (3.5)</td>
<td>330</td>
<td>Axial: (g_x = 2.052, g_y = 2.29) ((A_I = 60))</td>
</tr>
<tr>
<td>Plastocyanin</td>
<td>I (blue)/10.5 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>597 (4.9)</td>
<td>370</td>
<td>Axial: (g_x = 2.053, g_y = 2.26) ((A_I = 50)) Rhombic: (g_z = 2.042, g_y = 2.059, g_x = 2.226 (A_I = 63))</td>
</tr>
<tr>
<td>Laccase</td>
<td>I (blue)/60–140 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>607 (9.7)</td>
<td>785</td>
<td>Rhombic: (g_z = 2.030, g_y = 2.055, g_x = 2.300 (A_I = 43))</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td>II/145 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>614 (5.2)</td>
<td></td>
<td>Rhombic: (g_z = 2.036, g_y = 2.058, g_x = 2.227 (A_I = 45.8))</td>
</tr>
<tr>
<td>Laccase</td>
<td>II/60–90 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>788 (0.9)</td>
<td>782 ((P. versicolor)), 434 ((R. vernicifera))</td>
<td>Axial: (g_x = 2.053, g_y = 2.237) ((A_I = 200.6))</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td>II/145 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>760 (3.6)</td>
<td></td>
<td>Axial: (g_x = 2.053, g_y = 2.242) ((A_I = 199))</td>
</tr>
<tr>
<td>Galactose oxidase</td>
<td>II/68 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td></td>
<td>410</td>
<td>EPR silent</td>
</tr>
<tr>
<td>Laccase</td>
<td>III/60–90 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>330(2.7)</td>
<td>570 ((P. versicolor)), 390 ((R. vernicifera))&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EPR silent</td>
</tr>
<tr>
<td>Ascorbate oxidase</td>
<td>III/145 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>330 (2.0)</td>
<td></td>
<td>EPR silent</td>
</tr>
<tr>
<td>Tyrosinase</td>
<td>III/46 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>345 (20.0)</td>
<td>370&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EPR silent</td>
</tr>
<tr>
<td>Hemocyanin</td>
<td>III/4 × 10&lt;sup&gt;3&lt;/sup&gt; to 9 × 10&lt;sup&gt;6&lt;/sup&gt;, subunits from 75 × 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>350 (20.0)</td>
<td>&gt;800&lt;sup&gt;b&lt;/sup&gt;</td>
<td>EPR silent</td>
</tr>
</tbody>
</table>
geometry with a long bond for an axial ligand. Type-1 copper centers are typically characterized by an intense absorption at 600 nm ($\varepsilon=5000 \text{ M}^{-1}\text{cm}^{-1}$) as a result of Cys S-Cu$^{II}$ charge-transfer transition, $S_{\text{π}}$ to Cu $d_{x^2-y^2}$, giving this type of copper proteins a deep blue color, and hence the name. Absorption near 450 nm, giving rise to a green color, is also plausible for this type of copper proteins. Four small hyperfine splitting in the parallel region of an EPR spectrum is observable for the oxidized Cu$^{II}$ form, $A_|| < 80 \times 10^{-4} \text{ cm}^{-1}$.

Type-2 copper centers occur in enzymes assisting in oxidations and oxygenations such as galactose oxidase$^{39,40}$ and dopamine β-monooxygenase (DβM).$^{41}$ Type-2 copper centers are also found in the dinuclear Cu,Zn-superoxide dismutase (SOD). Type-2 copper centers are mononuclear and characterized by a weak absorption around 700 nm, and are commonly coordinated to His, Asp, or Tyr residues in a distorted 5-coordinated geometry. The EPR signal exhibits four hyperfine splitting in the parallel region, $A_|| \sim (130-180) \times 10^{-4} \text{ cm}^{-1}$. Type-2 copper proteins are also known as normal copper centers due to the lack of distinctive spectroscopic characteristics as compared to mononuclear Cu$^{II}$ complexes.

Type-3 copper centers are found in oxygen transport proteins, such as, hemocyanin$^{42,43}$, and in oxidases such as tyrosinase$^{44,45}$ and catechol oxidase$^{46,47,48}$. Type-3 copper proteins are EPR silent due to a coupled binuclear copper center. As a result of spin pairing, the two copper ions are antiferromagnetically coupled with an $S = 0$ ground state, $-2J > 600 \text{ cm}^{-1}$, resulting in the absence of an EPR single. The coupled copper centers are approximately 3.6 Å apart. Each Cu$^{II}$ atom is
coordinated by three His imidazole nitrogens, \( N_e \) in a tetragonal geometry. A hydroxyl group links the two Cu\(^{II} \) atoms in the \( met \) state, whereas a \( \mu-\eta^2:\eta^2 \)-peroxo is formed in the \( oxy \) state, which has attracted much attention.

### 1.4 Dioxygen Binding and Activation by Select Copper Proteins

Copper containing metalloproteins catalyze a large number of biologically important reactions and play key roles in dioxygen binding and activation.\(^{49} \) Copper-proteins that activate oxygen function as dioxygenases, monooxygenases, and oxidases. Hemocyanin reversibly binds oxygen, while tyrosinase and catechol oxidase binds and activates oxygen for subsequent substrate hydroxylation and or oxidation to its corresponding \( o \)-quinone product, respectively. Despite their different functions, hemocyanin, tyrosinase, and catechol oxidase exhibit similar dinuclear copper centers with strong magnetic interactions.

Hemocyanin, the dioxygen transport protein for invertebrates, arthropods, and mollusks, binds oxygen reversibly and forms a stable \( \mu-\eta^2:\eta^2 \) is Cu-O\(_2\) species in the \( oxy \) state, oxyhemocyanin (Figure 1.7).\(^{43} \) In the absence of O\(_2\), hemocyanin copper centers exist in the \( deoxy \) form, and bind dioxygen cooperatively.\(^{50} \) Each Cu(I) ion is coordinated to three histidine residues in a trigonal geometry at variable distances between the two Cu(I) ions depending on the studied species. Hydrophobic residues surround the copper coordination environment. The reaction with O\(_2\) generates a blue chromophore and rearrangement into a square-pyramidal coordination. Oxyhemocyanin exhibits unique spectral properties, magnetically
**Figure 1.7**: Side-on $\mu$-$\eta^2$-$\eta^2$ peroxo binding mode in hemocyanin. Oxy-Hc exhibits a characteristic LMCT at 350 nm, and $\nu_{\sigma-\sigma} = 750$ cm$^{-1}$. 50,51
coupled dicopper center, that have been studied extensively.\textsuperscript{52,53} The electronic absorbance spectrum of oxyhemocyanin has an intense absorbance at 345 nm (ε=20,000 M\textsuperscript{-1}cm\textsuperscript{-1}) and a less intense absorbance at 570 nm (ε=1000 M\textsuperscript{-1}cm\textsuperscript{-1}).\textsuperscript{55}

The rR spectrum exhibits a peroxide O–O stretching vibration at 740-750 cm\textsuperscript{-1} characteristic of a side-on μ-η\textsuperscript{2}:η\textsuperscript{2} binding mode.\textsuperscript{2} The two Cu(II) ions in oxyhemocyanin are strongly coupled with distances varying between 3.3 and 3.9 Å depending on the studied species. Magnus and co-workers reported the X-ray crystal structure of oxyhemocyanin, \textit{Limulus polyphemus}, at 2.4 Å resolution (PDB: 1OXY).\textsuperscript{54,55}

Hemocyanin and catechol oxidase show no significant similarities in sequence nor the overall folding of the protein, except for the very similar catalytic copper core in both proteins.\textsuperscript{27} In the presence of dioxygen, catechol oxidase catalyzes the oxidation of catechols to their corresponding o-quinones products also known as catecholase activity (Figure 1.8). Catechol oxidase is a ubiquitous enzyme found in a number of plants and fruits including potato, apple, and spinach, which is responsible for the browning reaction observed in cut or injured plants when exposed to O\textsubscript{2}.\textsuperscript{24,45} The molecular weight of catechol oxidase varies between 38-45 and 55-60 kDa depending on the tissue and organism. Each of the copper atoms, Cu\textsubscript{A} and Cu\textsubscript{B}, is coordinated by three His imidazole nitrogen, N\textsubscript{ε}. CuA is coordinated to His-88, His-109, and His-118, and Cys-92 hydrogen bonds to His-109. CuB is coordinated to His-240, His-244, and His-274.\textsuperscript{13,46} The native \textit{met} form of catechol oxidase from \textit{Lycopus europaeus} and \textit{Ipomoeas batatas} exhibits a μ–OH bridged dicopper (II) center with a short Cu–Cu distance of 2.9Å based on EPR, X-ray, and
Figure 1.8: Schematic representation of tyrosinase activity.
UV-Vis spectroscopy. The oxy form, \( \mu-\eta^2:\eta^2 \) peroxo-dicopper (II), has characteristic maximum absorption at 343 nm, \( \varepsilon = 6500 \, \text{M}^{-1}\text{cm}^{-1} \), and 580 nm, \( \varepsilon = 450 \, \text{M}^{-1}\text{cm}^{-1} \), corresponding to LMCT transition of peroxo-to-Cu(II).

Tyrosinase is another well-studied oxygenases catalyzing the hydroxylation of monophenols to their corresponding di-phenols (phenolase or cresolase activity), and the two-electron oxidation of di-phenols or catechols to their corresponding o-quinones (catecholase activity). Catecholamine neurotransmitters like DOPA, epinephrine, and norepinephrine are part of the tyrosinase metabolic pathways, hence, the activity of tyrosinase is of significant importance in medicine and biology. Tyrosinase is found in plants, animals, and fungi. In plants it is responsible for the brown color observed in cut or injured plants when exposed to \( \text{O}_2 \). In animals and fungi, tyrosinase is responsible for generation of the melanin precursor DOPA-quinone. The active site structure is very similar to the dinuclear site in catechol oxidases and hemocyanins.

The catalytic mechanism of tyrosinase is proposed on biochemical studies and model systems. In the proposed mechanism (Figure 1.9), tyrosinase is present in different redox forms, deoxy, oxy, and met, during its catalytic cycle. The reduced deoxy diCu(I) form binds \( \text{O}_2 \) and generates the oxy form in a \( \mu-\eta^2:\eta^2 \) peroxo-dicopper (II) binding mode, followed by destabilization of the O–O and activation as a result of substrate interaction. The met form of the diCu(II) site is bridged with a hydroxide ion.
Figure 1.9: Schematic representation of the proposed mechanism of tyrosinase.
1.5 Biomimetic of Copper-Dioxygen Centers

Biomimetic copper model systems that react with dioxygen provide an opportunity to probe, in detail, the biological activity of native enzymes. The structural and mechanistic complexities of copper-dioxygen species in copper centers have attracted much attention in the field of bioinorganic chemistry both in model systems and protein intermediates (Figure 1.10). Of the pioneers are Nobumasa Kitajima, Edward Solomon, Kenneth D. Karlin, William B. Tolman, and Shinobu Itoh. Their efforts in synthetic model systems and intermediates have led to significant contribution in mechanistic studies involving copper dioxygen chemistry. Cu–O₂ species are key reaction intermediates in copper proteins that bind and/or activate oxygen. The different functions within such copper proteins are largely attributed to the coordination environment of the Cu–O₂ species, donor ligands, geometry, and coordination mode of oxygen. The imidazole group of histidine is the most common ligand in copper proteins. In biomimetic studies, nitrogen-containing strong donor ligands, such as pyridines, are often incorporated to mimic imidazole of histidine, which has been proposed to assist the stabilization of copper-oxygen intermediates. Figure1.10 summarizes possible modes for Cu–O₂ binding in model systems.

Synthesis and structural characterization of type-3 biomimetic copper centers have been the focus of numerous studies due to their unique spectroscopic features and characteristic O₂ binding modes. Model systems of type-3 copper proteins are relevant to hemocyanin, dioxygen carrier protein, and to copper
Figure 1.10: Select superoxo and peroxo Cu–O₂ binding modes found in model systems and Cu–proteins.
proteins that activate O\textsubscript{2} for subsequent incorporation into substrates, such as tyrosinase.\textsuperscript{64,65,66} The dicopper center in hemocyanin\textsuperscript{66} was used as a motif for ligand design in model systems. A similar dicopper center is also found in tyrosinase\textsuperscript{64} and catechol oxidase\textsuperscript{24}, where biomimetic efforts are aimed toward understanding dioxygen binding and activation. Kitajima reported the first crystal structure of a model system mimicking the side-on \(\mu-\eta^2:\eta^2\)-bridging peroxo dicopper (II) species found in type-3 copper proteins.\textsuperscript{67,68,69} The characterized complex, \([\text{Cu}(\text{HB}(3,5\text{-iPr}_2\text{pz})_3)]_2(\text{O}_2)\), where (3,5-iPr\textsubscript{2}pz\textsubscript{3} is hydrotris(3,5-diisopropyl-1-pyrazolyl)borate), exhibited characteristic spectroscopic features associated with the \(\mu-\eta^2:\eta^2\) coordination mode (Figure 1.11).\textsuperscript{68,70,71,84} In the proposed mechanism, a di-Cu(I) center binds O\textsubscript{2} to form a side-on bound peroxo species and then hydroxylates the xylyl moiety (Figure 1.12A).\textsuperscript{68,70,71} Other early examples include an asymmetrical end-on peroxo species in \([\text{Cu}_2^{2+}\ (\text{XLO}^-)(\text{O}_2)]^+\), \textsuperscript{72} \(\mu\)-1,2-peroxo species in \([[(\text{TMPA})\text{Cu}^{2+}]_2(\text{O}_2)]^2+\), where TMPA is tris(2-pyridylmethyl)amine,\textsuperscript{73} and a side-on \(\mu-\eta^2:\eta^2\) peroxo species in \([\text{Cu}_2^{2+}\ (\text{N}_4)(\text{O}_2)]^2+\), where N\textsubscript{4} is an alkyl chain \((\text{CH}_2)_4\).\textsuperscript{74} Analogues of \([[(\text{TMPA})\text{Cu}^{2+}]_2(\text{O}_2)]^2+\) and \([\text{Cu}_2^{2+}\ (\text{N}_4)(\text{O}_2)]^2+\) were shown to exhibit variable stability and reactivity.\textsuperscript{75,76,77}

Karlin and co-workers synthesized and characterized a number of tridentate model compounds using pyridine-based ligand systems. Oxygenation rates were first studied for \([\text{Cu}_2^2(\text{R-KYL-H})]^{2+}\) systems serving as models for hemocyanin and tyrosinase, where KYL is m-xylyl linker. In one of the models, a two bis[2-[2-pyridyl]ethyl]amine (PY2) are linked by a spacer xylyl group, R = H. Upon binding to oxygen, it forms an end-on peroxo dicopper (II) moiety (Figure 1.12B).
Figure 1.11: $[\text{Cu(HB(3,5-iPr_2pz)_3)]_2(O_2)\text{] prepared by N. Kitajima.}^{70,84}$
Figure 1.12: (A) Proposed mechanism of a monooxygenase model system by Karlin and co-workers. (B) Tridentate ligands, MePY2 and N₄PY2.⁷⁸
Other reports of tridentate ligand systems include a methylbis[2-(2-pyridyl)ethyl]amine, MePY2 (Figure 1.12B). MePY2 reacts with Cu(I) to form a [(MePY2)Cu(CH3CN)]+ complex, and forms a [{{(MePY2)Cu}2(μ-η²-η²-peroxo)}]2+ upon dioxygen binding forming a side-on peroxo copper (II) species. In an alternative ligand system, PY2 was synthesized and characterized in the presence of a variable (CH₂)n linker (Figure 1.12B). Depending on the length of the (CH₂)n linker, oxygenation of the Cu(I) complexes leads to non-planar or planar side-on peroxo cores. In the presence of a (CH₂)₄ linker, N₄PY2, and upon oxygenation, a [Cu₂(N₄PY2)(O₂)]⁺² peroxo species is formed.

Tolman and co-workers used amine-based ligand systems, such as, 1,4,7-triisopropyl-1,4,7-triazacyclononase (L^{iPr₃}), and 1,4,7-tribenzyl-1,4,7-triazacyclononane (L^{Bn₃}), and identified the bis-μ-oxo dicopper(II) binding mode. The formation of a [Cu₂(μ-O)₂]⁺² with L^{iPr₃} or L^{Bn₃} as ligands activate C-H bonds for subsequent cleavage and oxidation. They characterized a bis-μ-oxo-dicopper (III) species, with Cu–Cu distance ~2.8 Å, using tridentate substituted triazacyclononane as ligands. A similar species was characterized by Stack and co-workers using ethylenediamine-based ligands. Formation of bis-μ-oxo-dicopper (III) species versus side-on μ-η²-η² peroxo dicopper (II) species is influenced by reaction conditions and ligand environment. Both species were found to be under dynamic equilibrium, and coexist in the crystalline state and in theoretical calculations.

Biomimetic of galactose oxidase, a mononuclear type-2 copper center, has emerged to characterize its unique structural features and radical-based oxidation mechanism. Some model systems revealed a distinct coordination of Cu(II) to
**Figure 1.13:** Proposed mechanism of a galactose oxidase model system by Stack and co-workers.\(^{84,85}\)
phenoxyl radicals, forming Cu(II)-(O)-phenol species, upon oxidation of phenolato-copper(II) compounds. Stack and co-workers characterized tetridentate model systems converting benzylic and allylic alcohols to their corresponding aldehydes (Figure 1.13). In the proposed mechanism in the presence of O₂, the Cu(I)-phenol complex forms a Cu(II)-phenoxyl radical species bound to a hydroperoxo group which interacts with a benzylic or allylic alcohol and oxidizes it to its corresponding aldehyde.

In summary, the design and incorporation of Cu sites in synthetic models continue to rapidly advance. Bioinspired model systems provide a unique opportunity enabling detailed characterization of active sites in the native enzymes using small molecules as probes. A number of copper dioxygen binding modes have been found in synthetic ligand systems. Ligand environment and solvent are important factors influencing the variable Cu-O₂ binding modes, including superoxo end-on η¹-, superoxo side-on η², peroxo end-on μ-η¹:η¹-O₂²-, peroxo side-on μ-η²:η²-O₂²-, trans-μ-1,2-peroxo, or a bis-μ-oxo. Biomimetic copper model systems that react with oxygen provide an opportunity to probe in detail the biological activity of native enzymes. Characterization of model systems mimicking the catalytic activity and spectroscopic features of monooxygenase and dioxygenase reactions are relevant to deducing reaction mechanisms in biological systems and to various industrial processes.
1.6 Michaelis-Menten Enzyme Kinetics and Simple Inhibition Pattern

Steady state saturation kinetics is discussed herein in sight of the oxidative and inhibition studies presented within chapters two and four, respectively. The field of enzymology is centered on understanding the biochemical nature and activity of enzymes. One of its branches is enzyme kinetics, centered on factors affecting the rates of enzyme-catalyzed reactions. A number of factors influence enzyme catalyzed reactions including enzyme concentration, ligand(s) concentration(s), pH, ionic strength, and temperature. For instance, varying ligand concentrations, i.e. substrate and inhibitor, at fixed enzyme concentration can give insight into reaction rate, kinetic mechanism, and inhibition pattern. A detailed understanding of enzyme kinetics in vitro is crucial to understanding the activity of an enzyme and its regulation in vivo.

The first general rate equation for enzyme-catalyzed reactions was brought about by Henri in 1903, where the initial reaction rate was directly proportional to the enzyme concentration and increased in a non-linear manner with increasing substrate concentration up to a maximum rate. Later, Michaelis and Menten confirmed Henri’s work and modified the mechanism as shown in equation (1.1) with the rate law shown in equation (1.2),

\[ E + S \rightleftharpoons ES \overset{k_2}{\underset{k^{-1}}{\rightarrow}} E + P \quad (1.1) \]

\[ v = \frac{V_{\text{max}} [S]}{K_m + [S]} \quad (1.2) \]
where $K_m$, the Michaelis constant, is defined as $(k_{-1} + k_2)/k_1$, and $V_{\text{max}}$ represents the maximum velocity of an enzyme at saturating substrate concentration, $V_{\text{max}} = k_{\text{cat}}[E]$. The measure of the catalytic activity of a particular enzyme (the breakdown of ES into E + P) is given by the rate constant, $k_{\text{cat}}$. It measures the number of substrate molecules that are turned over per enzyme per unit time (seconds), also known as the turnover number.

$$V_{\text{max}} = k_{\text{cat}}[E]_t \quad (1.3)$$

In steady-state approximation of an enzyme catalyzed reaction, equation 1.1, the total concentration of enzyme, $[E]$, is equal to the initial concentration of enzyme, $[E]_o$, plus the concentration of the enzyme-substrate complex, $[ES]$.

$$[E]_t = [E]_o + [ES] \quad (1.4)$$

The rate of formation of the ES complex is dependent on the forward and reverse reactions: formation of the ES complex from E and S, and dissociation of the ES complex into enzyme and substrate, respectively.

$$K = \frac{k_{-1}}{k_1} = \frac{[E] + [S]}{[ES]} \quad (1.5)$$

$K$ is the dissociation constant of the ES complex. Rearranging equation (1.5) and substituting $[ES]$ from equation (1.4) into equation (1.3) gives:

$$K_m = \frac{k_{-1} + k_2}{k_1} \quad (1.6)$$
$K_m$ is a constant for a particular enzyme-catalyzed reaction and can be used as means of comparing different enzymes as well as comparing the specificity of different substrates to a particular enzyme, to a certain extent. Hence, a ratio of $k_{cat}/K_m$ is measure of the enzyme’s catalytic efficiency.

In enzyme-catalyzed reactions that display Michaelis-Menten kinetics, a plot of initial velocity of versus the substrate concentration gives a hyperbolic curve. At very low substrate concentration the $v$ versus $[S]$ plot is essentially linear following *first-order kinetics*, where $v$ is directly proportional to $[S]$. Consider $[S] \ll K_m$, $[S]$ in the denominator of equation (1.2) may be ignored and $v = k[S]$, where $k$ is defined as the *first-order* rate constant equivalent to $V_{max}/K_m$. The *first-order* rate constant, $k$, is an approximation of the fraction of substrate converted into product per unit time, $\frac{-d[S]}{dt} = v = k[S]$. At very high substrate concentration, $v$ is essentially independent of $[S]$ following *zero-order kinetics*. Consider $[S] \gg K_m$, $K_m$ in the denominator of equation (1.2) may be ignored and $v = V_{max}$.

The Michaelis-Menten equation can also be represented as a linear form. In 1934, the Lineweaver-Burk double reciprocal plot,\textsuperscript{94} $1/v$ versus $1/[S]$, was introduced as a linear form, $y = mx + b$, of the Michaelis-Menten equation:

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (1.7)$$

where $K_m$ and $V_{max}$ can be obtained from the plot of $1/v$ versus $1/[S]$. The slope, $y$- and $x$-intercept are equal to $K_m/V_{max}$, $1/V_{max}$, and $-1/K_m$, respectively.
In the presence of an inhibitor, the rate of an enzyme-catalyzed reaction is decreased. In simple inhibition systems, in the presence of a single substrate and a single inhibitor, enzyme inhibitors are classified into three types or patterns: competitive, uncompetitive, and noncompetitive. Mixed type inhibition is also known. In enzyme-catalyzed reactions displaying Michaelis-Menten kinetics, the presence of an inhibitor affects the reaction rate and thereby alters the $K_m$ and $V_{max}$ values according to inhibition type. In the presence of an inhibitor, the $K_m$ and $V_{max}$ are referred to as apparent $K_m$ or $K_{m'}$ and apparent $V_{max}$ or $V_{max'}$, respectively.

In competitive inhibition, the inhibitor and the substrate compete for the same site, where inhibitor and substrate are mutually exclusive. In a Michaelis-Menten enzyme-catalyzed reaction, the initial reaction rate is proportional to the steady-state concentration of the enzyme-substrate complex, $v = k_2[ES]$. In competitive inhibition, the initial reaction rate is unaffected by the presence of low concentration of inhibitor, but an increased concentration of substrate is required to reach the same enzyme-substrate complex concentration present in the absence of an inhibitor. Hence, the $K_m$ is increased while the $V_{max}$ remains the same as compared to $K_m$ and $V_{max}$ in the absence of an inhibitor. The increase in $K_m$ is as a result of the distribution or competition between the substrate and inhibitor to the same enzyme site. Accordingly, in a competitive inhibition, the total enzyme concentration is present in three forms: free enzyme, enzyme-substrate complex, and enzyme-inhibitor complex, EI. The enzyme-inhibitor complex can be defined as
terms of the inhibition constant, \( K_i \) where \( K_i = [E][I]/[EI] \). Thus, similar equation 1.2, the rate equation for a competitive inhibition is given below:

\[
\nu = \frac{V_{\text{max}}[S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \quad (1.8)
\]

In uncompetitive inhibition, the inhibitor binds reversibly to the enzyme-substrate complex forming an inactive enzyme-substrate-inhibitor complex, ESI. The inactive ESI complex is present at any inhibitor concentration. Even at higher substrate concentrations, the enzyme will form ES and ESI. Consequently, in the presence of an uncompetitive inhibitor, \( V_{\text{max}} \) and \( K_m \) are decreased relative to \( V_{\text{max}} \) and \( K_m \) in the absence of the inhibitor. The rate equation, steady-state assumptions, in the presence of an uncompetitive inhibitor is given below:

\[
\nu = \frac{V_{\text{max}}[S]}{K_m + [S]\left(1 + \frac{[I]}{K_i}\right)} \quad (1.9)
\]

The reciprocal form is given below:

\[
\frac{1}{\nu} = \frac{K_m}{V_{\text{max}}[S]} + \frac{1}{V_{\text{max}}\left(1 + \frac{[I]}{K_i}\right)} \quad (1.10)
\]

Similar to competitive inhibition, for uncompetitive inhibition a plot of \( 1/\nu \) versus \( 1/[S] \) gives a slope equal to \( K_m/V_{\text{max}} \), and an \( x \)-intercept is equal to \( -1/K_m \), in the presence of the inhibitor and \( -1/K_m \) in the absence of the inhibitor. Alternatively, the \( y \)-intercept, \( \frac{1}{V_{\text{max}}} \left(1 + \frac{[I]}{K_i}\right) \), is increased by a factor of \( \left(1 + \frac{[I]}{K_i}\right) \) giving
parallel plots, displacement of the plot in the presence of increasing concentration of inhibitor increases as compared to the plot in the absence of inhibitor, the control. Plotting the y-intercept, taken from the reciprocal plot, versus the inhibitor concentration gives a line with a slope of $1/K_i V_{max}$, y-intercept of $1/V_{max}$ and x-intercept of $-K_i$. Similarly, plotting the x-intercept, $-1/K_m$, versus the inhibitor concentration gives a line with a slope of $1/K_i K_m$, y-intercept of $1/K_m$ and x-intercept of $-K_i$.

In noncompetitive inhibition, the inhibitor and the substrate bind independently at different sites on the enzyme. The inhibitor binds to the free enzyme and to the enzyme-substrate complex. Likewise, the substrate binds to the free enzyme and to the enzyme-inhibitor complex, the latter results in an inactive enzyme-substrate-inhibitor complex. The inhibition constant is thus dependent on the formation of enzyme-inhibitor and enzyme-substrate-inhibitor complex, $K_i=[E][I]/[EI]=[ES][I]/[ESI]$. Consequently, at any substrate concentration, the inactive enzyme-substrate-inhibitor complex forms and the observed $V_{max}$ is less than the $V_{max}$ in the absence of inhibitor. The $K_m$ remains the same since the substrate has equal affinity to enzyme-substrate and enzyme-substrate-inhibitor complex. Accordingly, the rate for a noncompetitive inhibitor is given by:

$$v = \frac{V_{max} [S]}{K_m \left(1 + \frac{[I]}{K_i} \right) + [S] \left(1 + \frac{[I]}{K_i} \right)} \quad (1.11)$$

The reciprocal form of the rate equation is given by:
\[ \frac{1}{v} = \frac{K_m}{V_{\text{max}}} \left( 1 + \frac{[I]}{K_i} \right) \frac{1}{[S]} + \frac{1}{V_{\text{max}}} \left( 1 + \frac{[I]}{K_i} \right) \]  

(1.13)

where the slope and y-intercept are increased by a factor of \( 1 + \frac{[I]}{K_i} \) in comparison to the slope in the absence of the inhibitor, \( K_m/V_{\text{max}} \). Consequently, \( K_i \) for a noncompetitive inhibitor can be calculated from the y-intercept, \( \left( 1 + \frac{[I]}{K_i} \right)/V_{\text{max}} \), or the slope, \( K_m \left( 1 + \frac{[I]}{K_i} \right)/V_{\text{max}} \), of a plot of \( 1/v \) versus \( 1/[S] \). The x-intercept is unaffected by the presence of a noncompetitive inhibitor, \(-1/K_m\). Plotting the y-intercept, taken from the reciprocal plot, versus the inhibitor concentration gives a line with a slope of \( 1/(K_i V_{\text{max}}) \), y-intercept of \( 1/V_{\text{max}} \), and x-intercept of \(-K_i\). Similarly, plotting the slope, \( K_m/V_{\text{max}} \), versus the inhibitor concentration gives a line with a slope of \( K_m/(K_i V_{\text{max}}) \), y-intercept of \( K_m/V_{\text{max}} \), and x-intercept of \(-K_i\).

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CHAPTER 2: STRUCTURAL CHARACTERIZATION OF A BETA-TURN DESIGNED METALLOPEPTIDE: \textsuperscript{1}H NMR AND CD SPECTROSCOPY

2.1. Introduction

The structure and function of metalloproteins and metallopeptides is often regulated or modulated by coordinated metal ions exhibiting specific interactions with biomolecules.\textsuperscript{1, 2, 3} Conformational changes induced by the folding of metalloproteins and metallopeptides give insight on structural and functional information.\textsuperscript{4} Metal ions exist as cofactors in a large number of proteins and are necessary for structural stability and catalytic transformations.\textsuperscript{5} The folding induced by metal ions in zinc figure domains represent a classical example of structural stability induced by metal ions, where the zinc ions crosslink α−β domains promoting the formation of a defined structure.\textsuperscript{6} In the absence of zinc, the protein is in unfolded state. The conformation of zinc figure domains is further modulated upon interacting with DNA in order to fit in its major groove.\textsuperscript{7}

Metal ions can be incorporated into peptides by metallochaperones through protein−protein interaction or directly from the cellular pool.\textsuperscript{8} Metallothionein plays an important role in zinc homeostasis through its translocation and regulation in the cell, where high zinc concentrations may be toxic.\textsuperscript{9} Metallothionein can further
regulate the flow of copper in the cell and prevent poisoning from cell exposure to toxic metals, cadmium and mercury.\textsuperscript{9} In its apo form, the protein can bind up to seven equivalents of Zn\textsuperscript{2+} or Cd\textsuperscript{2+} and up to six equivalents of Cu\textsuperscript{+} ions, forming metal–thiolate clusters.\textsuperscript{10,11} The binding of metal ions induces conformational changes where the protein changes from a random coil conformation into a folded two domain structure.\textsuperscript{12,13} Metallothionein has been shown to bind neuronal receptors initiating pathways leading to neurite survival.\textsuperscript{14,15,16}

Calcium–binding proteins represent a good example of reversible binding of metal ions, a process needed for calcium signaling.\textsuperscript{17} Of such proteins is calmodulin, an EF–hand calcium binding protein.\textsuperscript{18} Each of its N-terminal and C-terminal lobes binds up to two calcium ions via its helix-loop-helix domains.\textsuperscript{19} Calmodulin binds calcium ions with different affinities inducing significant conformational changes by coupling of its N- and C- terminal lobes.\textsuperscript{20} The induced conformational change is necessary for the function of calmodulin and its interaction with proteins and enzymes.\textsuperscript{21,22} An alternative example for metal–induced conformational changes necessary for the function of the metalloprotein can be demonstrated by carboxypeptidase A. A pancreatic proteolytic zinc-bound metalloenzyme, with a globular shape, that catalyzes the hydrolysis of peptide bonds at the C-terminal end of protein and peptide substrates, and is specific to hydrophobic residues.\textsuperscript{23,24} Its zinc active site is involved in stabilization of the intermediate, deprotonation of coordinated water molecule, and electrostatic interactions necessary for recognition of the C-terminus amino acid in the substrate. The metalloenzyme is also active with
zinc substitution into Co^{2+}, Ni^{2+}, Fe^{2+}, and Mn^{2+}, while it produces an inactive enzyme towards all substrates when Cu^{2+} is substituted.\textsuperscript{25,26}

Superoxide dismutases\textsuperscript{27,28} (SODs) are metalloenzymes containing Cu^{2+}, Zn^{2+}, Fe^{2+}, Mn^{2+}, and Ni^{2+}.\textsuperscript{29,30,31} The ubiquitous family of superoxide dismutases catalyzes the dismutation of superoxide anion to dioxygen and hydrogen peroxide. Hence, SODs function as defense systems against oxidative stress; specifically superoxide anion radicals mediated damage in cells.\textsuperscript{32,33} Of the most abundant proteins in cells is the Cu– and Zn–containing SOD, CuZnSOD, a beta–barrel dimeric protein. Each of its monomers contains one bound Cu^{2+} and Zn^{2+} ion, and an intrasubunit difulfide bond necessary for proper folding.\textsuperscript{29} Misfolding of the metalloprotein, and the presence of mutants, are linked to amyotrophic lateral sclerosis (ALS), a neurodegenerative disease affecting motor neurons characterized by the accumulation of misfolded SOD1 deposits in affected cells.\textsuperscript{34} The disease is associated with aggregation and amyloid fibril\textsuperscript{35} formation of SOD1.\textsuperscript{29,33}

Alzheimer’s disease represents a major neurodegenerative diseases associated with protein misfolding and aggregation. Plaques and fibrils in the brain of Alzheimer’s disease patients are aggregates of Aβ peptide and are associated with the pathogenesis of the disease.\textsuperscript{36,37,38} The Aβ peptide is a 40-42 amino acid peptide formed by proteolytic cleavage of the amyloid precursor protein (APP) by β- and γ-secretase.\textsuperscript{39} Conformational changes of Aβ peptide are associated with the binding of transition metal ions copper, zinc, and iron, forming the insoluble fibrils and plaques.\textsuperscript{40,41,42} The Aβ fibrils adopt a beta sheet structure, whereas Aβ in micelles and during Aβ fibrillogenesis adopt alpha helical structures.\textsuperscript{43,44,45}
The design of protein or peptidic scaffolds with metal anchoring sites is essential for probing and understanding fundamental aspects that dictate confirmations, structures, active sites, and functions in metalloproteins.\textsuperscript{46, 47, 48, 49, 50} The significant progress in the field of protein and peptide design led to a range of application not limited to fundamental biochemistry research, but also to advances in biotechnology\textsuperscript{51, 52} and medicinal chemistry\textsuperscript{53} research.\textsuperscript{54} Efforts are put forth in redesigning native proteins and engineering desired functions and metal-binding sites into its scaffold to impart new or improved properties.\textsuperscript{55} The de novo design has also acquired much attention in the field. Metalloprotein and peptidic scaffolds are designed to exhibit predicted structures and functions, based on the understanding of native proteins and using sequences that are not necessarily related to native proteins.\textsuperscript{56}

A major challenge in metallopeptide design, and more specifically in minimalistic systems, is to create stable scaffolds with characteristic secondary structures, as seen in native metallopeptides, into which specific functions can be introduced.\textsuperscript{57, 58, 59, 60}

The design of β structures can be illustrated by a designed bell-shaped β-sandwich peptide with two 32-residue β-sheets that can assemble to one another by metal binding to His residues incorporated at the end of the turns.\textsuperscript{61} The design of short peptides exhibiting β-hairpin conformations has been demonstrated by a 12-mer, RGITVNGKTYGR, exhibiting a loop at the NG and a type I’ β-turn across VNGK, characterized by means of NMR and CD spectroscopy.\textsuperscript{62} A similar example is
presented by a decamer, IYSYNGKTWT, forming a tight β-hairpin with a loop at NG and exhibiting a type I’ β-turn across YNGK.63

The design of β-turn and β-hairpin structures is frequently associated with the presence of a Pro residue at the loop/turn,64 thus promoting folding. Gly-Pro residues, among others, are also common in promoting a loop/turn formation when centrally located in the peptide sequence.65,66 This can be demonstrated by a series of designed peptides derived from the N-terminus of ubiquitin, MQIFVKSXXKTITLKV-NH₂, where XX was a variation of D- and L- Pro-Ala or Ala-Pro residues.67 A two residue loop, Pro-Pro, located centrally in a designed KFVPPLFV peptide sequence shows a strong type II’ β-turn within a β-hairpin structure, based on observed NOEs.68 The turn was expanded with an Ala residue, Pro-Pro-Ala, to give a three-turn loop as opposed to the two-turn loop of Pro-Pro.69

In this study, the design and characterization of a de novo octamer, Ac-RHHPPHHE-NH₂ (Figure 2.1), exhibiting a β-turn structure and a di-metal center is presented. The characterized peptide is a 10-residue β-turn, designed with di-Pro residues towards the center of the peptide, known to facilitate β-turn structures by restricting conformations of neighboring residues. An Arg residue at its N-terminus and a Glu residue at its C-terminus were incorporated into the peptide design to further refine a β-turn via charge interactions at the terminus. Two di-His residues were incorporated as metal anchoring sites, which further refines the overall structure. The multi-imidazole environment within the peptide is suitable for Cu²⁺ and Zn²⁺ band Co²⁺ binding. Hence, mono- and di-metal centers are likely to form.
Figure 2.1: Structure of the peptide sequence RHHPPHHE–CONH$_2$. 
Co$^{2+}$ has been demonstrated to be an excellent probe for studying Cu$^{2+}$, and Zn$^{2+}$-bound metalloproteins by $^1$H NMR spectroscopy. This can be demonstrated by Co$^{2+}$ substitution into Cu-Zn-SOD, to form Cu-Co-SOD.$^{70}$ In the native enzyme, the signals near of nuclei near the bound Cu$^{2+}$ ions are too broad to be detected, while the signals associated with the bound Zn$^{2+}$ ions are buried within the protein signals and thus difficult to detect. The use of Co$^{2+}$ as a paramagnetic probe facilitates characterized of metal binding sites as a result of characteristic hyperfine shifted signals attributed to the metal-bound form, which are under chemical exchange with the metal-free form and thus can be easily assigned by means of various paramagnetic NMR techniques.$^{71,72,73}$

The metallo-peptides are characterized in terms of select structural, spectroscopic, and catalytic properties. The $^1$H NMR assignment of the peptide Ac-RHHPHHHE-NH$_2$ is presented together with its solution structure based on $^1$H NMR derived distance constrains. Metal binding and solution structure of the metallopeptide was also investigated by means of $^1$H NMR techniques, circular dichroism, molecular dynamics, and conformational search calculations.

2.2. Experimental

2.2.1. Materials

The investigated peptides, Ac-RHHPHHHE-NH$_2$ and Ac-RHHPHHHEF-NH$_2$ were purchased from GenScript. Methanol, CuSO$_4$, and sodium phosphate
monohydrate were from Fischer Scientific. Deuterated d$_6$-DMSO and phosphate buffer were from Sigma-Aldrich. All aqueous solutions were prepared using deionized water with resistivity of 18.2-MΩ obtained from a Millipore Milli-Q system.

2.2.2. Circular Dichroism

Circular dichroism experiments were performed on AVIV Model 215 circular dichroism spectrometer equipped with temperature control in a 1 mm path-length quartz cuvette. In a typical assay, 300 μM peptide sample was prepared in 20 mM phosphate buffer, TFE, or in different ratios of phosphate buffer to TFE. Spectra were recorded within the far-UV region in the range of 190-260 nm, in triplicate runs. The output of the spectra is given in ellipticity, θ, measured in millidegrees, which is then converted into molar ellipticity, [θ], on the spectrometer, [θ] = θ/(c × l). The molar ellipticity is in units of deg cm$^2$ dmol$^{-1}$, thus, it is given by [θ] = θ/ (10 × c × l), where c is the molar concentration of the sample, and l is the path length in cm. The molar concentration, c, is dependent on the mean residue molar concentration, given by $c = (1000 \times n \times c_g)/M_r$, where n is the number of peptide bonds present, $c_g$ is the concentration in g/ml, and $M_r$ is the molecular weight of the peptide sample. The spectra shown are after subtraction of the blank, solvent system.
2.2.3. NMR Techniques

All NMR spectra were recorded on either a Varian Unity INOVA 600 spectrometer equipped with triple resonance probe, a Varian Unity INOVA 500 spectrometer equipped with triple resonance probe, or on Varian Direct-Drive 500 spectrometer equipped with cold probe exhibiting five times the sensitivity compared to a non-cold probe. The spectrometers were available in the NMR facility of the chemistry department at the University of South Florida. $^1$H NMR resonances of the apo peptide were assigned by means of various homonuclear 2D techniques, COSY, TOCSY, and NOESY$^{74,75}$ All samples were prepared in 99.9% d$_6$-DMSO, and $^1$H chemical shifts were referenced to the internal d$_6$-DMSO signal at 2.5 ppm. A line-broadening of 0.5 for diamagnetic systems and up to 10 Hz for paramagnetic systems was introduced to enhance signal to noise ratio by exponential multiplication prior to Fourier transformation. Metal binding studies of the designed peptide were carried out by various paramagnetic NMR techniques and by monitoring chemical shift perturbations. Delay times, recycle times, and mixing times where adjusted as necessary and maybe found in the figure captions of the spectra. A typical concentration of apo peptide varied between 1.0–4.0 mM per sample, in a total volume of 400-500 μL. Up to 1 mM transition metal stock solutions were prepared to avoid possible increase in the water peak (3.4 ppm in d$_6$-DMSO) due to metal titration. Copper and zinc stock solutions were prepared in water, and cobalt solution was prepared in d$_6$-DMSO.

2D $^1$H-$^1$H COSY and TOCSY: Of the simplest 2D NMR experiments is $^1$H-$^1$H COSY; it provides details of through-bond spin-spin correlation of coupled nuclei. 2D
$^1$H-$^1$H Double Quantum Filtered COSY (DQF-COSY) uses double quantum transition to filter out protons that do not experience $J$-coupling between other protons, thus simplifying the spectrum of a complex system. The pulse sequence of DQF-COSY is similar to that of COSY. In DQF-COSY, two 90° pulses replace the delay time in a COSY pulse sequence. The first 90° pulse converts the single quantum states to double quantum states, and the second 90° pulse returns the double quantum states to detectable single quantum magnetization. TOCSY experiment enables the identification of through-bond spin-spin couplings within a spin system. The experiment was run at 80 ms mixing time. COSY and TOCSY experiments are useful in determining connectivities between adjacent nuclei within a spin system.

2D $^1$H-$^1$H NOESY and EXSY: A NOESY experiment uses through space correlation of dipolar interactions of spins for correlation of protons that are within 5 Å apart in space, monitored by the appearance of cross peaks in the 2D contour plot. The intensity of observed cross peaks is proportional to the distance between two given protons. The amino acids in the 1° sequence of a peptide or protein, for example, will show NOE cross peaks due to their close proximity in space when folded in 3° structure. The intensities of the NOE cross peaks can be classified as strong, medium, and weak to determine relative distance constrains. Since the intensities of NOE cross peaks are inversely proportional to the sixth power of the distance, a more precise distance constrain can be calculated given that there is a known reference inner-proton distance between two protons ($r_{ref}$) and its corresponding NOE cross-peak volume ($a_{ref}$), the distance between the two protons “a” and “b” can be calculated according to $r_{ab} = r_{ref} (a_{ref}/a_{ab})^{1/6}$. The NOESY
experiments were acquired at 200, 400, and 500 ms mixing times, and up to $4K \times 4K$ data points.

2D EXSY experiments are crucial for structural determination of metal-bound peptide, which also applies to studies of proteins and small ligand molecules in general. EXSY has the same pulse sequence as two-dimensional NOESY experiment ($\tau_1$-$90^\circ$-$t_1$-$90^\circ$-$\tau_m$-$90^\circ$-FID). The second and third $90^\circ$ pulses are separated by the mixing time during which magnetization is exchanged between the spins. Since the metal-bound peptide complex is expected to be undergoing chemical exchange with the apo peptide, signal assignment can be achieved by the use of saturation transfer two-dimensional EXSY experiment. EXSY experiments were acquired with $256 \times 256$ data points, and a mixing time between 5–50 ms. The FIDs were zero-filled up to $4K \times 4K$ data points and processed with $45–60^\circ$ sine-squared-bell window function in both dimensions prior to Fourier transformation.

*Spin-lattice relaxation time measurements:* In the presence of paramagnetic metal ions, relaxation rates and hyperfine-shifted signals due to interactions of the paramagnetic metal with a peptide center provide details on the metal-peptide interactions (metal-to-proton distance constraint) enabling the determination of the metal binding site within the peptide.

2.2.4. Molecular Dynamics and Conformational Search

Calculations were carried out using Maestro, an interface for all Schrödinger software. Structures were determined using NMR derived distance constrains of apo and metal-bound peptide. Cross-peak intensities in the NOESY spectra were
classified into strong, medium, and weak. In conformational search, a single structure with the lowest energy is obtained. In molecular dynamics, a number of structures are obtained with the lowest possible energy plus a deviation set to ±20 kJ/mol.

2.3. Results and Discussion

2.3.1. ¹H NMR assignment

Signal assignment of the ¹H NMR spectrum of the apo peptide, RHHPPHHE, (Figure 2.2) was determined by homonuclear 2D ¹H-¹H COSY, TOCSY, and NOESY experiments. Signal assignment is crucial for elucidating secondary structure, and metal coordinating site. Herein, structure of the apo and metal-bound peptide is characterized in addition to any induced structural and or conformational changes induced by the metal-bound peptide complex. The ¹H NMR spectrum was assigned by means of standard sequential assignment methods.⁷⁷

2D ¹H-¹H COSY and TOCSY spectra: The first stage involved the identification of different spin systems present in the peptide sequence. This was feasible by COSY and TOCSY experiments, which are useful in determining connectivities between adjacent nuclei within a spin system. The COSY spectra (Figure 2.3) provide details of through-bond spin-spin correlation of coupled nuclei. This can be illustrated in the COSY spectra, where the backbone NH proton of Glu at 8.24 ppm, shows cross peak to the αH proton of Glu at 4.16 ppm. The αH proton of Glu at
Figure 2.2: $^1$H NMR spectra of RHPPHHE in $d_6$-DMSO at 500 MHz and 25°C.
Figure 2.3: 2D $^1$H-$^1$H gDQ COSY spectra of RHHPPHE in $d_6$-DMSO at 500 MHz and 25°C.
4.16 ppm shows cross peak to its βH proton, which in turn shows cross peak to its γH proton (Figure 2.4). In the TOCSY spectra (Figure 2.5) the identification of through-bond spin-spin coupling within a spin system is feasible, as detailed later. For Glu, the NH proton in the TOCSY spectra shows cross peaks to the αH, βH, and YH protons; hence, coupling within a spin-system is observed. The COSY spectra complement the TOCSY spectra in identifying direct scalar connectivities. The spin system of each different amino acid within the peptide sequence, RHHPPHHE, was identified based on cross peaks within the TOCSY spectra, as detailed below.

**Arginine spin system:** In the TOCSY spectra, the Arg spin system is identified through cross peaks between its εNH proton to its YH, βH, and δH protons (Figure 2.6, dash line). A strong, larger intensity, cross peak was observed for its δH proton, which supports the assignment of the diagonal peak to the εNH rather than the backbone NH proton of Arg. In the aliphatic region of the spectra, connectivities between Arg αH, βH, γH, and δH, are clearly identified (Figure 2.7, dash lines). Its side-chain ηH11 ηH12 protons show connectivities to its ηH21 ηH22 protons (Figure 2.8, solid).

**Glutamine spin system:** In the TOCSY spectra, the Glu-8 spin system is identified through cross peaks between its diagonal backbone NH peak to its αH, βH, and YH protons (Figure 2.6, solid line). The two βH protons of Glu are typically shifted slightly upfield as compared to its YH proton. In the aliphatic region of the spectra the diagonal αH proton of Glu shows cross peaks to the γH and βH protons (Figure 2.7, solid). Cross-peaks between the diagonal γH and βH protons are also shown.
Figure 2.4: 2D $^1\text{H}-^1\text{H}$ gDQ COSY spectra of RHHPPHHE labeled with connectivities between Glu backbone NH to its $\alpha$–H, $\alpha$–H to two $\beta$–H, and both $\beta$–H to $\gamma$–H.
Figure 2.5: 2D $^1$H-$^1$H TOCSY spectrum of RHHPPHHE. Spectrum acquired with 80 ms mixT in d6-DMSO at 600 MHz and 25°C.
Figure 2.6: The NH region of 2D $^1\text{H}$-$^1\text{H}$ TOCSY spectrum of RHHPPHHE. The spectrum shows the spin systems of the amino acid residues, four H (dotted), E (solid), and R (dashed). Each crosspeak is labeled with its corresponding $\alpha$H, $\beta$H, $\gamma$H, or $\delta$H.
**Figure 2.7:** The aliphatic region of 2D $^1$H-$^1$H TOCSY spectrum of RHHPPHHE. Connectivities between Arg (dash), Glu (solid), and His (dotted) diagonal cross peaks to its corresponding $\beta$H, $\gamma$H, or $\delta$H is shown.
Figure 2.8: Connectivities within the NH region of the TOCSY spectrum showing cross-peaks between His δH and εH imidazole ring protons and Arg side-chain ηH protons.
Histidine spin system: The four-histidine residues were similarly assigned through cross peaks between the backbone NH proton to its $\alpha$H, and $\beta$H protons (Figure 2.6). Its imidazole ring protons were assigned through connectivities within the NH region of the spectrum (Figure 2.8). The imidazole $\epsilon$H protons show connectivities to the $\delta$H ring protons.

Proline spin system: The two Pro are the only residues without NH protons in the peptide. The $\alpha$H, $\beta$H, $\gamma$H, and $\delta$H protons of Pro give rise to cross-peaks with characteristic spin system in the TOCSY spectra. The two Pro residues are identified through cross peaks between the diagonal $\alpha$H peaks to its corresponding $\beta$H, $\gamma$H, and $\delta$H cross peaks (Figure 2.9). The assignment of Pro $\beta$H is further confirmed from an overlay between the COSY and TOCSY spectra within a select region of the spectra (Figure 2.10).

2D 1H-1H NOESY spectra: The COSY and TOCSY spectra allowed the identification of all spin systems in the RHHPPHHE peptide sequence. The second stage of sequential assignment involves the determination of connectivities between adjacent amino acids within the peptide sequence. This is feasible by NOESY experiment, which uses through space correlation of dipolar interactions of spins for correlation of protons that are within 5 Å apart in space. The NOESY spectra acquired at 200 ms (Figure 2.11) shows NOE connectivities, cross peaks, enabling the determination of amino acids adjacent in sequence. To facilitate the assignment of NOE correlations, the assigned TOCSY spectra (Figure 2.5) was overlaid on the NOESY spectra (Figure 2.11), as shown (Figure 2.12). Connectivities between backbone NH protons and $\alpha$H protons of adjacent amino acids can be identified by a
Figure 2.9: The α–H region of 2D $^1$H–$^1$H TOCSY spectrum of RHHPPHHE. The two Pro residues (dot, and solid) are identified through cross peaks between diagonal αH protons to βH, γH, and δH protons.
Figure 2.10: Select region of the α–H region of an overlay between the 2D $^1$H–$^1$H TOCSY (green) and COSY (red/blue) spectra of RHHPPHHE. The two Pro residues (dot, and solid) are identified through connectivities between the (αH, βH) cross peak and to the diagonal αH protons to βH, which in turn show cross peaks to γH protons.
Figure 2.11: 2D $^1$H-$^1$H NOSEY spectra of RHHPPHHE acquired with 200 ms mixN in $d_6$-DMSO at 600MHz and 25°C.
Figure 2.12: Overlay of TOCSY (green) and NOESY (red-orange) spectra of RHHPHHE from Figures 2.5 and 2.11.
sequential walk from one (NH, αH) cross peak to another within the NH region of the spectra (Figure 2.13). In the spectra, the (NH, αH) of Glu-8 (G-8) shows NOE to the (NH, αH) of His-7 (H7), which shows NOE to the (NH, αH) of His-6 (H6), which in turn shows NOE to the αH of Pro-5 (P-5) (Figure 2.12). It is also feasible to assign the spectra by other NOEs observed within the NH region. In the spectra (Figure 2.14), the Glu-8 backbone NH proton shows strong NOE to its αH proton (9) and to an αH proton of His (4). This His residue can thus be assigned to His-7. The NOESY spectra (Figure 2.10) also show a much weaker NOE between the NH of Glu-8 and the βH protons of His-7. In Figure 3.6.1, His-6 can be assigned based on the observed NOE between the NH of His-7 and its αH (3) and the αH of an adjacent His, His-6 (13). Similarly, the εNH proton of Arg shows cross peaks to an αH and βH protons of a His residue, and was thus assigned as His–2 in the RHHPPPHHE peptide sequence.

The residues adjacent to Pro can be easily identified. The NH protons exhibiting strong cross peaks to αH protons of Pro can be assigned to either His–3 or His–6 residues. The NH of a His shows cross peak to its αH proton (11) and to an αH proton (5) of Pro (Figure 2.15). The NH proton of His also shows cross peaks to the βH protons of Pro but with a weaker intensity. The His residue with NH proton showing cross peaks to αH and βH protons of Pro, and to αH, and βH of His–2 was assigned to His–3, and the Pro was thus assigned to the adjacent Pro-4 residue. NOE correlation is observed between the αH of His-3 (11) and the βH of Pro-4 (16), and for the αH of Pro-4 (5) to the βH of Pro-5 (8), (Figure 2.15). Pro-5 and His–6 residues were similarly assigned. The imidazole ring protons were assigned based
Figure 2.13: Sequential walk within the NH region of an overlay of the TOCSY (green) and NOESY (orange) spectra from Figure 2.12. The spectra show connectivities between NH, α–H protons of Pro-5, His-6, His-7, and Glu-8.
**Figure 2.14:** NH region of an overlay of the TOCSY (green) and NOESY (orange) spectra shown in Figure 3.6. The spectra reveals NOE correlations of His-3 NH to its $\alpha$H (11) and Pro-4 $\alpha$H (5), His-6 NH to its $\alpha$H (12) and Pro-5 $\alpha$H (8), His-7 NH to its $\alpha$H (3) and $\alpha$H of His-6 (13), and of Glu-8 NH to its $\alpha$H (9) and $\alpha$H of His-7 (4).
**Figure 2.15:** α-H region of an overlay of TOCSY and NOESY spectra. The spectra show NOE correlations of His-3 αH (11) to Pro-4 βH (16), Pro-4 αH (5) to Pro-5 βH (15).
on observed cross peaks between ring protons and the αH and βH protons of each 
His residue. At this stage, the complete sequential assignment of the peptide 
sequence was reached. Complete assignment of the $^1$H NMR signals of the peptide is 
necessary to obtain structural information and deduce the metal binding site. A list 
of the signal assignment is given in Table 2.1.

2.3.2 Chemical Shift Index

The chemical shift data obtained from signal assignment of the NMR spectra 
can give information on the secondary structure of the peptide. Chemical shift 
index, CSI, is a simple method used to define the secondary structure of proteins and 
peptides using backbone chemical shift data.\textsuperscript{78, 79} The method is based on 
differences between the chemical shifts of αH and βH protons relative to the 
chemical shift values of a random coil for each specific residue. Residue-specific 
chemical shifts, of a random coil, can be obtained from the biological magnetic 
resonance data bank, BMRDB.\textsuperscript{80} The CSI method assigns a (+1, 0, −1) index for 
chemical shifts. In a given residue, if a downfield αH shift is greater than 0.1 ppm, 
relative to its equivalent chemical shift value in a random coil, then it is given a +1 
value. Similarly, if an upfield αH shift is greater than 0.1 ppm, the residue is given a 
−1 value. A value of 0 is given to a residue exhibiting an upfield or downfield 
chemical shift less than 0.1 ppm of a random coil. The CIS values of RHHPPHHE are 
shown in Table 2.1, (Figure 2.16). Four or more consecutive αH with CSI of −1 
define an alpha helix, and three or more αH with CSI of +1 define a beta sheet.\textsuperscript{81} All
Table 2.1: Signal Assignment of $^1$H NMR spectrum of RHHPPHHE in d$_6$-DMSO.

Average $^1$H resonances of random coil structures and CSI index value are shown.

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<th>$^1$H Random Coil</th>
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Figure 2.16: Chemical shift index plot for $\alpha$–H for RHHPPHHE peptide. Four or more consecutive -1 values represent $\alpha$–helix, three or more +1 values represent $\beta$–sheet structures, and all other regions can be designated as coil.
other regions can be designated as coil, which is attributed to all other structures, including loops, turns, and random coils. Based on the CSI method, the chemical shifts of the peptide RHHPPHHE suggest the presence of a coil structure, which may include loops, turns, or random coil.

2.3.3. Structure calculations

To gain structural information of the peptide in solution, further NOESY experiments were necessary. The NOESY experiments were acquired at longer mixing times of 400 and 600 ms to obtain long range through space NOE correlations (Figure 2.17, 2.18), respectively. Both NOESY spectra showed similar NOEs, which can be attributed to the short peptide sequence and folding of the peptide. Select NOE correlations within the NH region of the spectra are shown (Figure 2.19), where the backbone NH of His-3 shows a medium range NOE to the δH (a), αH (a'), and βH of Arg-1. The backbone NH of His-5 shows strong NOE to the βH proton of Glu (f), while the backbone NH of His-2 shows a weaker NOE to the βH proton of Glu (f), (Figure 2.19). The data may suggest a folded structure, where Glu-8 shows strong NOEs to His-2. Further, the δH proton of Pro-4 shows strong NOE to the backbone NH proton of His-5 (b), and weak range NOE to the backbone NH proton of His-2 and Glu-6. A list of all NOE correlations used in structural calculations is shown in Table 2.2. Protons that are within 5 Å apart in space give rise to cross-peaks or NOEs on a NOESY spectrum indicative of protons that are within 5 Å apart in space. The spectra gave rise to 55 different NOEs and were used as distance constrains to obtain structural information of the peptide in solution.
Figure 2.17: 2D $^1$H-$^1$H NOSEY spectra of RHHPPHHE acquired with 400 ms mixN in d6-DMSO at 400MHz and 25°C.
Figure 2.18: 2D $^1$H-$^1$H NOSEY spectra of RHHPPHHE acquired with 600 ms mixN in d6-DMSO at 600 MHz and 25°C.
Figure 2.19: NOE connectivities of a select NH region of the 2D $^1$H-$^1$H NOSEY spectra of RHHPPHHE acquired with 400 (left) and 600 (right) ms mixN. Spectra are labeled with NOEs between the NH of His-3 and $\delta$H (a) and $\alpha$H (a’) of Arg-1, and between the NH of His-5 and His-2 to $\beta$H of Glu (f). NOEs to $\delta$H of Pro-4 (b, d, e) and Pro-5 (c) are shown.
### Table 2.2: List of TOCSY and NOESY correlations of RHHPPHHE.

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Initially, the peptide structure was calculated in the absence of any distance constrains. Molecular dynamic calculations of the peptide sequence, RHHPPHHE, give rise to a random coil structure (Figure 2.20, A), with energy value of \(-818\) kJ/mol. Random coil structures are highly dynamic with no preference to any secondary or tertiary structures. Further, in the presence of the NMR derived constrains, NOE correlations (Table 2.3), molecular dynamic calculation give rise to a \(\beta\)-turn structure (Figure 2.20, B). This supports the initial design, where the presence of Pro residues towards the center may force or facilitate a \(\beta\)-turn like structure in the presence and or absence of coordinated metal ions. The chemical shift assignment of the peptide also supports a structure that deviates from \(^1\)H chemical shifts of random-coil structures (Table 2.1).

The presence of constrains refines the structure. Molecular dynamic calculations provide a single structure with the lowest energy. Maestro Schrödinger also allows structure calculations by running conformational search in the presence or absence of any set constrains. In conformational search calculations, a group of structures are obtained, the structure with the lowest energy plus all structures that vary within a set energy deviation from the lowest energy structure. In the presence of the NOE distance constrains obtained from the NOESY spectra, conformational search calculation resulted in 94 structures within 20 kJ/mol of energy, with the lowest structure having \(-1355\) kJ/mol (Figure 2.21). The combined 90 structures had an average RMSD value of 1.5 Å. The data suggest a well-defined structure, exhibiting a \(\beta\)-turn character, with the turn seen around the HPPH segment of the peptide. The turn is attributed to the Pro residues restricting the
**Table 2.3:** NOE connectivities of RHHPPHHE at mixN of 200, 400 and 600 ms. Each row shows the NOEs associated with the highlighted proton.

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**Figure 2.20:** Molecular dynamic calculation of RHHPPHHE, shown as stereo relaxed-eye. (A) in the absence and (B) in the presence of NMR derived distance constrains. The amino acids are colored by atom type with carbon as green, hydrogen as white, oxygen as red, and nitrogen as blue.
Figure 2.21: Backbone superposition of 90 structures, as stereo relaxed-eye, obtained from conformational search calculations of RHHPPHHE using the NMR derived distance constrains (PE = $-1355 \text{ kJ/mol}$, RMSD = 1.5 Å). The backbone is colored gray, and the amino acids are colored by atom type with carbon as green, oxygen as red, and nitrogen as blue. Hydrogen atoms are omitted for clarity.
conformation of neighboring amino acid residues. The structure of the peptide may be altered upon metal coordination, thus further characterization of the peptide, in its metal-bound form, was necessary. The His residues within the peptide sequence, RHHPPHHE, serve as metal anchoring sites. The molecular dynamic calculations of the apo peptide, in the presence of distance constrains, indicate the close proximity of His-3 to His-7, and His-2 to His-6 within the folded peptide. To elucidate the metal binding site and any induced structural changes upon its binding, metal coordination and solution structure of the metallopeptide was investigated by means of $^1$H NMR and molecular dynamics calculations.

2.3.4. Monitoring metal binding by means of $^1$H NMR

CKET BINDING: Herein, the coordination of Cu$^{2+}$, Co$^{2+}$, and Zn$^{2+}$ to the peptide is investigated. Structural characterization of the metal-binding site(s) was essential in understanding the overall folding of the peptide, and the catalytic oxidative activities of the metallopeptide complex when bound to the redox-active Cu$^{2+}$ ions. The interactions due to the binding of paramagnetic transition metal ions Cu$^{2+}$ and Co$^{2+}$ with the peptide is studied via $^1$H NMR experiments. Paramagnetic transition metal ions cause line broadening and hyperfine-shifted $^1$H signals upon interacting with the ligand, which is associated with the correlation time and the metal's electronic relaxation rate. Stoichiometric titration of transition metal ions was monitored by means of $^1$H NMR, through mapping of chemical shift perturbation, hyper-fine shifted signals, and relaxation time measurements.
Figure 2.22: Structures display the backbone and His residues obtained from conformational search calculations of RHHPPHHE using the NMR derived distance constrains of the apo peptide, shown as stereo relaxed-eye. The calculations show the proximity of His-2 and His-6, and His-3 and His-7.
In paramagnetic systems, unpaired electron cause fast paramagnetic relaxation of neighboring nuclei and affect the line-width of resonance lines in the spectrum. In the case of Cu$^{2+}$ ions, line broadening is within 1000-5000 Hz, thus addition of Cu$^{2+}$ ions to a molecule will cause signals in the $^1$H spectrum to be very broad and difficult to distinguish. Thus, small trace amounts of Cu$^{2+}$, up to 0.1 equivalents, were titrated to apo peptide to avoid complete line broadness of the $^1$H NMR spectrum (Figure 2.23). Monitoring changes in chemical shift due to Cu$^{2+}$ interaction with the peptide, RHHPPHHEF, is indicative of protons in near proximity of the metal binding site (Figure 2.23). The imidazole $\varepsilon$H of His-3, His-2, and His-6 were shifted up to 0.1 ppm as a result of Cu$^{2+}$ titration. The imidazole $\delta$H of His-3, His-2, and His-7 were also shifted up to 0.1 ppm, with the largest shift observed for His-2. The backbone NH protons of His-3 and His-6, and to a less extent His-2 and Glu-8, were also shifted indicative of the close proximity to the metal center. The observed shift of $\alpha$H of His-3, along with the $\varepsilon$H and backbone NH, is indicative of Cu$^{2+}$ coordination to the $\delta$NH. His-2 is likely coordinated by its $\varepsilon$NH, as a result of the observed imidazole $\varepsilon$H and $\delta$H shifts. Hence, monitoring changes in the NMR spectra of apo peptide and upon copper titration is indicative of copper coordination to the peptide. However, to elucidate metal-peptide coordination unambiguously, cobalt is used as a paramagnetic probe to elucidate metal-peptide coordination.

**Cobalt Binding:** Compared to Cu$^{2+}$, which causes signal broadening due to long enough relaxation time, Co$^{2+}$ exhibits less pronounced line broadening and shows hyperfine-shifted NMR signals of coordinated ligands. Thus, cobalt is used as
Figure 2.23: $^1$H NMR spectra of Cu$^{2+}$ titration into RHHPPHHE, apo peptide (green), 0.07 equiv Cu$^{2+}$ (red), 0.1 equiv Cu$^{2+}$ (blue), in d$_6$-DMSO at 500MHz and 25°C. Select region is shown in each of the spectra (a) 7.5-9.5 ppm, (b) 6.5-7.5 ppm, (c) 4.4-4.9 ppm, and (d) 0.5-9.5 ppm.
a paramagnetic probe to study metal binding to peptide systems due to its fast
electronic relaxation and characteristic hyperfine shifted\textsuperscript{83} signals that can be
correlated to bound protons in the diamagnetic region of the spectrum \( \sim 13 \) ppm.\textsuperscript{84}
The \( ^1\text{H} \) NMR spectrum of \( 2\text{Co}^{2+} \) complex of \( \text{RHHPPHHE} \) (\( 2\text{Co} – \text{RHHPPHHE} \)) shows
eight hyperfine-shifted signals in a window of about 100 ppm (Figure 2.24). Shifted
signals are a result of exchange between the metal-bound and meta-free form of the
studied system; in the presence of chemical exchange, protons near the metal
binding site show shifted signals.\textsuperscript{85} Upon addition of 1 equivalent of \( \text{Co}^{2+} \) to
\( \text{RHHPPHHEF} \), a complex is formed which exhibits six hyperfine-shifted signals (A, B,
C/D, F, H, G in Figure 2.22). Upon addition of the second equivalent of \( \text{Co}^{2+} \) to
\( \text{RHHPPHHEF} \), two new hyperfine-shifted signals appear on the spectra (C/D, E, in
Figure 2.24) and peak A, B, and F are enhanced in intensity.

Since none of the shifted signals disappear or shift upon addition of a second
equivalent of \( \text{Co}^{2+} \), it can be said the binding of the second \( \text{Co}^{2+} \) ion does not alter the
binding site of the first coordinating \( \text{Co}^{2+} \) ion. It may alter the overall secondary
structure of the peptide but not the coordinating sites. Shifted protons attributed to
the imidazole NH protons of the His residues coordinated to the \( \text{Co}^{2+} \) in the peptide
can be easily identified as NH protons disappear in the presence of \( \text{D}_2\text{O} \) solution due
to exchange. The signals labeled A, B, C, and E were identified as NH signals in
Figure 2.25. The observed hyperfine shifted signals are assigned based on chemical
exchange between the free and bound form of \( \text{Co}^{2+} \)-peptide complex. In the EXSY
spectrum, eight of the hyperfine-shifted signals can be assigned as a result of
appearance of cross-peaks between hyperfine shifted signals and their diamagnetic
**Figure 2.24:** $^1$H NMR spectra of 2Co$^{2+}$ complex of RHHPPHHE in d6-DMSO, at 500MHz and 25°C. The insets show the paramagnetically shifted signals with a larger vertical scale, for clarity.
Figure 2.25: Select region of the $^1$H NMR spectra of the Co$^{2+}$–RHHPPHHEF complex in d6-DMSO, at 500MHz and 25°C, showing hyperfine shifted signals associated with the binding of 1 Co$^{2+}$ (top), 2 Co$^{2+}$ (middle) equivalents per peptide. The asterisks indicate solvent-exchangeable NH signals. Partial to full disappearance of H$_2$O exchangeable signals (Bottom).
counterpart (Figure 2.26, 2.27), (Table 2.3). The shifted signal at 48.69 ppm (E) is in exchange with 8.98 ppm, and the shifted signal at 47.24 ppm (F) is in exchange with 7.5 ppm, attributed to $\varepsilon$H and $\delta$H of His-3. The broad shifted signal at 50.0 ppm (D) is attributed to the imidazole ring protons $\delta$H of His-6. In the presence of paramagnetic metal ion, the imidazole ring protons, $\varepsilon$H, and $\delta$H, in the metal-bound state are shifted outside the diamagnetic region, downfield between 50 to 70 ppm. Herein, the shifted signals attributed to protons near the metal center are observed within 45 to 70 ppm, consistent with His binding in several metalloproteins. The imidazole ring protons, $\varepsilon$H, and $\delta$H, are typically broad when $\varepsilon$NH is coordinated to the metal center, and the $\delta$H is typically sharp when $\delta$NH is coordinated to the metal center. The signals (G) and (H) are show exchange with the $\alpha$H and $\beta$H protons of His-3.

Cross peaks as a result of chemical exchange near the diamagnetic region of the EXSY spectrum (Figure 2.27) are attributed to imidazole NH ring protons show exchange with water (1, 2, 3, and 4), and with imidazole ring protons (5, 6, 8, 7). Protons associated with the metal binding site are not detected due to deprotonation upon metal coordination. The results suggest Co$^{+2}$ coordination through its $\delta$ NH of His-2, and $\varepsilon$ NH of His-6 at the first site and through $\varepsilon$ NH of His-3, and $\delta$ NH of His-7 at the second metal binding site.

**Zinc Binding:** Structural changes induced by the binding of the diamagnetic Zn$^{2+}$ ions are characterized by changes in chemical shifts and NOEs. Zn$^{2+}$ binding to RHHPPHHE induced chemical shift changes up to 0.14 ppm (Figure 2.28). Monitoring changes in the $^1$H chemical shift suggests Zn$^{2+}$ coordination near $\varepsilon$2H
Figure 2.26: 2D $^1$H-$^1$H EXSY spectrum of $2\text{Co}^{2+}$-RHHPPHHE complex. Spectrum acquired with $256 \times 256$ data points, recycle time of $108 \text{ ms}$, mixN of $20 \text{ ms}$, in d6-DMSO at $25^\circ\text{C}$.
Table 2.3: Assignment of cross-peaks in the EXSY spectra of Co-RHPPHHEF.

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<th>Crosspeak</th>
<th>Assignment</th>
<th>Apo</th>
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<td>B 59.0</td>
<td>8.96</td>
<td>His-6/His-2 εH</td>
<td>8.82/8.86</td>
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<tr>
<td>C 58.0</td>
<td>7.35</td>
<td>His-2 δH</td>
<td>7.27</td>
</tr>
<tr>
<td>D 50.0</td>
<td>7.37</td>
<td>His-6 δH</td>
<td>7.30</td>
</tr>
<tr>
<td>E 48.7</td>
<td>9.06</td>
<td>His-3 εH</td>
<td>8.91</td>
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<td>His-7 εH</td>
<td>7.45</td>
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<td>G −14.0</td>
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<td>H −1.60</td>
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<td>His-3 αH</td>
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Figure 2.27: Expanded region of the 2D EXSY spectra showing exchange near the diamagnetic region.
**Figure 2.28:** $^1$H NMR spectra of Zn$^{2+}$ titration into RHHPPHHE in d6-DMSO at 500MHz and 25°C. Equivalent of Zn$^{2+}$ titrated, from bottom to top spectrum, 0.0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, and 5, respectively.
and $\delta H$ of His-3, $\varepsilon NH$ of Arg, $\varepsilon 2H$ of His-6, and $\delta H$ of His-7, at 8.72, 7.39, 7.95, 8.78, and 7.21 ppm, respectively, for the first equivalent of Zn$^{2+}$. The $\delta H$ of His-6, and the $\delta H$ of His-2 are affected most by the addition of the second equivalent, at 7.29, and 7.32 ppm, respectively. The data may suggest Zn$^{2+}$ coordination to $\varepsilon NH$ of His-3 and $\delta NH$ of His-7 at the first site, and to $\delta NH$ of His-6 and $\delta NH$ or $\varepsilon NH$ of His-2 at the second site, which can be further confirmed by the use of Co$^{2+}$ as a paramagnetic probe.

To obtain structural information of the Zn$^{2+}$-bound peptide complex, 2D TOCSY (Figure 2.29) and NOESY (Figure 2.30) experiments were performed. The spectra were assigned by and by comparison to the apo peptide assignment. A list of TOCSY and NOESY connectivities are shown in Table 2.4. The NOESY spectrum acquired at 400 ms (Figure 2.30) shows through space connectivities between amino acids, which uses through space correlation of dipolar interactions of spins for correlation of protons that are within 5 Å apart in space. The NH region (Figure 2.31) of the NOESY spectrum (Figure 2.30) correlations between the NH protons of the peptide can be identified. The numbers on the spectrum (Figure 2.31) can be correlated to the chemical shifts in Table 2.4. The backbone NH proton of Arg-1 (55) shows NOE to the backbone NH proton of His-3 (26), which in turn shows NOE its $\delta H$ (18) and to the $\delta H$ of His-6 (52), (Figure 2.30). This suggests the close proximity of His-3 and His-6 within the folded state of the peptide, NOEs between the NH and $\delta H$, respectively. Further, the $\varepsilon 2H$ of His-3 (30) shows NOE to the $\delta H$ of His-2 (18) and His-6 (52), which supports the close proximity of His-3 and
**Figure 2.29:** 2D $^1$H-$^1$H TOCSY spectrum of diZn$^{2+}$–RHPPHHE. Spectrum acquired with 80 ms mixT in d$_6$–DMSO at 500 MHz and 25°C.
Figure 2.30: $^1$H-$^1$H NOESY spectrum of diZn$^{2+}$–RHPPHHE complex at 500 MHz and 25°C.
His-6, (Figure 2.31).

The backbone NH of His-6 (50) shows NOE to the backbone NH of His-7 (60), which in turn shows NOE to the backbone NH of Glu-8 (70), hence the connectivities between the backbone NH protons of adjacent amino acids in the peptide sequence, RHHPPHHE (Figure 2.31). The backbone NH of His-6 (50) also shows NOE to its δH (52). The backbone NH of Glu-8 (70) shows NOE to the ε2H (64) and δH of His-7 (62), and to the δH of His-2 (18), (Figure 2.31). Hence, the close proximity between Glu-8 and the imidazole ring of His-2. The backbone NH of His-7 (60) shows NOE to δH of His-6 (52), (Figure 2.31). NOEs within the NH–αH region of the NOESY spectrum can give rise to important structural information. Within the NH–αH region of the NOESY spectrum (Figure 3.32), the backbone NH protons of His-6 (50), Glu-7 (70), and His-7 (60) show weak NOEs to the αH of Glu-8 (68). The εNH of Arg-1 (8) also shows NOE to the αH of Glu-8 (68). Hence, the N- and C-terminus of the peptide are in close proximity, within 5 Å, in the folded state of the peptide. Further, the spectrum (Figure 3.32) shows NOEs between the δH of His-3 (31) and the αH of Glu-8 (68) and Pro-5 (43). NOE between δH of His-6 (52) and the αH of Pro-4 (48) is also shown (Figure 3.32). The NOE connectivities are used as distance constrains for structural calculations. A summary of NOE connectivities is shown in Table 2.4 and 2.5.

The structure and potential metal-binding sites were determined by running conformational search calculations of the apo peptide, using the NMR-derived constraints (Table 2.5 and 2.6) of the Zn^{2+}-bound peptide complex (Figure 2.32). The conformational search calculations give rise to 34 structures within 20 kJ/mol
Figure 2.31: The NH region of the diZn²⁺−RHHPPHHE NOESY spectra acquired with 400 ms mixN. The spectrum shows NOE connectivities for Arg-1 NH (55), His-3 ε2H (30), His-3 NH (26), His-6 NH (50), Glu-8 NH (70), His-7 NH (60), NH-7 ε2H (64), His-3 δH (31), His-2 δH (18), His-6 δH (52), and His-7 δH (62).
**Figure 2.32:** NOE connectivities of a select NH–αH region of the 2D $^1$H–$^1$H NOSEY spectra of diZn$^{2+}$–RHHPPHHE acquired with 400 ms mixN. Spectrum is labeled with NOEs between the αH of Glu-8 (68) and the backbone NH of His-6 (50), Glu-8 (70), and His-7 (60), and the εNH of Arg-1 (8). NOEs are also shown between the δH of His-3 (31) and the αH of Glu-8 (68) and Pro-5 (43). NOE between δH of His-6 (52) and the αH of Pro-4 (48) is also shown.
Table 2.5: List of TOCSY and NOESY correlations of Zn-bound RHPPPHE complex.

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Table 2.6: NOE connectivities for RHHPPHHHE. Each row shows the NOEs associated with the highlighted proton.

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<th>Observed NOE</th>
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</tr>
<tr>
<td>δH Arg₁</td>
<td>εH His₇</td>
</tr>
<tr>
<td>εNH Arg₁</td>
<td>ε1H His₃</td>
</tr>
<tr>
<td>εH His₃</td>
<td>αH Arg₁</td>
</tr>
<tr>
<td>NH His₇</td>
<td>ε1H His₃, εH His₈</td>
</tr>
<tr>
<td>ε₁H His₇</td>
<td>δH, εNH Arg₁</td>
</tr>
<tr>
<td>δH His₇</td>
<td>αH, βH Glu₈</td>
</tr>
<tr>
<td>εH His₇</td>
<td>γH Arg₁, αH Glu₈, βH His₃</td>
</tr>
<tr>
<td>NH Glu₈</td>
<td>αH, βH Glu₈, βH His₃</td>
</tr>
</tbody>
</table>
of energy, and an RMSD value of 1.5 Å. The data clearly suggests the formation of a beta-turn like structure. Similar structure was obtained in the absence of metal (Figure 2.18).

To further characterize the binding site, metal ions were incorporated into the molecular dynamic calculations of the peptide calculated in the presence of derived NMR constrains (Figure 2.33, 2.34). The data suggest the binding to two metal ions, with His-2 and His-6 occupying one site, and His-3 and His-7 occupying a second site. The characteristic structure of the peptide promotes metal binding to His residues that are not necessary adjacent to one another in sequence, His-His, which would be preferred or suggested in a random coil like structure. In the calculation of the solution structure of (metallo)RHPPHHE, the molecule was considered to exist as a single monomeric species, where the observed NOEs were not identified as a result of a dimeric interaction. However, this does not influence the overall folding and calculated structure of the peptide.

2.3.5 Circular dichroism spectra of (metallo)RHPPHHE

Circular dichroism was used for structural studies of the metal-free and metal-bound peptide, despite its much lower resolution compared to NMR. Far-UV CD spectroscopy in the range of 180–260 nm provides information on peptide secondary structure. The spectrum is a result of backbone amide-amide interactions within the peptide, amide chromophore, which estimates the different classes of secondary structures. Conformational changes as a result of ligand-
**Figure 2.33**: Backbone overlap of 34 structures, as stereo relaxed-eye, obtained from conformational search calculations of RHHPPHHE using the Zn-bound NMR derived distance constrains. The RMSD between structures is 1.5 Å, with potential energy of ~1565 kJ/mol. The backbone is colored gray, and the amino acids are colored by atom type with, oxygen as red, nitrogen as blue, and carbon as yellow in His and as green in Glu, Arg, and Pro residues.
Figure 2.34: Molecular dynamic calculations of 2-to-1 Zn-to-RHHPPHHE ratio using the Zn-bound NMR derived distance constraints. Three different orientations are shown with a stereo relaxed-eye view. The amino acids are colored by atom type with, carbon as green, oxygen as red, and nitrogen as blue. Coordinated zinc ions are depicted as cyan spheres.
peptide binding may also be investigated using CD. Herein, the CD spectra were recorded for the metal-free and metal-bound form of the peptide in order to elucidate secondary structure of the peptide and any induced conformational changes as a result of metal coordination (Figure 2.35 and 2.36), respectively. The data are summarized in Table 2.7.

The CD spectrum of the peptide, RHHPPHHE, shows a clear negative band at 204 nm and two slightly negative shoulders at 226 and 235 nm, in phosphate buffer (Figure 2.35, dashed trace). The spectra suggest the presence of random coil, while the shift in its observed bands may be attributed to the presence of secondary structure in equilibrium with the random coil structure. Random coil structures exhibit a characteristic minimum around 195 nm and a weak positive shoulder around 212 nm.86 In the presence of 80% TFE, similar spectrum is observed (Figure 2.35, solid trace). TFE is known to promote and stabilize α-helical formation characterized by an intense positive band around 192 nm, and two less intense negative bands around 208 and 222 nm.87 The short peptide sequence may be attributed to lack of well-defined α-helical structure even the presence of 80% TFE in solution. The relatively small deviation in the spectrum between the apo peptide in phosphate and in 80% TFE may suggest that the peptide backbone has some flexibility in the metal-free form. The NMR data is not in agreement with the CD spectra suggesting the presence of a β-turn structure for the apo peptide. A similar CD spectrum, of a random coil with a negative band at 200 nm, was observed for a 16-mer exhibiting a native β-hairpin structure in solution based on NMR data.88
Figure 2.35: Far-UV CD spectra of 300 μM RHHPPHHE peptide. Spectra were acquired in 20 mM phosphate buffer (solid trace), and in 4:1 TFE:20 mM phosphate buffer (dashed trace) at 25°C.
Figure 2.36: Far-UV CD spectra of 300 μM Cu²⁺–RHHPPHHE complex. Spectra were acquired in 4:1 TFE:20 mM phosphate buffer (top), and in 20 mM phosphate buffer (bottom) at 25°C, with 1 Cu²⁺ ( _ _ ), 2 Cu²⁺ (---), and 3 Cu²⁺ (....) equivalents per peptide.
**Table 2.7:** List of bands in the CD spectrum of (Cu)-RHHPPHHE peptide. A list of bands in characteristic structural features is also presented.

<table>
<thead>
<tr>
<th>Sample</th>
<th>( \lambda_1 ) (nm)</th>
<th>([\theta]_1 \times 10^3)</th>
<th>( \lambda_2 ) (nm)</th>
<th>([\theta]_2 \times 10^3)</th>
<th>( \lambda_3 ) (nm)</th>
<th>([\theta]_3 \times 10^3)</th>
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<td>RHHPPHHE(^a)</td>
<td>204</td>
<td>-14</td>
<td>226</td>
<td>-0.89</td>
<td>235</td>
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</tr>
<tr>
<td>RHHPPHHE(^b)</td>
<td>203</td>
<td>-11</td>
<td>229</td>
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<tr>
<td>Cu-RHHPPHHE(^a)</td>
<td>211</td>
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<td>232</td>
<td>-1.8</td>
<td>190</td>
<td>2.3</td>
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<tr>
<td>2Cu-RHHPPHHE(^a)</td>
<td>205</td>
<td>-2.6</td>
<td>231</td>
<td>-1.3</td>
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<td>-6.6</td>
<td>229</td>
<td>-6.4</td>
<td>193</td>
<td>13</td>
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<td>(\alpha)-helix(^89)</td>
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<td>222</td>
<td>-</td>
<td>193</td>
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<td>(\beta)-sheet(^89)</td>
<td>218</td>
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<td>195</td>
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<td>(\beta)-turn(^90)</td>
<td>207</td>
<td>-/+</td>
<td>227</td>
<td>-</td>
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a: Sample prepared in 20 mM phosphate buffer  
b: Sample prepared in 4:1 TFE: 20 mM phosphate buffer
In the presence of bound-Cu$^{2+}$, CD spectra suggest metal-induced structural changes to the apo peptide. The CD spectra of Cu$^{2+}$ binding to the peptide were monitored in the absence and presence of TFE (Figure 2.36). In the presence of TFE, the CD spectra of the Cu$^{2+}$–peptide complexes exhibits a characteristic fold, with two negative bands around 206 and 229 nm, and a positive band around 193 nm (Figure 2.36a). Despite the presence of TFE, the complex did not fold into an α-helical conformation suggesting an alternative stable conformation for the peptide. The observed negative bands around 206 and 229 nm are characteristic of β-turn structures.$^{91}$ The turn has been classified as a type II’ turn.$^{92}$ Similar spectra features were obtained for up to 3 equivalents of bound-Cu$^{2+}$ ions per peptide. The CD data suggest that pronounced structural changes, to the apo peptide, are primarily induced by metal coordination to the first site, upon binding of the first equivalent of Cu$^{2+}$ ions. Nevertheless, the CD spectra of the apo form similar to that of a β-hairpin structure, as discussed earlier.

Similarly, structural changes, to the apo peptide, are primarily induced by metal coordination to the first site, upon binding of the first equivalent of Cu$^{2+}$ ions, in phosphate buffer, the CD spectra of the Cu$^{2+}$–peptide complexes, suggests the formation of β-turn structure, where negative bands at 211 and 232 ppm are observed in the presence of one equivalent of bound-Cu$^{2+}$ per peptide. A slight shift in CD bands is observed in the presence of two equivalents of bound-Cu$^{2+}$ per peptide, negative bands at 205 and 231 nm. In the presence of one equivalent of bound-Cu$^{2+}$, the spectra exhibit a negative band at 221 ppm, a less intense negative band at 232 ppm, and a positive band at 190 ppm is also observed. The CD spectra
suggest the presence of β-turn structure. Type II β-turn structures have a characteristic CD spectra, negative band at 222-227 nm and positive band within 196-202 ppm, and type II’ β-turn exhibit two negative bands at 218-222 nm 200-2012 nm. The characteristic features of the spectra clearly suggest the formation of a type II’ turn. Based on the findings, the metallopeptide adopts a β-turn structure in phosphate buffer, in the presence or absence of TFE. The data is in agreement with a β-turn structure of RHHPHHE, as calculated based on NMR derived constrains (Figure 2.33), in d6-DMSO.

2.4 Concluding Remarks

The successful design of a de novo octamer is achieved. The peptide is folded with two potential metal binding sites. The findings in this study suggest that the designed de novo octamer has a characteristic β-turn secondary structure based on NMR derived constrains and molecular dynamic calculations. The results herein suggest important implications in model peptide design since model peptides are often unstructured in solution due to its very short sequence. In the presence of metal, the solution structure exhibits a β-turn structure as well. The metal is likely coordinated to the imidazole NH of His-3 and His-7 at the first site, and His-2 and His-6 at the second site, as supported by the NMR data.

The diPro residues were essential in facilitating a β-turn structure, while the oppositely charged side chains of Arg and Glu support the turn at the N- and C-terminus. Variation in the amino acids at the turn, HPPH, to HGPH, HPGH, or a
single Pro in HPH is likely to alter the observed \( \beta \)-turn, which in turn may alter the activity. Incorporating an amino acid between the di-His site, HH to HAH, is likely to alter the metal binding site within the peptide sequence to the His residues adjacent to one another in sequence.

Studying several peptides analogous and a mononuclear metal center address possible alternatives. To improve the function of this or other systems, one can make systematic changes in the amino acid sequence, favoring greater stability, altering binding sites, or affecting catalytic activity as need for a particular purpose.

2.5 List of References


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CHAPTER 3: INVESTIGATING CATECHOLASE ACTIVITY OF A DESIGNED METALLOPEPTIDE MODEL SYSTEM

3.1. Introduction

Metalloenzymes regulate an array of biological activities including oxidases, oxygenases, dismutases, and nitrogenases.\(^1\) The fundamental biological functions of metalloenzymes have stimulated much interest into probing the nature of the metal-active site.\(^1,2\) One common approach to probing the active site(s) in metalloproteins is by the design of synthetic analogues.\(^3,4,5\) The design of protein or peptidic scaffolds with metal anchoring sites can give direct insights on fundamental aspects that dictate confirmations, structures, active sites, function, and mechanism in metalloproteins. Metalloprotein and peptidic scaffolds are designed to exhibit predicted structures and functions, based on our understanding of native proteins and using sequences that are not necessarily related to native proteins.\(^6,7\)

Copper proteins have attracted much attention in the field due to its unique spectroscopic features, and structural and mechanistic complexities, refer to Chapter 1.\(^1,2,8,9\) Copper-dioxygen complexes are of special interest when coupled with the oxidation of organic substrates.\(^10,11\) Synthetic analogs of dinuclear copper centers have been extensively studied as structural and functional models of type 3
copper centers, tyrosinase and catechol oxidase, refer to section 1.5. A number of model systems for catechol oxidase have been reported, where the dicopper center is typically coordinated to a nitrogen containing bi-nucleating ligands, and the oxidative activities monitored using 3,5-di-tert-butylcatechol (DTBC) as a substrate. The catechol oxidase activity was also reported for peptide systems, with histidine as a coordinating ligand for copper. Copper coordination to His imidazole side chains is found in the active site of a number of metalloproteins. Metal coordinating His residues are also found in metallopeptides associated with neurodegenerative diseases such as Alzheimer's and prion disease. Copper coordination to the histidine rich antimicrobial peptides histatins has been suggested, and shown to exhibit catecholase activity.

Herein, the oxidative activity of the metallopeptide was investigated toward the oxidation of catechol-containing substrates in the presence of a mono- and di-
Cu$^{2+}$ centers. The Cu-peptide complexes were shown to exhibit substrate specificity and oxidative activities following Michaelis Menten enzyme-like kinetics. A few fold increase in the catalytic efficiency was observed in the presence of a di-Cu$^{2+}$ center. However, substrate-binding studies suggest mononuclear catalysis.
3.2. Experimental

3.2.1. Materials

The investigated peptides, (Ac)-RHHPPHHHE-NH$_2$ and (Ac)-RHHPPHHHEF-NH$_2$ were purchased from GenScript. The substrates Catechol, THB, and 3,5-DTBC, and HEPES buffer were obtained from Sigma-Aldrich; 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate from Acros (Fairlawn, NJ); Methanol, CuSO$_4$, and sodium phosphate monohydrate were from Fischer Scientific. All aqueous solutions were prepared using deionized water with resistivity of 18.2-MΩ obtained from a Millipore Milli-Q system. Unless otherwise noted, all the studies were in 0.1 M HEPES pH 7.04 and 0.15M NaCl, small amount of Chelex resin was added to buffer stock solution to avoid metal contamination. DTBC containing assays were prepared in mixed solvent of 50:50 methanol to HEPES pH 7.04 due to low solubility of DTBC in buffer solvents.

3.2.2. Ligand Binding and Kinetic Studies

Ligand binding and kinetic measurements were carried out on a Varian CARY50 Bio UV-Vis spectrophotometer equipped with a temperature control, in a 1 cm path-length quartz cuvette. In a typical ligand (copper) binding assay, ligand is titrated into 300-600 μM of the apo peptide sample in increments ranging between 0.1-0.2 equivalents with respect to the peptide in 100 mM HEPES buffer pH 7.0, unless otherwise noted. Electronic spectra were collected for the apo peptide and
after each increment addition of the ligand, monitoring the increase in dd and or CT transition as a function of ligand concentration.

In a typical kinetic assay, a range of concentrations varying between 0.25 to 6 mM of substrate (catechol) is added to a fixed amount of catalyst, 10 μM, in HEPES buffer pH 7.0, unless otherwise noted. The change of absorbance over time, up to 3 minutes, was monitored at 500 nm as a result of o-quinone formation in the presence of MBTH, where catechol and dopamine have a molar absorptivity, $\varepsilon$, of 32,000 M$^{-1}$ cm$^{-1}$, and $\varepsilon = 17,230$ M$^{-1}$ cm$^{-1}$ for catechin, and $\varepsilon = 32,000$ M$^{-1}$ cm$^{-1}$ for epicatechin. The absorbance per minute readings were converted to rates in units of mM/s, and the rates were fitted to appropriate rate laws and rate constants by the use of Sigma Plot 10.0. Similar assays were carried out in the presence of fixed amount of catalyst and H$_2$O$_2$ and varying amount of substrate, and at fixed amount of catalyst and substrate but varying amounts of H$_2$O$_2$. In the presence of DTBC as a substrate, the reaction was monitored at $\lambda = 400$ nm, $\varepsilon = 1900$ M$^{-1}$ cm$^{-1}$.

3.3. Results and Discussion

3.3.1. Metal Binding

Spectrophotometric titrations of the peptide solution with copper were carried out to obtain information about copper binding and its affinity towards the peptide. Copper titration into apo peptide exhibits d–d transition at 600 nm, indicative of metal-peptide complex formation (Figure 3.1). A metal-to-ligand charge-transfer transition around $\lambda = 300$ nm is also observed as a result of increase
Figure 3.1: Optical titration of Cu$^{2+}$ into RHHPPHHE in HEPES pH 7.0 at 25°C.
in intensity as a function of metal concentration. The observed d-d transition around 600 nm is characteristic of a distorted octahedral geometry at the metal binding site.

The increase in absorbance as a result of metal titration can be fit into metal-ligand binding affording an affinity constant, $K_a$, of $4.8 \times 10^5$ M (Figure 3.1). The data suggest the presence of a 2-to-1 metal-to-peptide stoichiometry, which is further supported by the NMR studies from chapter 2.

3.3.2. Metallopeptide–Substrate Interactions

The formation of metallopeptide-substrate adducts is monitored by the use of DCC as a substrate. Spectrophotometric titrations of DCC into Cu–RHHPPHHE complex is monitored by changes in the electronic spectrum as a result of adduct formation with complex–DCC (Figure 3.2). In comparison to catechol, DCC exhibits a much larger redox potential owing to its electron withdrawing substituents, and thus DCC is difficult to oxidize. It is oxidized into its corresponding o-quinone about 200-fold slower than catechol with respect its $k_{cat}$, and may, thus, be used for binding studies. A 0.15 mM solution of diCu-RHHPPHHE was slowly titrated with DCC in HEPES buffer pH 7.0 at 25°C. Changes in the electronic spectrum as a result of DCC titration were monitored in a wavelength range of 200-800 nm. Upon DCC titration, the increase in intensity at ~ 410 nm is attributed to complex formation between DCC and diCu-RHHPPHHE complex, while the increase in intensity at ~ 300 nm is attributed to the presence of an increasing concentration of DCC (Figure 3.2).
**Figure 3.2:** Formation of metallopeptide–substrate adducts. (A) Electronic spectrum of DCC titration into diCu²⁺:RHHPPHHE complex (0.15 mM) in HEPES pH 7.0 at 25°C. Plot of the change in molar absorptivity as a function of DCC at 410 nm (B), and 300 nm (C). The data was fit into a 1-to-2 metallopeptide-to-substrate per Cu²⁺ site (solid line), and to the Hill equation (dotted line).
A plot of molar absorptivity at 410 nm versus the concentration of DCC titrated into the dicopper-peptide complex shows saturation at 2 equivalent of DCC affording $K_D$ of $0.27 \pm 0.09$ mM, (Figure 3.2, solid line). The data was fit into a 1–to–2 metallopeptide–to–substrate per Cu$^{2+}$ site. The spectra features are indicative of substrate binding, and the presence of a 1–to–1 stoichiometry of metal center–to–substrate.

Further, the plot appears to be sigmoidal in nature suggesting the possibility of a consecutive or cooperativity in the binding of DCC to the active sites. The data were fit to the Hill equation and afford a Hill coefficient, $\theta$, of 2.4 (Figure 3.2, dotted lines). Hence, the data may suggest positive cooperativity between the binding of DCC to the metal active site(s).

3.3.3. Oxidative Activities:

Oxidative activities of the complex towards catechol containing substrates were probed (Figure 3.3). The initial rates of the oxidation of catechol containing substrates were determined by monitored the change in absorbance over time at $\lambda_{max}$, as a result of o-quinone formation (Figure 3.4). Reactions were monitored at fixed complex concentration while varying substrate concentration. At low substrate concentration the reaction is first-order with respect to the substrate, and at higher concentrations the reaction is zero order and saturation is observed. The data are consistent with enzyme-like saturation kinetics. Catechol is oxidized aerobically in the presence of 1:1 and 2:1 Cu$^{2+}$–RHHPPHHE complex in HEPES pH 7.
Figure 3.3: Structures of select catechol containing substrates.
Figure 3.4: Schematic representation of catechol oxidation, in O$_2$ from air, into its corresponding o-quinone product.
at 25°C (Figure 3.5B). The observed catalytic activity towards catechol oxidation was approximately less than three-fold higher in the presence of two versus one coordinated copper ion(s) to the peptide, affording $k_{cat}/K_m$ of 38 and 14 M$^{-1}$s$^{-1}$, respectively. The activity of catechol oxidase from gypsywort (*Lycopus europaeus*) exhibits a catalytic efficiency of 32,000 M$^{-1}$s$^{-1}$ towards catechol oxidation,$^{26}$ and 917,200 M$^{-1}$s$^{-1}$ from *I. batatas*.$^{27}$ The reactions herein exhibited similar $K_m$ values of 0.24 ± 0.03 mM and 0.27 ± 0.2 mM, respectively. While, in the presence of one Cu$^{2+}$ center the reaction affords a $k_{cat}$ of $(3.7 ± 0.1) \times 10^{-3}$ s$^{-1}$, and a $k_{cat}$ of $(9.1 ± 0.2) \times 10^{-3}$ s$^{-1}$ in the presence of two Cu$^{2+}$ centers. Hence, the observed decrease in $K_m$ and increase in $k_{cat}$ may be attributed to a decrease in the substrate’s relative dissociation constant from the ES complex. The results herein, are not necessary consistent with the formation of a coupled dinuclear center as in the case of catechol oxidase and other model systems.$^{28,29,30}$ The oxidation of catechol to form o-quinone, by the diCu$^{2+}$ center, exhibits a $20 \times 10^5$ fold increase in rate acceleration with respect to $k_{cat}$, as compared to its aerobic auto-oxidation in the absence of catalyst, $k_o = 4.47 \times 10^{-7}$ s$^{-1}$. $^{31}$

In the presence of H$_2$O$_2$, significant increase in the catalytic efficiency of Cu–RHHPPHHE complexes towards catechol oxidation is observed (Figure 3.5A). The observed catalytic activity towards catechol oxidation, in the presence of a fixed H$_2$O$_2$ concentration, 34 mM, was approximately two-fold higher in the presence of two versus one coordinated copper ion(s) to the peptide, affording $k_{cat}/K_m$ of 72 and 28 M$^{-1}$s$^{-1}$, respectively. The reactions exhibited similar $k_{cat}$ values of $(3.3 ± 0.2) \times 10^{-2}$ s$^{-1}$, and $(2.6 ± 0.3) \times 10^{-2}$ s$^{-1}$, in the presence of 1:1 and 2:1 Cu–RHHPPHHE
Figure 3.5: Catechol Oxidation by 3μM diCu–RHPPHHEF (●) and Cu–RHPPHHEF (○) in HEPES pH 7.0 at 25°C. Reactions were carried out in the presence (A) and absence (B) of fixed 34 mM H₂O₂.
complex respectively (Figure 3.5A). To further analyze the role of H$_2$O$_2$, the concentration of catechol was fixed while varying the concentration of H$_2$O$_2$, while exhibits a saturation profile following enzyme-like kinetics. The data may suggests a similar mechanism to catechol oxidase$^{32,33}$ where the oxidation of catechol by Cu-RHHPPHHE was observed in the absence and presence of H$_2$O$_2$. This was further investigating as a bi-substrate mechanism to deduce the influence between the substrates, catechol and H$_2$O$_2$. The Hanes analysis allows the calculation of the substrates’ apparent binding equilibrium constants, and, and an intrinsic binding constant for one of the substrates.$^{34}$

For the bi-substrate reaction, the rate of catechol oxidation was determined at various H$_2$O$_2$ concentrations (Figure 3.6A). The data was further analyzed by Hanes plots of $[\text{Catechol}]/v_0$ versus [Catechol] for each of the H$_2$O$_2$ concentrations (Figure 3.6B), according to the following equation:

$$\frac{[\text{Catechol}]}{v_0} = \frac{1 + \frac{K_{\text{App}}^{\text{H}_2\text{O}_2}}{[\text{H}_2\text{O}_2]}}{V_{\text{max}}} [\text{Catechol}] + \frac{K_{\text{App}}^{\text{Catechol}}}{V_{\text{max}}} \left(1 + \frac{K_{\text{int}}^{\text{H}_2\text{O}_2}}{[\text{H}_2\text{O}_2]}\right)$$

(3.1)

where $v_0$ is the experimental velocity, and $V_{\text{max}}$ is maximum velocity. $K_{\text{Catechol}}^{\text{App}}$, and $K_{\text{H}_2\text{O}_2}^{\text{App}}$ are the apparent dissociation constants for catechol and H$_2$O$_2$, respectively. $K_{\text{H}_2\text{O}_2}^{\text{App}}$ is the intrinsic dissociation constant for H$_2$O$_2$ from its bound form. The constants are calculated from secondary plots of the slope and y-intercept versus
Figure 3.6: Saturation profiles and Hanes analysis of catechol oxidation by 2Cu$^{2+}$--RHPPHHHE in the presence H$_2$O$_2$. (A) Saturation plots in the presence of H$_2$O$_2$ fixed at 0, 1, 2, 4, 8, 16 mM, from bottom to top, respectively. (B) Re-plot of data from (A). (C) Re-plot of slope and y-intercept from (B) with respect to $1/[H_2O_2]$. Plot of [catechol]/Rate versus [Catechol] from (A).
1/[H₂O₂] (Figure 3.6C), according to the following equations:

\[
y - \text{intercept} = \frac{K_{\text{catechol}}^{\text{App}}}{V_{\text{max}}} + \left(\frac{K_{\text{catechol}}^{\text{App}}K_{\text{H₂O₂}}^{\text{Int}}}{V_{\text{max}}}\right) \frac{1}{[\text{H₂O₂}]} \quad (3.2)
\]

\[
slope = \frac{1}{V_{\text{max}}} + \left(\frac{K_{\text{H₂O₂}}^{\text{App}}}{V_{\text{max}}}\right) \frac{1}{[\text{H₂O₂}]} \quad (3.3)
\]

The data herein, afford \( K_{\text{catechol}}^{\text{App}} \) of \( 15.4 \times 10^{-3} \) M, of \( K_{\text{H₂O₂}}^{\text{App}} \) \( 1.5 \times 10^{-3} \) M, and \( K_{\text{catechol}}^{\text{Int}} \) of \( 1.5 \times 10^{-3} \) M. The calculated ratio of \( K_{\text{catechol}}^{\text{App}} / K_{\text{H₂O₂}}^{\text{Int}} \) is 1.7 and 1 for \( K_{\text{H₂O₂}}^{\text{App}} / K_{\text{H₂O₂}}^{\text{Int}} \) suggest the presence of an interaction between the two substrates.³⁵

**Job Plot:** The interaction or preferred stoichiometry between the metallopeptide complex and the substrate, catechol, is studied by the Job plot method (Figure 3.7). Job plot is a continuous variation method for determining the optimum interaction or stoichiometry of metal-to-ligand (optical) or catalyst-to-substrate (mechanistic) ratios.³⁶,³⁷ It has been previously used in determining metal-to-drug stoichiometry in complex systems, among others.³⁸ Herein, in the mechanistic Job plot, the reaction rate as a function of complex mol fraction \( (X_c) \) or substrate mol fraction \( (X_s) \) was measured with a constant total concentration of \( ([C] + [S]) \) of 50 μM. The concentration of complex and substrate was varied, while keeping the overall total concentration constant at 50 μM. The reaction rate reaches
Figure 3.7: Mechanistic Job plot at a constant 50 μM concentration of Catechol and complex in HEPES pH 7.0 at 25°C. (A) Cu-RHHPPHHE. (B) diCu-RHHPPHHE complex in the presence (●) and absence (○) of 6.4 mM H₂O₂.
a maximum in the plot reflecting the interaction or stoichiometry of the complex–substrate in solution. The investigation of the reaction mechanism by the Job plot method is feasible due to the presence of equilibrium between the substrate and complex to form the complex-substrate complex during the pre-equilibrium stage of catechol oxidation. The Job plot will reflect a 1:1, or 2:1 complex-to-substrate stoichiometry for reactions following mononuclear or dinuclear mechanisms, respectively. The observed rate of catechol oxidation was plotted against the mole fraction of the complex, \(X_c\), \([\text{complex}]/([\text{complex}]+\text{catechol})\). In the presence of a mononuclear Cu\(^{2+}\) site in Cu–RHHPPHHE complex, the plot shows an increase in the rate of the reaction, which reaches a maximum at \(X_c \sim 0.5\), followed by a decrease (Figure 3.7A). The data suggest interaction between the substrate and complex in a 1:1 ratio, reflecting mononuclear catalysis.

In the presence of two Cu\(^{2+}\) equivalents per peptide, a maximum rate is observed on the mechanistic Job plot around \(X_c \sim 0.33\) (Figure 3.7B). In the presence of a dinuclear center, the dicopper center would interact with a single substrate, and would show an \(X_c \sim 0.5\) in a Job plot. Herein, the observed \(X_c \sim 0.33\) is indicative of a single complex interacting with two substrates suggesting the presence of two mononuclear active sites, each bound to a single substrate. This is may suggest two mononuclear active site with a different proposed mechanism from that of catechol oxidase, where two substrates are bound to the diCu active site per catalytic cycle. In the presence of \(H_2O_2\), the Job plot exhibits a maximum at \(X_c \sim 0.33\), which again indicates a 1:1 stoichiometry between catechol and each active site in diCu–RHHPPHHE complex, reflecting mononuclear catalysis. The data
verifies the stoichiometry obtained from DCC binding. The observed binding stoichiometry is comparable to the metallopeptide antibiotic bacitracin showing a 1:1 catechol–(Cu–bacitracin) stoichiometry, and suggesting mononuclear catalysis.\textsuperscript{39}

\textit{Substrate Specificity:} Specificity towards substrates presents a topic of interest in enzymes. In catechol oxidase, only \textit{ortho}-catechols are oxidized into their corresponding quinone product.\textsuperscript{40} The oxidative activities of the complexes studied herein were monitored against different catechol containing substrates. In the presence of a 1:1 or 2:1 Cu–peptide complex, the oxidative activity was monitored in the presence of dopamine, catechin, epicatechin, or DTBC as substrate. Similar to the observed reaction in the presence of catechol as a substrate, the reactions herein exhibit saturation profile at higher concentrations of substrates consistent with pre-equilibrium kinetics.

The oxidation of dopamine in the presence of a single Cu\textsuperscript{2+} center in Cu\textsuperscript{2+}–RHHPPHHEF, affords a second-order rate constant \( k_{\text{cat}}/K_m \) of 4 M\textsuperscript{−1}s\textsuperscript{−1}, and about three-fold increase to 11 M\textsuperscript{−1}s\textsuperscript{−1} in the presence of two Cu\textsuperscript{2+} centers (Figure 3.8A). This can be compared to a two-fold increase in the presence of catechol as a substrate, which may suggest a slight cooperativity between the metal centers in the presence of dopamine as a substrate. A similar \( K_m \) value is observed for catechol and dopamine oxidation by the diCu\textsuperscript{2+}–peptide complex, 0.24 and 0.30 mM, and a slightly higher \( k_{\text{cat}}/K_m \) of 14 and 11 M\textsuperscript{−1}s\textsuperscript{−1}, respectively. In the presence of \( \text{H}_2\text{O}_2 \), 32 mM, an increase in the catalytic efficiency of the diCu–peptide is observed towards dopamine oxidation, affording \( k_{\text{cat}}/K_m \) of 40 M\textsuperscript{−1}s\textsuperscript{−1}, which exhibits a two-fold
Figure 3.8: Saturation profiles of (A) Dopamine, (B) catechin, (C) epicatechin, and (D) DTBC oxidation by 3 μM Cu²⁺–RHHPPHHE in 1:1 (●) and 2:1 (○) ratios in HEPES pH 7.04* at 25°C. *DTBC oxidation was carried out in 50:50 MeOH:HEPES pH 7.04 due to the low solubility of DTBC water.
decrease as compared to catechol as a substrate, under the same conditions. The obtained kinetic parameters are summarized in (Table 3.1).

Catechin and epicatechin are stereoisomers of one another, and may give some insight regarding any stereo specificity towards the active sites. Catechin is efficiently oxidized into its o-quinone product by the metallopeptide complexes (Figure 3.8B). In the presence of a diCu\(^{2+}\) complex, the second-order rate constants affords a four-time increase in catalytic efficiency as compared to the presence of a single mononuclear Cu\(^{2+}\) site, \(k_{\text{cat}}/K_m\) of 50 versus 12 M\(^{-1}\)cm\(^{-1}\), respectively. The observed increase in catalytic efficiency in the presence of a diCu complex may suggest influence between the Cu\(^{2+}\) sites towards catechin oxidation. The observed \(K_m\) value in the presence of a diCu\(^{2+}\) center is a three-fold smaller in reference to a mono-Cu\(^{2+}\) center. Catechin oxidation by the diCu\(^{2+}\) complex exhibits four to five-fold increase in catalytic efficiency as compared to catechol oxidation, under the same conditions, suggesting an increase in substrate specificity towards catechin.

Similarly, epicatechin oxidation into its corresponding o-quinone product by mono- and di- Cu\(^{2+}\)-RHHPPHHEF complexes exhibits saturation profile consistent with pre-equilibrium like kinetics (Figure 3.8B). In the presence of a diCu\(^{2+}\) complex, epicatechin exhibits about a two-fold increase in catalytic efficiency as compared to its stereoisomer, catechin, \(k_{\text{cat}}/K_m\) of 86 versus 50 M\(^{-1}\)s\(^{-1}\), respectively. A list of kinetic parameters is presented in Table 3.1. Epicatechin oxidation by the complex affords a \(K_m\) of 0.10 mM, as compared to 0.14 mM for catechin. The data
Table 3.1: Kinetic parameters of observed oxidative activities against various catechol-containing substrates catalyzed by 1:1 and 2:1 Cu²⁺–RHHPPHHE complexes in HEPES pH 7.04 at 25°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cu:RHHPPHHE</th>
<th>$k_{cat}/K_m$ (M⁻¹s⁻¹)</th>
<th>$k_{cat}$ (s⁻¹)</th>
<th>$K_m$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Catechol</td>
<td>1:1</td>
<td>14 (3.7 ± 0.1) x 10⁻³</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>38 (9.1 ± 0.1) x 10⁻³</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1:1*</td>
<td>28 (2.6 ± 0.3) x 10⁻²</td>
<td>0.94</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1*</td>
<td>72 (3.3 ± 0.2) x 10⁻²</td>
<td>0.47</td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>1:1</td>
<td>4 (2.0 ± 0.1) x 10⁻³</td>
<td>0.52</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>11 (3.2 ± 0.0) x 10⁻³</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1*</td>
<td>40 (3.3 ± 0.8) x 10⁻²</td>
<td>0.84</td>
<td></td>
</tr>
<tr>
<td>Catechin</td>
<td>1:1</td>
<td>12 (4.7 ± 0.2) x 10⁻³</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>50 (7.0 ± 0.2) x 10⁻³</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Epicatechin</td>
<td>1:1</td>
<td>10 (3.7 ± 0.3) x 10⁻³</td>
<td>0.44</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1</td>
<td>86 (9.0 ± 0.2) x 10⁻³</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>DTBC</td>
<td>1:1*</td>
<td>54 (7.6 ± 0.0) x 10⁻²</td>
<td>1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2:1*</td>
<td>191 (8.2 ± 0.0) x 10⁻²</td>
<td>0.43</td>
<td></td>
</tr>
</tbody>
</table>

* In the presence of fixed H₂O₂ concentration of 32 mM.

* In 50:50 MeOH:HEPES pH 7.04.
does not suggest any stereo selectivity of the complex towards catechin or epicatechin oxidation. While, the large catalytic efficiency observed herein suggests substrate specificity towards catechin and epicatechin as compared to catechol and dopamine. The data may suggest the presence of influence between the two metal centers, as evident by more than eight-fold increase (10 to 86 M\(^{-1}\)s\(^{-1}\)) in catalytic efficiency in the presence of a diCu\(^{2+}\) center. The differences in catalytic efficiency may also be attributed to the two active sites acting differently, where a monomeric reaction mechanism is suggested based on DCC binding and Job plot. Due to the presence of an influence between the two monomeric metal active sites, the observed activity is not additive to the activity of two monomeric sites, as observed herein. The catalytic efficiency increases a few fold in the presence of two versus one metal center (Table 3.1).

Since variations in the observed kinetic parameters using epicatechin, catechin, and dopamine as substrates may also be attributed to structural differences, a different substrate was necessary to clearly point out any influence between the metal centers. Further, in the presence of catechol as a substrate, the \(k_{\text{cat}}/K_m\) in the presence of one versus two metal centers was slightly over a two-fold increase, 14 and 38 M\(^{-1}\)s\(^{-1}\), respectively. The catechol oxidase activity in copper containing model systems has been determined primarily by the oxidation of DTBC.\(^{41,42}\) Thus, 3,5-DTBC was used as a substrate, despite the slightly different conditions due to its low solubility in water. In the presence of 3,5-DTBC, reactions were carried out in 4:1 MeOH:HEPES buffer pH 7.04, where as all other reactions were carried out in HEPES buffer 7.04. The formation of the \(o\)-quinone product,
DTBQ, was monitored ~ 408 nm. The mono- and di-Cu\(^{2+}\) complexes of catalyzed the formation of DTBQ very efficiently, exhibiting k\(_{\text{cat}}\)/K\(_{m}\) of 54, and 191 M\(^{-1}\) s\(^{-1}\), respectively (Figure 3.8D). About a four-fold increase in catalytic efficiency is observed in the presence of a diCu\(^{2+}\) center. The data may suggest the presence of an influence between the two sites in catalyzing DTBC oxidation. The activity of the studied metallopeptide is higher than those of other simple model systems.\(^{43}\)

The oxidative activities presented herein exhibit an increase in catalytic efficiency, k\(_{\text{cat}}\)/K\(_{m}\) in the presence of a diCu\(^{2+}\) center (Table 3.1). The reactions afford a smaller Michaelis Menten constant, K\(_{m}\), for the diCu\(^{2+}\) center as compared to the mono-Cu\(^{2+}\) center, and a larger k\(_{\text{cat}}\) value. The decrease in K\(_{m}\) implies a higher relative substrate affinity to the ES complex, whereas the increase in k\(_{\text{cat}}\) is associated with an increase in S turnover into P. Since K\(_{m}\) is a combined term, K\(_{m}\)= (k\(_{-1}\)k\(_{\text{cat}}\))/k\(_{1}\), the decrease in K\(_{m}\) and increase in k\(_{\text{cat}}\) may also be associated with a decrease the relative substrate dissociation constant of the ES complex, k\(_{d}\)=k\(_{-1}\)/k\(_{1}\).

The catecholase activity of model systems is often monitored using 3,5-DTBC as a substrate. The low redox potential of DTBC makes it easily oxidized and monitored at \(\lambda_{\text{max}}\) 400 nm. The bulkier tert-butyl groups prevent side reactions and polymerization of the resulting quinone.\(^{44}\) A list of kinetic parameters of various model systems is shown in Table 3.2. The ligand environment and various conditions experimental conditions including temperature, pH, solvent, and degree of oxygenation are important factors contributing to the overall activity of the different model systems.
Table 3.2: Comparison of kinetic parameters for catechol* and DTBC oxidation by catechol oxidase model systems.

<table>
<thead>
<tr>
<th>Complex</th>
<th>$k_{\text{cat}}$ (s$^{-1}$)</th>
<th>$K_m$ (mM)</th>
<th>$k_{\text{cat}}/K_m$ (M$^{-1}$s$^{-1}$)</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$-Aβ$_{20}$</td>
<td>0.41</td>
<td>0.78</td>
<td>526</td>
<td>HEPES pH 7.0</td>
</tr>
<tr>
<td>Cu$^{2+}$-Aβ$_{20}$ *</td>
<td>0.15</td>
<td>0.35</td>
<td>429</td>
<td>HEPES pH 7.0</td>
</tr>
<tr>
<td>Cu$^{2+}$-Bacitracin*</td>
<td>0.007</td>
<td>3.3</td>
<td>2</td>
<td>HEPES pH 7.0</td>
</tr>
<tr>
<td>Cu$^{2+}$-Histatin*</td>
<td>0.011</td>
<td>0.59</td>
<td>186</td>
<td>HEPES pH 7.0</td>
</tr>
<tr>
<td>Cu$^{2+}$-SgAP</td>
<td>1.45</td>
<td>0.44</td>
<td>3295</td>
<td>HEPES pH 7.0</td>
</tr>
<tr>
<td>Cu$^{2+}$-P1</td>
<td>0.065</td>
<td>0.16</td>
<td>406</td>
<td>MeOH:MES pH 6.0</td>
</tr>
<tr>
<td>[Cu$_2$((22)pr4pz)(CO$_3$)(H$_2$O)$_2$]$_2$</td>
<td>0.12</td>
<td>0.18</td>
<td>667</td>
<td>MeOH</td>
</tr>
<tr>
<td>[Cu$_2$(L$_1$)(OH)(H$_2$O)(EtOH)]$^+$</td>
<td>0.059</td>
<td>0.24</td>
<td>246</td>
<td>MeOH</td>
</tr>
<tr>
<td>([Cu$_2$(L$_2$)(CF$_3$SO$_3$)]$_2$</td>
<td>0.033</td>
<td>2.9</td>
<td>11</td>
<td>MeOH</td>
</tr>
<tr>
<td>1-[CuBMPM(OAc)$_2$]$^+$</td>
<td>0.25</td>
<td>6.4</td>
<td>39</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>[Cu$_2$BMPM(μ-OH)]$^{2+}$</td>
<td>0.024</td>
<td>1.5</td>
<td>16</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>[Cu$_2$L$_1$(μ-OH)]$^{2+}$</td>
<td>0.006</td>
<td>8.8</td>
<td>1</td>
<td>CH$_3$CN</td>
</tr>
<tr>
<td>Catechol oxidase*</td>
<td>2293</td>
<td>2.5</td>
<td>917,200</td>
<td>Phosphate buffer pH</td>
</tr>
</tbody>
</table>

* Catechol as a substrate.

Abbreviations: P1: 4-vinylpyridine (4Vp) and acrylamide (Ac) with an average repeating unit of 4Vp$_3$Ac$_4$. SgAP: Streptomyces griseus aminopeptidase. L$_2$OH: 1,3-bis{N,N-bis(2-[2-Pyridyl]ethyl)}amino-2-hydroxypropane. pr4pz: (9,22-dipropyl-1,4,9,14,17,22,27,28,29,30-decaazapentacyclo[22.2.1.1$^{4,7}$,1$^{11,14}$,1$^{17,20}$]triacontane-5,7(28),11(29),12,18,20(30),24(27),25-octaene). H-BPMP: 2,6-Bis[bis{pyridin-2-ylmethyl-amino}methyl]-4-methylphenol. HL$_1$: 2,6-bis{bis[2-pyridylmethyl]aminomethyl}-4-fluorophenol. HL$_2$: 4-bromo-2,6-bis(4-methylpiperazin-1-ylmethyl)phenol.
As compared with other catecholase model systems (Table 3.2), the studied complex herein exhibits moderate catalytic efficiency. When compared to Cu$^{2+}$–Bacitracin which exhibits a mononuclear reaction mechanism$^{39}$, the diCu$^{2+}$ complex herein affords a significant increase in catalytic efficiency, 2 vs 38 M$^{-1}$ cm$^{-1}$, respectively.

3.4 Concluding Remarks

The successful design of a folded de novo octamer is achieved. The peptide is folded, binds diCu(II), exhibits oxidative activities, and to the best of my knowledge, it represents the first designed $\beta$-turn minimalistic peptide with a diCu(II) center. Structure-function studies on its oxidative activities show that in the presence of a dinuclear copper center, the metallopeptide shows an clear influence between the two metal centers as evident by the enhancement in activity as compared to reaction catalyzed by a mononuclear copper center. The minimalistic design and the incorporation of di-Cu centers in a characteristic $\beta$-turn structure promote the observed influence between two copper centers.

Incorporating an amino acid between the di-His site, HH to HAH, is likely to alter the metal binding site within the peptide sequence to the His residues adjacent to one another in sequence, which may in turn alter the oxidative activities. Studying several peptides analogous and a mononuclear metal center address possible alternatives. To improve the function of this or other systems, one can
make systematic changes in the amino acid sequence, favoring greater stability, altering binding sites, or affecting catalytic activity as need for a particular purpose.

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16380–16381.


CHAPTER 4: STRUCTURE-FUNCTION CORRELATIONS OF PHOSPHATE CONTAINING INHIBITORS TOWARDS THE OXIDATIVE ACTIVITY OF METALLO-\(\text{A}\beta\) COMPLEX: KINETICS AND \(^{31}\text{P}\) NMR RELAXATION STUDIES

4.1 Introduction—Metallo–Beta Amyloid in Alzheimer’s disease

Alzheimer’s disease is the most common cause of dementia in elderly. It is an irreversible and progressive brain disease that destroys memory and thinking skills ultimately leading to neuronal death. Characterized by the formation of senile amyloid plaques and neurofibrillary tangles, the onset of the disease is believed to occur years prior to its diagnosis.\(^1\)\(^,\)\(^2\)\(^,\)\(^3\) Deposition of beta-amyloid (\(\text{A}\beta\)) plaques in an AD brain is associated with the pathology of the disease.\(^4\)\(^,\)\(^5\) The main constituent of the amyloid plaques is \(\text{A}\beta\) peptide.\(^6\)\(^,\)\(^7\) Hence, \(\text{A}\beta\) is one of the most studied molecules in AD research. However, the cause and effect of amyloid plaques in AD is still under debate.\(^8\)\(^,\)\(^9\) Among other suggested hypotheses are genetic defects\(^10\), free radical mediated processes\(^11\),\(^12\),\(^13\) defective membrane metabolism\(^14\), and trace element-induced neurotoxicity.\(^15\),\(^16\) In 2013, the Alzheimer’s Association reported an estimate of 5.2 million Americans affected with the disease, 5 million over the age of 64. This number is expected to rise to 7.1 million by 2025. In an effort to find a cure, many researchers have focused on the amyloid cascade of the disease.
Senile plaques found in the hippocampus and neocortical regions in brains of AD patients are aggregates of Aβ, a 4.3-kD peptide part of a much longer transmembrane protein detained in chromosome 21. Aβ is a 40-42 amino acid peptide (DAEFRHDSGY_{10} EVHHQKLVF_{20} AEDVGSNKGA_{30} IIGLMVGGVV_{40} IA) formed by proteolytic cleavage of the amyloid precursor protein (APP) by β- and Y-secretase. APP is a transmembrane glycoprotein expressed at high levels in the brain. The N-terminus domain of Aβ is rich in hydrophilic residues, while the C-terminus domain is rich in hydrophobic residues. Under physiological conditions Aβ_{42} forms neurotoxic β-sheet secondary structure. The Aβ_{20} fragment of the full length peptide forms α-helical or unordered structure depending on the conditions.

The primary component of Aβ in cerebrospinal fluid is the free soluble Aβ_{1-40}, present at a concentration of 5 nM. The primary component in amyloid plaques is Aβ_{1-42} peptide aggregates suggested to induce oxidative stress and neurotoxicity, which has been studied extensively. Formation of Aβ aggregates and its accumulation as plaque deposits in AD brain is also affected by transition metal ions, copper, zinc, and iron, an area of intense focus in AD research.

High cytoplasmic concentrations of free metal ions are toxic. Physiological pathways have thus evolved to accurately transport and distribute free metal ions to their target proteins, a process that was shown to be less efficient with aging. Metal ions are proposed to play a central role in the pathogenesis of the disease, where upon binding, metal ions induce Aβ toxicity. Amyloid senile plaques from
AD brain subjects were found to contain 25.0 ± 7.8 μg/g of copper, 69.0 ± 18.4 μg/g of zinc, and 52.5± 13.7 μg/g of iron.40 Aβ aggregates are induced by metal ions and found to contain up to millimolar amount of Cu²⁺ (0.4 mM), Zn²⁺ (1 mM), and Fe³⁺ (1 mM).41,42

Since Aβ aggregation is enhanced in the presence of metal ions and in order to shed light onto the pathology of the disease, many researchers have focused on understanding the metal binding site and its associated chemistry. The coordination environment of transition metal ions to Aβ has been extensively studied.43,44 The metal binding site is located within the N-terminus domain of Aβ where metal-anchoring histidine residues are found. It has been shown that Cu²⁺−Aβ forms a stable complex around physiological pH with the N-terminal amino group of Asp-1 and the side chain imidazole group of His-6, His-13, and His-14 as the anchoring ligands for Cu²⁺ adopting a distorted square planar geometry.45 The interaction of either Asp-1, Glu-11 or water molecule with the metal center has been suggested. 46 Electronic spectrum of Cu²⁺−Aβ exhibits a typical tetragonally distorted octahedral environment with a λ_{max} 600 nm of ε ~100 M⁻¹cm⁻¹ consistent with many Cu⁺² complexes in solution.47 The ^1H NMR spectra of the Co²⁺−Aβ₂₀, where Co²⁺ is used as a paramagnetic shift reagent and close in coordination to the Cu²⁺ adduct, revealed Co²⁺ coordination to imidazole NH protons of His-6, His-13, and His-14, in d₆−Me₂SO. 47 The NMR study was consistent with Raman spectroscopic studies of metal coordination to the three His residues.48 Cu²⁺−Aβ forms a stable complex affording a dissociation constant of 10⁻¹⁰ to 10⁻¹¹ M.49
The hydrophobic region within the C-terminus residues 29-42, is not involved in metal binding.\textsuperscript{50,51} The binding of Cu\textsuperscript{2+} binding to the full-length peptide A\textsubscript{\beta}\textsubscript{40} was essentially identical to fragments of A\textsubscript{\beta} (A\textsubscript{\beta}\textsubscript{16}, A\textsubscript{\beta}\textsubscript{20}, and A\textsubscript{\beta}\textsubscript{28}) suggesting that the metal anchoring sites are within the N-terminal region of the peptide.\textsuperscript{52, 53}

Neurotoxicity of A\textsubscript{\beta} as a result of its aggregation, metal binding, and oxidative stress\textsuperscript{54} has been studied quite extensively and linked to AD pathology.\textsuperscript{55} Redox-active divalent transition metal ions have been associated with oxidative stress and generation of ROS including hydrogen peroxide, superoxide, and hydroxyl radicals.\textsuperscript{41} The chemistry of redox-active metallo-A\textsubscript{\beta} complexes and its induced oxidative stress has been an area of intense focus in AD research. A\textsubscript{\beta} coordinated to Cu\textsuperscript{2+} and Zn\textsuperscript{2+} have been extracted from post-mortem AD brains.\textsuperscript{56} The regulation of the redox activity of metal ions is essential for the biological activity of numerous enzymes, whereas its misregulation is closely associated with a number of neurodegenerative diseases including AD, Wilson’s disease, and Menken’s disease.\textsuperscript{57}

A\textsubscript{\beta} induces oxidative stress when bound to copper and iron\textsuperscript{58} associated with generation of reactive oxygen species including hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})\textsuperscript{59}, superoxide (O\textsubscript{2}\textsuperscript{-}), and hydroxyl radical (OH\textsuperscript{-}) via Fenton chemistry\textsuperscript{60} (Cu\textsuperscript{+} + H\textsubscript{2}O\textsubscript{2} → Cu\textsuperscript{2+} + OH\textsuperscript{-} + OH\textsuperscript{-} or Fe\textsuperscript{2+} + H\textsubscript{2}O\textsubscript{2} → Fe\textsuperscript{3+} + OH\textsuperscript{-} + OH\textsuperscript{-}). In the presence of copper and iron, A\textsubscript{\beta}\textsubscript{40} was shown to reduce Cu\textsuperscript{2+} to Cu\textsuperscript{+} and similarly Fe\textsuperscript{3+} to Fe\textsuperscript{2+} and generate H\textsubscript{2}O\textsubscript{2}.\textsuperscript{61,62,63,64} The presence of redox active metals in the more reduced oxidation state (Cu\textsuperscript{+} and or Fe\textsuperscript{2+}) and H\textsubscript{2}O\textsubscript{2} would lead to conversion of H\textsubscript{2}O\textsubscript{2} to hydroxyl radicals via Fenton chemistry. Neurotoxicity of A\textsubscript{\beta} may be a result of ROS.
generation in the form of free radicals or $\text{H}_2\text{O}_2$. The imbalance of redox-active 
Cu$^{2+}$ and Fe$^{3+}$ in AD brain has been associated with an increase in lipid 
peroxidation, protein and DNA oxidation. In efforts to treat and prevent 
ROS-mediated damage, recent focus in AD chemistry has been correlating metal-
centered pathways to the homeostasis of AD.

Further, aggregates of Aβ are directly linked to its cell toxicity, where a 
number of studies focused on investigating the neurotoxicity of a range of Aβ$_{40}$ 
peptide fragments. Formation of stable neurotoxic Aβ aggregates within the C-
terminus regions of Aβ$_{40}$ peptide, amino acids 25-35 and 29-35 have been 
previously reported. Understanding the nature of aggregation and oxidative stress 
within AD and the (bio)chemistry of the metals involved in amyloidogenesis may 
thus shed light on the disease.

In enzymatically controlled pathways or biological systems there are 
mechanisms to protect against oxidative stress. Misregulation of such systems 
leads to oxidative stress. In the presence of an oxidizing agent such as hydrogen 
peroxide a compound can be oxidized leading to the formation of a new unfavorable 
reactive product, reactive oxygen species. Oxidative stress associated with the 
pathology of the disease is linked to the presence of metal ions in senile plaques of 
AD brain. Copper has received considerable attention in AD research due to its 
presence in high concentrations bound to Aβ, and due to its involvement in redox 
cycling and generation of ROS. Copper binds monomeric Aβ peptide with an
affinity constant of $10^9$–$10^{10}$ M$^{-1}$. However, the driving force for copper accumulation in senile plaques remains unclear.

In an effort to understand some of the chemical processes of metallo-Aβ, the redox-chemistry of Cu$^{2+}$-Aβ in the presence and absence of H$_2$O$_2$ was previously investigated showing metal-centered pre-equilibrium kinetics towards the oxidation of catechol and catecholamine substrates consistent with the mechanism of Type-3 copper oxidases, catechol oxidase.$^{81, 82, 83}$ Cu$^{2+}$–Aβ complex was also shown to exhibit double-stranded oxidative plasmid DNA cleavage.$^{81}$ The rates of the observed oxidative activities are significant and add to the importance of continuous research into defining the role of metals in the pathology of the disease. Antioxidants$^{84}$ with metal chelating ability may serve as inhibitors of the redox activity of metallo-Aβ, which in turn may attenuate its neurotoxicity. Studies on the structural and functional roles of metal ions in Alzheimer’s disease, particularly of metallo-Aβ aggregation and plaque formation, and oxidative stress may shed light on the pathology of the disease. The search for therapeutics in AD research is increasing as the number of people affected with this disease is at rise.

Binding of metal ions to Aβ induces amyloid plaques formation and oxidative stress, a driving force to the pathogenesis of the disease.$^{85, 86}$ Metallo-Aβ has thus been a target in AD therapeutics.$^{87}$ Potential drug candidates include metal chelators and small molecules capable of binding to the active site of metallo-Aβ, thereby, inhibiting the redox activity of bound copper and iron ions. Another important factor is the ability of the drug to cross the blood brain barrier, which
generally requires a small hydrophobic molecule. Recent data suggest an important role of the blood brain barrier in AD therapeutics.\textsuperscript{88} Clioquinol,\textsuperscript{89} which crosses the blood brain barrier, is an antibiotic shown to be potent against Aβ deposition in transgenic mice models.\textsuperscript{90} with metal chelating ability towards Cu and Zn.\textsuperscript{87,91,92} Despite its potent effects, phase II/III of clinical trails were stalled due to the presence of toxic, di-iodo 8-hydroxy quinolone, impurity upon large-scale chemical synthesis.\textsuperscript{93,94} Metal chelating compounds are among promising therapeutics in AD research, and are emerging as important drug targets for a number of diseases associated with misregulation of metal ions.\textsuperscript{95,96,97} The oxidative activities of Cu\textsuperscript{2+}–Aβ complexes toward neurotransmitters and DNA cleavage have been demonstrated. Inhibition of the oxidative reactions may attenuate the observed oxidative stress in AD, providing an alternative therapeutic or prevention of the disease.

Vitamin B6 compounds are potent inhibitors against ROS species, suggested to inhibit tyrosinase activity by scavenging ROS species.\textsuperscript{98} Vitamin B6 exists in three different forms: pyridoxamine (PM), pyridoxine (PN) and pyridoxal (PL) (Figure 4.1). All three forms are eventually converted to the active form pyridoxal 5-phosphate (PLP) by pyridoxal kinase and are utilized as a cofactor for over 140 enzymes,\textsuperscript{99} some of which are involved in amino acid and monoamine neurotransmitter synthesis.\textsuperscript{100} In the methionine/glutathione transsulfuration pathway B6 converts homocysteine into cysteine to ultimately produce glutathione, which is an important reducing agent in humans. In addition to its regulatory roles, vitamin B6 has also been shown to serve as an antioxidant.\textsuperscript{101,102}
Figure 4.1: Structure of vitamin B6 compounds: PM, PN, PL, and PLP.
Nutritional deficiency of vitamin B6 can lead to insufficient insulin and altered hormone production. Its recommended daily intake is 2 mg and can be obtained from various vegetables, fish, and some fruits, whereas its tolerable daily upper limit is 100 mg for adults set by the US FDA, 25 mg set by the EU SCF, and 10 mg in the UK. In transgenic mouse models, nutritional deficiency of vitamins B6, B9, and B12 has been linked to an increased risk of AD. Herein, the research goal focused on screening B6 compounds and other naturally occurring compounds as potential therapeutics or inhibitors targeting metallo-Aβ induced oxidative stress. Monitoring the degree potency, of selected compounds, as inhibitors against Cu²⁺-Aβ catalyzed oxidation of catechol and derivatives to their corresponding o-quinone products will allow determination of the inhibition constant in vitro. Further, a structure-function correlation between various inhibitors may give insight for further drug design and discovery. The effect of phosphate derivatives of B6 compounds were similarly investigated to probe the role of the phosphate moiety against Cu²⁺-Aβ catalyzed oxidation of catecholamine.

Further, the role of (bis)phosphate moieties in various naturally occurring compounds as potential inhibitors against the oxidative stress caused by Cu²⁺-Aβ complexes will be discussed. The binding of phosphate, and phosphate moieties to positively charged side chain residues, such as, arginine and lysine has been suggested in literature. In metallo-Aβ, Arg-5 and Lys-16 are in close proximity to the metal center, and it is plausible that mutations of Arg-5 and or Lys-16 may attenuate the overall activity of the inhibitor. Herein, to gain further insight on the interaction of phosphate moieties with metallo-Aβ₂₀, two mutants of Aβ₂₀
substituting Arg-5 and Lys-16 by Ala were synthesized. The amino acid sequence of the Arg-5 mutant, $Aβ_{20(R5A)}$, is DAEFAHDSGY$_{10}$ EVHHQKLVFF$_{20}$, while the Lys-16 mutant, $Aβ_{20(K16A)}$, has the following sequence DAEFRHDSGY$_{10}$ EVHHQALVFF$_{20}$.

The mutants designed herein serve as probes for possible charge interactions between phosphate and the side chains of Arg-5 and Lys-16 in $Aβ_{20}$.

Phosphate moieties within the studied compounds may exhibit electrostatic interactions with the side chains of Arg and Glu residues. Consequently, mutations of Arg or Glu residues may shed light on the interaction of phosphate with (metallo)-$Aβ$. Mutations of Arg and Glu residues close to the metal binding site of $Aβ_{20}$ may also affect the redox activity of $Cu^{2+}$–$Aβ_{20}$ complexes. $Cu^{2+}$–$Aβ$ complexes were previously shown to catalyze the oxidation of catechol containing substrates to their corresponding o-quinone products.$^{81}$ Herein, the ability of $Cu^{2+}$–$Aβ$ complexes to catalyze the oxidation of catechol containing substrates was used as means of evaluating the effectiveness of various compounds as inhibitors against the observed oxidation.

4.2 Experimental

4.2.1 Materials

The 1–20 fragments of $Aβ$, and its Arg-5 to Ala-5 mutant, $Aβ_{20(R5A)}$, and Lys-16 to Ala-16 mutant, $Aβ_{20(K16A)}$, were synthesized at the Peptide Center of the University of South Florida. The identities of the peptides have been confirmed with
a Bruker matrix-assisted laser desorption ionization time-of-flight mass spectrometer. Vitamin B6 compounds pyridoxamine, pyridoxine, and pyridoxal, and the substrates dopamine, THB, and DTBC, and HEPES buffer were obtained from Sigma-Aldrich; 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate from Acros (Fairlawn, NJ); methanol, CuSO$_4$, pyridoxal-5-phosphate, and sodium phosphate monohydrate were from Fischer Scientific. ATP, ADP, NADPH, thiamine, TPP, phosphate, pyrophosphate were purchased from Sigma-Aldrich. All aqueous solutions were prepared using deionized water with resistivity of 18.2-MΩ obtained from a Millipore Milli-Q system. Unless otherwise noted, all the studies were in 0.1 M HEPES pH 7.4 with a small amount of Chelex resin to avoid metal contamination. DTBC containing assays were prepared in mixed solvent of 50:50 methanol to HEPES pH 7.4 due to low solubility of DTBC in buffer solvents.

4.2.2 Substrate Oxidation and Inhibition Assays

All spectra were acquired on a Varian CARY50 Bio-UV-Vis spectrophotometer in a 1 cm path length cuvette at 25°C. Solutions were freshly prepared for each experiment. In a typical assay, several concentrations of substrate (Dopamine, THB, or DTBC) ranging from 0.1 to 14.0 mM were incubated with a constant concentration of Cu$^{2+}$–Aβ (varied between 2-17 µM) and buffered with 0.1 M HEPES pH 7.4 in a final volume of 1.0 mL. The formation of the corresponding o-quinone product was monitored at 480 nm for dopamine oxidation ($\varepsilon = 3,300 \text{ M}^{-1} \text{ cm}^{-1}$), at 410 nm for DTBC oxidation ($\varepsilon = 1,910 \text{ M}^{-1} \text{ cm}^{-1}$), and at 500 nm for THB oxidation ($\varepsilon = 32,500 \text{ M}^{-1} \text{ cm}^{-1}$ as MBTH adduct) for up to 10 min, and
the rates were determined by the change in absorbance over time. Rates were fit to appropriate rate laws and rate constants determined by the use of SigmaPlot 11.0.

The inhibitory effect of various compounds towards substrate oxidation catalyzed by Cu$^{2+}$–Aβ complexes was analyzed by steady-state kinetics where the initial reaction rate was measured as a function of time. The effect of inhibitors on substrate oxidation was measured at constant concentrations of substrate (varied between 1–5 mM) and Cu$^{2+}$–Aβ (varied between 2–17 µM) while varying the concentration of inhibitors (0.5–40 mM), from which the inhibition constant $K_i$ can be obtained via non-linear regression. The inhibition constant, $K_i$, is a measure of the potency of the inhibitor, given that it is the concentration of inhibitor required to reach half of the maximum inhibition. The lower the value of $K_i$, the greater is the degree of inhibition at any given substrate and inhibitor concentration. Lineweaver Burk plots were also obtained by varying the concentration of substrate at constant inhibitor and Cu$^{II}$-Aβ concentrations, from which the inhibition pattern and inhibition constant can be determined. The standard deviation from the plot fitting were used as the error margins in the obtained parameters.

4.2.3 NMR Spectroscopy

Spectra were acquired on INOVA spectrometer at $^1$H resonance of 500 MHz equipped with a 3 mm broadband probe with a $^{31}$P filter. An external neat phosphoric acid was used as a reference. The peptide Aβ$_{1-20}$ and its Cu$^{II}$ complex (150 µM) were prepared in 20 mM phosphate buffer in the presence of 30% D$_2$O. Phosphate containing inhibitors were gradually titrated into the apo and metallo-Aβ
and $^{31}$P signal was detected followed by $T_1$ measurement of the phosphate signal at various concentrations of the inhibitor, 0 to 50 mM. $T_1$ measurements were obtained using the inversion recovery pulse sequence ($\tau_1$-180°-$\tau_m$-90°-FID), where $\tau_1$, also known as d1 or the delay time, is varied in value up to approximately five times larger than the largest expected $T_1$ value. A non-linear regression is then carried out to fit the best $T_1$ value (in seconds, s). The peak intensities versus the $\tau_1$ values are fitted with a three parameters least-squares fitting program on the spectrometer to afford the $T_1$ values. A $T_1$ error of less than 10% is estimated based on fitting and repeated measurements.

The $^{31}$P relaxation rate ($T_{1p}^{-1}$) of phosphate is obtained by subtracting the relaxation rate of phosphate in the absence of metal, apo Aβ1-20, from the measure $T_1^{-1}$ value in the presence of Cu$^{2+}$-Aβ1-20 under the same conditions. The overall concentration of the complex can also affect the relaxation time of a particular system. Thus, the molar relaxivity in a paramagnetic system is equal to $T_{1p}^{-1}/[E]$, where [E] is the concentration of the paramagnetic metal ions present in the system. A plot of $^{31}$P NMR relaxivity versus phosphate concentration in the presence of Cu$^{2+}$-Aβ1-20 is fit to appropriate equation, see section 4.3.7, to give $T_{1M}^{-1}$ and the affinity constant $K_f$.

4.2.4 DNA Cleavage Assay

In a typical DNA cleavage assay, pUC18 plasmid DNA (100 ng) was incubated with Cu$^{II}$-Aβ20 (8.0 μM) complexes, 0.2% H$_2$O$_2$, and varying concentrations of a potential protecting agent such as zoledronic acid at 8-1024 μM in HEPES pH 7.0
containing 0.15 M NaCl. Control experiments were carried out in the absence of zoledronic acid. Reaction assays were incubated at 37°C for 15 min and then subjected to electrophoresis on 0.8% agarose gel stained with ethydium bromide. The gel was then viewed on a transilluminator and digitized.

4.3 Results and Discussion:

4.3.1 Effect of vitamin B₆ compounds:

*Pyridoxamine:* The effect of PM on dopamine oxidation, catalyzed by Cu²⁺–Aβ complexes, was probed by steady-state kinetics monitoring the change in reaction rate as a function of increasing [PM], at fixed 1 mM [dopamine]. PM inhibits the oxidation of dopamine by the Cu²⁺–Aβ complexes (Figure 4.2A). The inhibition constant, $K_i$, is obtained by fitting the rate as a function of PM concentration, according to equation (4.1) for a competitive inhibitor. The Cu²⁺ complex of the full-length peptide, Cu²⁺–Aβ₄₀ affords $K_i$ of 1.40 mM against dopamine oxidation in the presence of PM. Similarly, PM inhibits dopamine oxidation by the Aβ fragments, Cu²⁺–Aβ₂₀ and Cu²⁺–Aβ₁₆, affording $K_i$ of 1.40 and 1.03 mM, respectively. The reactions exhibit a relatively high IC₅₀ around 7–8 mM. The inhibition pattern of PM towards dopamine oxidation was further analyzed, suggesting a competitive inhibition pattern with $K_i$ of 1.42 mM against Cu²⁺–Aβ₄₀ (Figure 4.2B).

$$v = \frac{V_{max} [S]}{K_m \left(1 + \frac{[I]}{K_i}\right) + [S]} \tag{4.1}$$
Figure 4.2: Effect of PM on dopamine (1.0 mM) oxidation catalyzed by (●) 2.0 μM Cu²⁺–Aβ₄₀, (○) 3.0 μM Cu²⁺–Aβ₂₀, and (▼) 2.0 μM Cu²⁺–Aβ₁₆ in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Lineweaver-Burk plot of 1/rate versus 1/[dopamine] by Cu²⁺–Aβ₄₀ in the presence of PM (0.0, 2.0, and 4.0 mM from bottom to top trace).
**Pyridoxine:** The effect of PN on dopamine oxidation by Cu²⁺–Aβ complexes was similarly analyzed (Figure 4.3). PN is shown to inhibit the oxidation of dopamine by Cu²⁺–Aβ complexes with much less potency as compared to PM. Cu²⁺–Aβ₂₀ affords a $K_i$ of 8.29 mM, while Cu²⁺–Aβ₁₆ affords a $K_i$ of 5.56 mM. To confirm the relatively higher $K_i$ values for PN as opposed to PM, the effect of PN was monitored towards different substrates, DTBC and THB. PN inhibits DTBC and THB oxidation affording $K_i$ values of 5.05 and 8.67 mM, respectively. Further supports the relatively higher $K_i$ values towards PN inhibition. The inhibition pattern of PN was investigated towards DTBC oxidation by Cu²⁺–Aβ₂₀ to afford a competitive inhibition with $K_i$ of 5.05 mM (Figure 4.4).

**Pyridoxal and Pyridoxal-5-phosphate:** The effect of PL towards catecholamine oxidation by Cu²⁺–Aβ complexes was analyzed using THB and DTBC as substrates instead of dopamine (Figure 4.5). The aldehyde group of PL can react with the amino group of dopamine forming a Schiff base moiety. PL inhibits the oxidation of DTBC by Cu²⁺–Aβ₂₀ and Cu²⁺–Aβ₁₆ in a competitive manner and the reactions afford $K_i$ values of 1.12 and 0.67 mM, respectively. PL affords a similar $K_i$ value towards THB oxidation by Cu²⁺–Aβ₂₀, 2.8 mM.

**The role of phosphate:** The observed decrease in the $K_i$ value (1.12 to 0.2 mM) for PLP, as compared to PL, towards the inhibition of catecholamine substrates catalyzed by Cu²⁺–Aβ₂₀ complexes may be attributed to the phosphate group in PLP. Phosphate was previously shown to be a competitive inhibitor towards dopamine oxidation catalyzed by Cu²⁺–Aβ₂₀ with a $K_i$ of 4.7 mM. Further, the study
Figure 4.3: Effect of PN on dopamine (1.0 mM) oxidation catalyzed by (●) 17.0 µM Cu²⁺–Aβ₂₀ and (○) 3.0 µM Cu²⁺–Aβ₁₆ in 0.1 M HEPES pH 7.4 at 25 °C.
Figure 4.4: Effect of PN on DTBC (1.0 mM) (●) and THB (3.0 mM) (○) oxidation catalyzed by 2 μM Cu²⁺-Aβ16 in 0.1 M HEPES pH 7.4 at 25 °C.
Figure 4.5: Effect of PL on DTBC (3.0 mM) oxidation by 1.4 μM Cu$^{2+}$–Aβ$_{20}$ complex in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Lineweaver Burk plot of 1/rate versus 1/[DTBC] in the presence of fixed concentrations of PL at 0.0 (▼), 0.4 (○), and 1.2 mM (●).
investigated the influence of the redox-active agents NAD(P)⁺/NAD(P)H as it is expected to influence the redox properties of Cu²⁺-Aβ in vivo, wherein NADP(H) exhibited a more pronounced inhibition. Herein, phosphate inhibits the oxidation of DTBC affording a $K_i$ of 4.73 mM (Figure 4.6). The data suggests PLP acts as a bi-functional inhibitor toward the oxidation of DTBC by Cu²⁺-Aβ₂₀, where the phosphate group is also involved in the inhibition. To further deduce the inhibitory effect of phosphate in PLP, the effect of a 1-to-1 molar ratio of PL-to-phosphate was monitored towards the oxidation of DTBC catalyzed by Cu²⁺-Aβ₂₀. The reaction affords a $K_i$ of 0.80 mM, which is significantly higher than the $K_i$ of PLP, 0.20 mM (Figure 4.7). The decrease in the $K_i$ value of PL in the presence of phosphate, 1.12 to 0.80 mM, may indicate that PL and phosphate inhibit reactions synergistically, which is more pronounced in PLP, 0.20 mM, where PL and phosphate are covalently bound. The $K_i$ and IC₅₀ values are summarized in Table 4.1.

4.3.2 Effect of PLP, PL, and phosphate on Aβ₂₀ mutants:

Oxidative activities of Aβ₂₀ mutants: Possible charge interactions between phosphate and the side chains of Arg-5 and Lys-16 of Aβ₂₀ were probed using two different mutants of Aβ₂₀. A single amino acid mutation of either Arg-5 or Lys-16 to Ala, denoted as Aβ₂₀(R5A) and Aβ₂₀(K16A), respectively. Under physiological conditions, the guanidinium group of Arg and the amino group on the side chain of Lys are protonated and positively charged. The protonated side chain may exhibit charge interactions with the phosphate group of the inhibitor. Therefore, mutation of the
Figure 4.6: Effect of PLP on DTBC (3.0 mM) oxidation by 1.4 µM Cu$^{2+}$–Aβ$_{20}$ complex 0.1 M HEPES at pH 7.4 and 25 °C. Lineweaver Burk plot of 1/rate versus 1/[DTBC] in the presence of fixed concentrations of PLP at 0.0 (■), 0.13 (▲), and 3.0 mM (●).
Figure 4.7: Plot of percent activity of DTBC oxidation by Cu$^{2+}$-Aβ$_{1-20}$ as a function of increasing concentration of PLP (●), PL (▼), phosphate (■, up to 50 mM), and 1:1 PL:phosphate (♦) all in 1:1 MeOH:HEPES buffer pH 7.0 at 25 °C.
Table 4.1: Summary of the $K_i$ and IC$_{50}$ values for DTBC oxidation by Cu$^{2+}$–Aβ$_{20}$.

<table>
<thead>
<tr>
<th></th>
<th>PL</th>
<th>PLP</th>
<th>$H_2PO_4^-$</th>
<th>PL: $H_2PO_4^-$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_i$ (mM)</td>
<td>1.2</td>
<td>0.2</td>
<td>4.7</td>
<td>0.80</td>
</tr>
<tr>
<td>IC$_{50}$ (mM)</td>
<td>1.1</td>
<td>0.25</td>
<td>22</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Arg and Lys residue may give insight into phosphate interaction with Cu$^{2+}$–$\text{A} \beta_{20}$ and its influence on the overall oxidative activities. In the presence of DTBC as a substrate, Arg-5 and Lys-16 mutants of Cu$^{2+}$–$\text{A} \beta_{20}$ complex exhibit enzyme like saturation kinetics, monitored by the change in absorbance over time as a result of $o$-quinone formation (Figure 4.8). DTBC oxidation catalyzed by Cu$^{2+}$–$\text{A} \beta_{20}(R5A)$ affords $k_{\text{cat}} = 0.22 \pm 0.02$ s$^{-1}$, and $K_m= 8.8 \pm 1.4$ mM. Similarly, Cu$^{2+}$–$\text{A} \beta_{20}(K16A)$ complex catalyzes the oxidation of DTBC affording $k_{\text{cat}} = 0.24 \pm 0.04$ s$^{-1}$, and $K_m = 8.7 \pm 2.7$ mM. To probe the role of phosphate towards the inhibition discussed earlier, the interaction of Cu$^{2+}$–$\text{A} \beta_{20}$ mutants with PL, PLP, and phosphate is further investigated. Arg-5 and Lys-16 residues are near the metal binding site His-6, His-13, His-14 in Cu$^{2+}$–$\text{A} \beta_{20}$ and may influence the binding of phosphate or phosphate moiety to the complex.

*Pyridoxal, pyridoxal-5-phosphphate, and phosphate:* To probe the plausible interaction of phosphate with either of Arg-5 and Lys-16 in $\text{A} \beta_{20}$, the inhibitory effect of PL, PLP, and phosphate towards DTBC oxidation by Cu$^{2+}$–$\text{A} \beta_{20}$ mutants was analyzed. The reactions were monitored by steady-state kinetics, plotting the change in reaction rate as a function of inhibitor concentration. PL and PLP inhibit DTBC oxidation by Cu$^{2+}$–$\text{A} \beta_{20}(R5A)$ affording $K_i$ values of 2.1 and 0.4 mM respectively (Figure 4.9). The $K_i$ value is for the observed decrease in reaction rate in the presence of varying amount of a competitive inhibitor is fit to equation (4.1). The observed decrease in $K_i$ value in the presence of PLP as compared to PL (2.1 to 0.4 mM) is indicative of phosphate interaction with the Cu$^{2+}$–$\text{A} \beta_{20}(R5A)$ complex, despite
**Figure 4.8**: DTBC oxidation catalyzed by 5.0 μM (●) Cu^{2+}−Aβ_{20(R5A)}, and (○) Cu^{+2}−Aβ_{20(K16A)} complexes in 50:50 MeOH:HEPES buffer pH 7.4 at 25 °C.
Figure 4.9: Effect of PL (○) and PLP (●) on DTBC (3 mM) oxidation catalyzed by (A) Cu²⁺–Aβ₂₀(R5A) and (B) Cu²⁺–Aβ₂₀(K16A) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
Arg-5 mutation. While the Lys-16 mutant, Cu$^{2+}$–Aβ$_{20(\text{K16A})}$, is inhibited by PL and PLP toward DTBC oxidation with $K_i$ values of 6.4 and 0.31 mM, respectively (Figure 4.9).

The data does not suggest any specific interactions between the phosphate moiety in PLP and the Arg-5 or Lys-16 residues in Aβ$_{20}$. The observed differences in the $K_i$ values of Cu$^{2+}$–Aβ$_{20}$ and its mutants suggest that Arg-5 and Lys-16 residues may not be exclusively responsible for phosphate interaction in the binary Cu$^{+2}$Aβ-inhibitor or the ternary (Cu$^{2+}$–Aβ)–S–inhibitor complex. This is supported by the similar $K_i$ values, where PLP inhibition of DTBC oxidation catalyzed by the native Cu$^{2+}$–Aβ$_{20}$ complex affords $K_i$ of 0.20 mM, as compared to 0.4 and 0.31 mM for the mutants.

The interaction of phosphate with the Aβ$_{20}$ mutants was further investigated. Phosphate inhibits the oxidation of DTBC catalyzed by Cu$^{2+}$–Aβ$_{20(\text{R5A})}$ and Cu$^{2+}$–Aβ$_{20(\text{K16A})}$ affording $K_i$ values of 64 and 104 mM, respectively (Figure 4.10). The observed ~14–22 times decrease in the potency of phosphate towards the oxidation of DTBC in Cu$^{2+}$–Aβ$_{20}$ mutant versus the native form is indicative of a possible interaction between phosphate and Arg-5 or Lys-16.

4.3.3 Effect of nicotinamide adenine dinucleotide phosphate, NAD(P)H:

The redox-active agents NAD(P)$^+$/NAD(P)H are expected to influence the redox properties of Cu$^{2+}$–Aβ in vivo. A previous study suggested a decrease in the oxidative activities of Cu$^{2+}$–Aβ$_{20}$ towards dopamine in the presence of NAD(P)H,
Figure 4.10: Effect of phosphate on DTBC (3 mM) oxidation catalyzed by
$\text{Cu}^{2+}$–$\alpha_2$ (●) $\text{Cu}^{2+}$–$\alpha_2$(R5A) (○) $\text{Cu}^{2+}$–$\alpha_2$(K16A) (◆) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
where a decrease in the ratio of $\text{NAD}^+(\text{H})/\text{NADP}^+(\text{H})$ gave a more pronounced inhibition in terms of $k_{\text{cat}}$. The more pronounced inhibition was attributed to phosphate, which is in agreement of our current study; refer to section 4.3.1. The ratio of $\text{NAD}^+/\text{NADH}$ are influenced by changes in metabolism, which have been associated with neurodegenerative diseases. The metabolic changes may thus reflect neurochemical status under oxidative stress in AD. Herein, the influence of NADPH on the oxidative activities of $\text{Cu}^{2+}$–$\text{A}\beta_{20}$ and mutants is investigated to further elucidate the interaction of phosphate with metallo–$\text{A}\beta$ (Figure 4.11). NADPH inhibits the oxidation of DTBC by $\text{Cu}^{2+}$–$\text{A}\beta_{20}$ complex affording a $K_i$ of 13 mM. The influence of NADPH on the oxidative activities by $\text{Cu}^{2+}$–$\text{A}\beta_{20}$ mutants may give insight possible interaction between the phosphate moiety in NADPH and the Arg-5 and Lys-16 residues in $\text{A}\beta_{20}$. NADPH inhibition of the oxidative activities catalyzed by $\text{Cu}^{2+}$–$\text{A}\beta_{20(\text{R5A})}$, $\text{Cu}^{2+}$–$\text{A}\beta_{20(\text{K16A})}$ afford $K_i$ values of 14 and 10 mM, respectively. The similar $K_i$ values, compared to that for $\text{Cu}^{2+}$–$\text{A}\beta_{20}$, suggest a similar inhibition mechanism and does not suggest any specific interactions between the phosphate moiety and $\text{A}\beta_{20}$ mutants.

4.3.4 Effect of vitamin B$_1$ and pyrophosphate

To further investigate the role of phosphate and vitamin B compounds, vitamin B$_1$, thiamine, and its phosphate derivative were probed against the observed oxidative activities of $\text{Cu}^{2+}$–$\text{A}\beta_{20}$ complexes. Glucose metabolism is linked to brain function and AD, a process controlled by thiamine–dependent
**Figure 4.11:** The effect of NADPH on the oxidation of DTBC oxidation catalyzed by Cu$^{2+}$-Aβ$^{20}$ (●), Cu$^{2+}$-Aβ$^{20(5A)}$ (○), Cu$^{2+}$-Aβ$^{20(14A)}$ (♦) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
enzymes. Brain glucose metabolism is altered decades prior to the development of AD symptoms. Thiamine crosses the blood brain barrier and is phosphorylated to the active form thiamine pyrophosphate (TPP), required for thiamine-dependent enzymes. Thiamine deficiency alters the activity of thiamine-dependent enzymes and is linked to neurodegenerative diseases. It is attributed to neuronal death with many parallels to that of AD, including an increase of ROS related damage and oxidative stress. A decrease in the concentration of TPP and the activity of thiamine-dependent enzymes is observed in AD brains. Thiamine deficiency has also been attributed to accumulation of Aβ peptide due to an increase in β-secretase activity, which further enhances oxidative stress. Inhibition of γ-secretase activity has also been implicated in AD therapeutics, where γ and β secretases cleave APP into its insoluble Aβ fragments.

Previous studies suggest that supplementation of thiamine or derivatives may reverse pathological process attributed with AD. An administration of pharmacological dosages of thiamine, 3–8 mg per day, showed beneficial effects to AD associated dementia. A decrease in plaque formation and improvement in memory was observed in genetically engineered AD mice treated with the thiamine pro-drugs benfotiamine or sulbutiamine that can pass through the blood-brain-barrier more easily than thiamine. In the results described in this dissertation, the phosphate derivative of vitamin B₁, TPP, was found to inhibit the oxidation of DTBC by Cu²⁺–Aβ₂₀ affording $K_i$ value of 3.3 mM (Figure 4.12). Similar $K_i$ values were observed for the oxidation of DTBC by Cu²⁺–Aβ₂₀ mutants suggesting a similar
Figure 4.12: Effects of thiamine (○) and thiamine pyrophosphate (●) on DTBC oxidation catalyzed by Cu²⁺–Aβ20 complex in 50:50 MeOH:HEPES pH 7.4 at 25°C.
reaction mechanism. $\text{Cu}^{2+}-\text{A}β_{20(\text{R5A})}$ affords $K_i$ of 2.9 ± 0.7 mM, and $\text{Cu}^{2+}-\text{A}β_{20(\text{K16A})}$ affords $K_i$ of 3.5 ± 0.7 mM towards the oxidation of DTBC in the presence of TPP (Figure 4.13).

In the presence of thiamine, the reactions afford $K_i$ values of 8.4 and 13 mM for $\text{Cu}^{2+}-\text{A}β_{20(\text{R5A})}$ and $\text{Cu}^{2+}-\text{A}β_{20(\text{K16A})}$, respectively. A more pronounced inhibition is observed for thiamine-phosphate, which further supports the role of phosphate observed in the inhibition by PL versus PLP. The results are comparable to a previous study where the inhibitory effect of vitamin B1 towards the diphenolase activity of mushroom tyrosinase activity affords $K_i$ value 11.7 mM.\textsuperscript{131} The study reports an IC$_{50}$ of 15 and 20 mM towards the inhibition of monophenolase and diphenolase activity, respectively, of mushroom tyrosinase by vitamin B$_1$. The antioxidant effects of vitamin B$_1$ against ROS were previously demonstrated.\textsuperscript{132,133} The data suggest comparable potency of TPP and PLP, in terms of the $K_i$ values, towards inhibition of the observed oxidative activities, 3.3 and 1.1 mM, respectively (Table 4.2). The metal chelating ability of pyridoxal\textsuperscript{135} may be attributed to its increased potency as compared to thiamine. Further, the data does not suggest any direct inhibitory effect attributed to the presence of a monophosphate in PLP versus a diphosphate moiety in TPP. The influence of the pyrophosphate moiety in TPP towards the observed inhibition was further probed. Pyrophosphate is shown to inhibit the oxidation of DTBC by $\text{Cu}^{2+}-\text{A}β_{20}$ following a competitive inhibition pattern, as determined by the Lineweaver-Burk plot (Figure 4.14). Pyrophosphate
Figure 4.13: Effects of thiamine (left) and thiamine pyrophosphate (right) on DTBC oxidation catalyzed by $\text{Cu}^{2+}$-$\text{A}β_{20}$ (●) $\text{Cu}^{2+}$-$\text{A}β_{20}$(R5A) (○) $\text{Cu}^{2+}$-$\text{A}β_{20}$(K16A) (◆) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
Table 4.2: List of inhibition constants, $K_i$ (mM), for various inhibitors against dopamine (3 mM) oxidation by Cu$^{2+}$–Aβ complexes.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Complex:</th>
<th>Cu$^{2+}$–Aβ$_{20}$</th>
<th>Cu$^{2+}$–Aβ$_{20(A5)}$</th>
<th>Cu$^{2+}$–Aβ$_{20(A16)}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>1.2</td>
<td>2.1</td>
<td>6.4 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>Pyridoxal 5′–phosphate</td>
<td>0.2</td>
<td>0.4</td>
<td>0.31 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td>4.7</td>
<td>64</td>
<td>104 ± 11</td>
<td></td>
</tr>
<tr>
<td>NADPH</td>
<td>13 ± 2</td>
<td>14 ± 4</td>
<td>10 ± 2</td>
<td></td>
</tr>
<tr>
<td>Thiamine</td>
<td>17 ± 4</td>
<td>8.4 ± 2.4</td>
<td>13 ± 4</td>
<td></td>
</tr>
<tr>
<td>Thiamine pyrophosphate</td>
<td>3.3 ± 0.7</td>
<td>2.9 ± 0.7</td>
<td>3.5 ± 0.7</td>
<td></td>
</tr>
<tr>
<td>Pyrophosphate</td>
<td>2.4 ± 0.6</td>
<td>54 ± 25</td>
<td>50 ± 8.1</td>
<td></td>
</tr>
<tr>
<td>ADP</td>
<td>2.4 ± 0.4</td>
<td>2.5 ± 0.5</td>
<td>3.2 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>3.5 ± 1.0</td>
<td>4.9 ± 0.8</td>
<td>6.2 ± 0.4</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.14:** Lineweaver-Burk plot of DTBC oxidation catalyzed by Cu$^{2+}$-Aβ$_{20}$ complex at fixed pyrophosphate concentrations of 0, 1, 4, 8, 32 (from bottom up) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
exhibits approximately a two-fold increase in potency as opposed to phosphate in terms of \( K_i \) (2.4 vs. 4.7 mM). However, this was not reflected in the \( K_i \) values in the presence of TPP versus PLP, where TPP exhibits a three-fold decrease in potency, which may be attributed to its lack of metal chelating ability.

As compared to the native form, \( \text{Cu}^{2+} - \text{Aβ}_{20} \), the mutants exhibit similar \( K_i \) values in the presence of phosphate, PLP, pyrophosphate, or TPP (Table 4.2). The \( \text{Cu}^{2+} - \text{Aβ}_{20(\text{R5A})} \) complex consistently exhibits a slightly lower \( K_i \) values in comparison to the reactions catalyzed by the \( \text{Cu}^{2+} - \text{Aβ}_{20(\text{K16A})} \) complex. The data may suggest some specific interactions with the phosphate moieties and the Arg-5 side chain in \( \text{Aβ}_{20} \).

4.3.4 Inhibition by adenosine-diphosphate (ADP) and -triphosphate (ATP)

ATP is a signaling molecule present abundantly in the central nervous system.\(^{136,137}\) It was previously shown to exhibit protective effects against mediated \( \text{Aβ} \) cytotoxicity,\(^{138,139}\) where a decrease in ATP levels is observed over the course of AD. \textit{In vitro} studies suggest a decrease in the misfolding of \( \text{Aβ}_{42} \) protein in the presence of ADP and ATP, which is further supported by computational studies to reveal a strong interactions between ATP and Tyr-10 and Ser-26 residues of \( \text{Aβ}_{42} \) fibrils in solution.\(^{140}\) Herein, ATP and ADP inhibition of \( \text{Cu}^{2+} - \text{Aβ}_{20} \) towards the oxidation of DTBC was monitored. The decrease in the rate of DTBC oxidation by \( \text{Cu}^{2+} - \text{Aβ}_{20} \) is plotted as a function of ATP and ADP concentrations (Figure 4.15), and fit to equation 4.1. The reactions afford an IC\(_{50}\) of 3.4 for ATP and 2.8 mM for ADP.
Figure 4.15: Effect of ADP (○) and ATP (●) on DTBC oxidation catalyzed by Cu\(^{2+}\)-Aβ\(_{20}\) complex in 50:50 MeOH:HEPES pH 7.4 at 25°C.
The inhibition of DTBC oxidation by ADP affords an increase in potency with respect to $K_i$ values, 3.5 to 2.4 mM, as compared to ATP. The presence of a bulkier triphosphate moiety in ATP, as compared to the diphosphate found in ADP, may have slightly hindered its interaction with $\text{Cu}^{2+}$-$\text{A} \beta_{20}$ or with $(\text{Cu}^{2+}$-$\text{A} \beta)$–S binary complex. The observed $K_i$ value of $\text{Cu}^{2+}$-$\text{A} \beta_{20}$ in the presence of ADP, $2.4 \pm 0.7$ mM, was the same as the $K_i$ value in the presence of pyrophosphate, $2.4 \pm 0.7$ mM. Hence, the potency of ADP may be attributed to the presence of the bisphophate moiety.

Similarly, ADP inhibits the oxidation of DTBC by $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{R5A})}$ and $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{K16A})}$ complexes with $K_i$ of $2.5 \pm 0.5$ mM, and $3.2 \pm 0.64$ mM, respectively (Figure 4.16). The slight increase in the $K_i$ value for $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{R5A})}$ as opposed to $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{K16A})}$ is consistent with the previous findings presented herein. A protective effect of ADP against Aβ complex toxicity has been suggested. A recent study suggests a molecular mechanism of neuroprotection by extracellular ADP preventing neuronal apoptosis by activating cell antioxidant enzymes, while it also suggests protective effects against toxicity of Aβ1–42 peptide of AD. Further, ATP inhibits the oxidative activities catalyzed by Aβ mutants. The results show $K_i$ values of 4.9 and 6.2 mM, towards the inhibition of DTBC oxidation catalyzed by $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{R5A})}$ and $\text{Cu}^{2+}$-$\text{A} \beta_{20(\text{K16A})}$ complexes, respectively. The data suggest a more potent inhibition, up to two-time decrease in terms of $K_i$ value, for the reactions catalyzed by the native $\text{Cu}^{2+}$-$\text{A} \beta_{20}$ complex, which affords $K_i$ of 3.5 mM (Table 4.2). The results may further suggest an interaction between the phosphate moiety in ATP and the Lys-16 residue in Aβ since the mutant K16A decreases the inhibition.
Figure 4.16: Effects of ADP and ATP on DTBC oxidation catalyzed by Cu²⁺–Aβ₂₀

(●), Cu²⁺–Aβ₂₀(R5A) (○), Cu²⁺–Aβ₂₀(K16A) (◆) in 50:50 MeOH:HEPES pH 7.4 at 25°C.
potency of ADP. This may be further supported by a previously proposed structure of \( \text{Co}^{2+}-\text{A}\beta_{16} \) where Arg-5 poses an extended H-bonding framework suggested to stabilize the structure,\(^{47}\) which may in turn prevent its further interaction with other ligands.

4.3.5 Effect of a bisphosphonate drug, Zoledronic Acid

Bisphosphonates are a group of compounds with a characteristic central carbon atom attached to two germinal phosphoric acid moieties, which binds mineralized bone and is widely used to inhibit osteoclastic activities.\(^{142, 143}\) Herein, the inhibitory role of phosphate against metal-centered oxidative activities described above was further probed by monitoring the effect of zoledronic acid (Figure 4.17) towards \( \text{Cu}^{2+}-\text{A}\beta_{20} \) induced oxidative stress. The inhibitory effect of zoledronic acid was investigated by varying the concentration of zoledronic acid at fixed concentrations of substrate, DTBC (3.0 mM), and catalyst, \( \text{Cu}^{2+}-\text{A}\beta_{20} \) complexes (5 \( \mu \)M) (Figure 4.18A). The data was fit to equation (1) affording \( K_i \) of 2 \( \mu \)M. This affords \( \sim 100 \) times increase in potency as compared to our study with PLP as a competitive inhibitor affording \( K_i \) of 200 \( \mu \)M. This pronounced increase in potency could be partially attributed to a different inhibition mechanism.

*Inhibition of oxidative DNA cleavage:* It has been previously suggested that the neuropathology of AD may induce oxidative DNA damage and cell death.\(^{71}\) In the presence of 5 \( \mu \)M \( \text{Cu}^{2+}-\text{A}\beta_{20} \) complexes, DTBC oxidation is inhibited by zoledronic acid affording an \( K_i \) of 2.4 \( \mu \)M. Yet, the decrease in activity as a result of
Figure 4.17: Chemical structure of zoledronic acid.
Figure 4.18: Effect of Zoledronic Acid on DTBC oxidation (3.0 mM) by 5 μM Cu²⁺-Aβ₂₀ complex in 0.1 M HEPES at pH 7.4 and 25 °C. (B) Lineweaver Burk plot of 1/rate versus 1/[DTBC] in the presence of fixed concentrations of zoledronic acid at 0.0 (●), 0.04 (○), 0.02 (■), 0.002 (▼).
increase in zoledronic acid concentration does not approach 0% activity, which is observed in a competitive inhibitor. The inhibition pattern was determined by generating Lineweaver-Burk plots, in which the concentration of catalyst and inhibitor were kept constant while varying the concentration of substrate. Control experiments were performed in the absence of inhibitor. The inhibition of DTBC oxidation catalyzed by Cu$^{2+}$-Aβ$_{20}$ exhibit a noncompetitive inhibition pattern (Figure 4.18B), suggesting a binding site other than the metal active site. Zoledronic acid reaches a plateau $\sim$ 60% activity, suggesting that it may be deactivating the metal active site to a certain degree, thus inhibiting Cu$^{2+}$-Aβ$_{20}$ induced oxidative stress. Zoledronic acid has been suggested as a potent angiogenesis inhibitor with IC$_{50}$ ranging between 4.1-6.9 $\mu$M.$^{144}$

The results may also suggest differences in interactions and inhibition mechanisms between zoledronic acid and other phosphate moieties towards Cu$^{2+}$-Aβ$_{20}$ induced oxidation. Zoledronic acid is an imidazole containing bisphosphonate drug, currently known for the treatment of osteoporosis, hypercalcemia, bone cancer, and other related bone diseases. The observed decrease in activity (Figure 4.18) in the presence of zoledronic acid may be attributed to imidazole chelating or blocking the metal active site in Cu$^{2+}$-Aβ$_{20}$. The data suggest that the bisphosphate moiety can alter the overall oxidative activity.

The Cu$^{2+}$-Aβ complexes of AD exhibit concentration- and time-dependent oxidative activities against plasmid DNA monitored in vitro with agarose gel electrophoresis. Herein, the effect of zoledronic acid towards cleavage of plasmid
DNA in the presence of Cu$^{2+}$–Aβ$_{20}$ and H$_2$O$_2$ is presented. In the absence of zoledronic acid, where pUC18 plasmid DNA is extensively cleaved by 8.0 μM Cu$^{2+}$–Aβ$_{20}$ and 0.2% H$_2$O$_2$ (Figure 4.19, lanes 4) relative to the controls (lanes 1–3, wherein only the supercoiled, SC, plasmid DNA is detected). In the presence of various concentrations of zoledronic acids (Figure 4.19, lanes 5-12), the data suggest inhibition of oxidative plasmid DNA cleavage in a concentration-dependent manner. At 8 μM (lane 5) and up to 32 μM (lane 7), an intensity of sheared DNA appears on the gel. The data suggest a partial inhibition of DNA cleavage, as compared to the control in the absence of zoledronic acid (lane 4) where more shearing of the DNA is observed. As the concentration of zoledronic acid is increased to 64 μM (lane 8), a linear, LN, band of cleaved plasmid is observed on the gel. At 128 and 256 μM zoledronic acid (lanes 9 and 10), the oxidative DNA cleavage is further inhibited where nicked circular, NC, bands appear on the gel as compared to the extensive DNA cleavage in the absence of zoledronic acid. As the concentration of zoledronic acid is increased to 512 and 1024 μM (lanes 11 and 12), the detection of SC DNA is indicative of inhibition of oxidative DNA cleavage by zoledronic acid to some extents.

4.3.4 Relaxation studies by phosphorous NMR

The binding nature of phosphate to Cu$^{2+}$–Aβ complex may be further supported by $^{31}$P NMR relaxation, by measuring the paramagnetic contribution to $^{31}$P relaxation. Phosphate binding to Cu, Zn-superoxide dismutase was previously
Figure 4.20: Agarose gel showing the effect of zoledronic acid on oxidative plasmid DNA cleavage by Cu²⁺–Aβ₂₀ complex. Lanes 1-4 are the controls, 100-μM (1) pUC18 plasmid DNA only, (2) 512 μM zoledronic Acid and the plasmid, (3) 10 μM Cu²⁺–Aβ₂₀ and the plasmid, and (4) 10 μM Cu²⁺–Aβ₂₀/0.2% H₂O₂ and the plasmid. Lanes 5-12 contained 10 μM Cu²⁺–Aβ₂₀, 0.2% H₂O₂, and varying concentration of zoledronic acid (8, 16, 32, 64, 128, 256, 512, and 1024 μM, respectively). All reaction assays were in 0.1 M HEPES pH 7.0, and incubated at 37°C for 15 min.
determined by means of $^{31}$P NMR relaxation studies, affording affinity constant of 20–34 M$^{-1}$ and Cu$^{2+}$–phosphate distance of 5.4 Å.\textsuperscript{145} Alternatively, $^{31}$P relaxation was used to determine phosphate binding to the Co$^{2+}$ substituted derivative of \textit{Streptomyces griseus} aminopeptidase, sAP, where $^{31}$P NMR relaxation reveals the binding of one phosphate to sAP with metal-phosphate distance within 4.1–4.3 Å.\textsuperscript{146}

In the presence of Cu$^{2+}$–Aβ the relaxation time of the phosphate NMR signal of the inhibitor increases as a result of their interaction. The binding of phosphate to Cu$^{2+}$–Aβ is thus confirmed by changes in the relaxation time of the phosphate signal as a function of the inhibitor concentration. The relaxation rate is obtained by taking the inverse of the measured relaxation time, $1/T_1$. For paramagnetic metal centers, the apparent $T_1$ of a nucleus on the interacting ligand is denoted to as $T_{1P}$. When measuring $T_{1P}$ for a paramagnetic system, the diamagnetic contribution to $T_{1d}$, in the absence of the paramagnetic metal, must be subtracted from the apparent relaxation rate, $(1/T_{1P}) - (1/T_{1d})$, under the same conditions. The overall concentration of the complex can also affect the relaxation time of a particular system. Thus, the normalized relaxation rate (the relaxivity) in a paramagnetic system is equal to $(1/T_{1P} - 1/T_{1d})/[E]$, where [E] is the concentration of the paramagnetic metal ions present in the system. Herein, the apparent paramagnetic contribution toward $^{31}$P NMR relaxation rate $1/T_{1P}$ of phosphate was obtained by subtracting the relaxation rate of phosphate in the absence of the Cu$^{2+}$ complex, $1/T_{1d}$, under the same conditions. Significant $1/T_{1P}$ were obtained which reflect an increase in relaxation rate due to the interaction of phosphate with the paramagnetic Cu$^{2+}$ center in Cu$^{2+}$–Aβ complex.
For a single phosphate binding site, a plot of $^{31}\text{P}$ relaxivity, $(1/T_{1\text{P}} - 1/T_1)/[E]$, versus phosphate concentration in the presence of Cu$^{2+}$–Aβ complex (Figure 4.20) can be fit to the following equation to afford the relaxation rate of bound phosphate and the affinity constant $K_f$ for the binding.$^{147,148,149}$

$$\frac{T_{1\text{P}}^{-1}}{[E]} = \frac{T_{1M}^{-1} K_f}{1 + K_f [P_i]} \quad (4.2)$$

Since the relaxation rates are concentration dependent, the plot can be used to determine the affinity of phosphate to the paramagnetic center. The fitted data affords $K_f$ value of 6.7, 15.1, and 2.7 M$^{-1}$ for phosphate binding to Cu$^{2+}$–A$\beta_{20}$, Cu$^{2+}$–A$\beta_{20}(R5A)$, and Cu$^{2+}$–A$\beta_{20}(K16A)$, respectively (Figure 4.20). The $K_f$ values for the formation of the EI complexes can be converted into dissociation constants of 149, 66, and 359 mM, respectively. The obtained dissociation constants are different as compared to the $K_i$ values obtained from the kinetic inhibition measurements (Table 4.2) at pH 7.4. The observed difference may be attributed to phosphate moiety competing with the substrate in the kinetic studies, where phosphate exhibits a competitive inhibition pattern.

The fitting affords $T_{1M}^{-1}$ values of 3852, 1415, and 5850 s$^{-1}$ for the P-$^{31}$NMR signal of the bound phosphate in the phosphate complexes of Cu$^{2+}$–A$\beta_{20}$, Cu$^{2+}$–A$\beta_{20}(R5A)$, and Cu$^{2+}$–A$\beta_{20}(R16A)$, respectively (Figure 4.20). The $T_{1M}^{-1}$ values can be further related to Cu$^{2+}$–$^{31}$P distance, $r$, according to the Solomon equation$^{150}$ by
Figure 4.20: Dependence of $^{31}$P NMR relaxivity of phosphate in the presence of $\text{Cu}^{2+}$-$\text{Aβ}_{20}$ (●), $\text{Cu}^{2+}$-$\text{Aβ}_{20(\text{R5A})}$ (○), $\text{Cu}^{2+}$-$\text{Aβ}_{20(\text{R16A})}$ (▼) at pH 7.4 in the presence of 10% D$_2$O at 25°C. The complex concentrations were kept constant at 50 μM, while varying the phosphate concentrations 0.01–0.6 M.
considering a predominant dipolar relaxation mechanism, as follows:

$$T_{1M}^{-1} = Cr^{-6}f(\tau_c) \quad (4.3)$$

where $C$ represents a group of physical constants, and $f(\tau_c)$ is the correlation function which is dominated by the electronic relaxation time of Cu$^{2+}$ at $\sim 10^{-9}$ s at room temperature. By solving the equation with the determined $T_{1M}^{-1}$ values and the estimated correlation function value, (Cu$^{2+}$–Aβ$_{20}$)-phosphate distances can be estimated to be 22.7, 8.3, and 34.5 Å for phosphate binding to Cu$^{2+}$-Aβ$_{20}$, Cu$^{2+}$-Aβ$_{20(R5A)}$, and Cu$^{2+}$-Aβ$_{20(K16A)}$, respectively. Such large values may indicate that the phosphate is not directly bound to the metal center.

### 4.4 Concluding Remarks

Probing interactions between inhibitors and Cu$^{2+}$–Aβ complexes is an important factor for gaining information on structure–function relationship, which may aid in the design of more potent therapeutics to inhibit negative effects of Cu$^{2+}$–Aβ. Herein, the role of (bis)-phosphate moieties in various compounds as inhibitors against the oxidative stress caused by Cu$^{2+}$–Aβ complexes was investigated. Vitamin B6 and B1 compounds are competitive inhibitors towards the oxidation of catechol derivatives by Cu$^{2+}$–Aβ complexes, which may reflect the conditions under oxidative stress. The $K_i$ values of the inhibitors suggest structure–activity relationship since the phosphate derivatives of the inhibitors exhibit higher
potency (lower $K_i$ values) against the oxidation of catechol containing substrates catalyzed by Cu$^{2+}$–Aβ complexes. The interaction of the phosphate moiety with redox-active metallo-Aβ complexes may be used as basis for the development of drugs against AD induced oxidative stress.

Mutation of the Aβ$_{20}$ peptide at positions 5 and 16 give insight into the interactions of the Arg/Lys side chains with the (bis)-phosphate moiety in the various inhibitors. The data herein suggest that the phosphate derivatives of select compounds have a more pronounced inhibition than the compounds themselves towards the oxidation of DTBC catalyzed by Cu$^{2+}$–Aβ complexes. The slight differences in the inhibition against the Aβ$_{20}$ mutants as compared to the native Aβ$_{20}$ form suggest that the mechanism by which phosphate moieties inhibit oxidative stress induced by metallo-Aβ$_{20}$ may not be attributed to their direct interaction with the Arg or Lys side chains. Other mutants are needed for further investigations to reveal the role of the phosphate moieties in interaction with metallo-Aβ peptides.

The $^{31}$P NMR relaxation studies further supports the binding/interaction of phosphate with Cu$^{2+}$–Aβ$_{20}$ complexes. Correlation of (bis)-phosphate moiety binding/activity will allow for the design of more potent inhibitors toward the Cu$^{2+}$–Aβ induced oxidative stress. Since the oxidative activity of Cu-Aβ complexes and their oxidative damage toward neurotransmitters and biomolecules have been previously demonstrated, inhibition of the oxidative reactions may alleviate oxidative stress in the disease and provide alternatives for AD therapeutics and/or prevention. Research efforts and clinical trials are ongoing in many research
laboratories to find a cure and ultimately prevent progression of AD, and until then, AD remains a major public issue.

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