TOWARD IMPROVING METALLOENZYME INHIBITOR DESIGN: A THERMODYNAMIC STUDY OF SMALL MOLECULE INTERACTIONS WITH COPPER(II), COBALT(II) AND MANGANESE(II)

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Table of Contents

**CHAPTER 1: INTRODUCTION** ................................................................................................................. 9
  1.1. FIRST ROW TRANSITION METAL ION (II) ......................................................................................... 9
  1.2. COPPER ION (II) ............................................................................................................................... 10
  1.3. COBALT ION (II) ............................................................................................................................. 17
  1.4. MANGANESE ION (II) ..................................................................................................................... 20
  1.5. METALLOENZYMES (II) ............................................................................................................... 24
  1.6. N-LIGANDS .................................................................................................................................... 25
     16.1 DA2Im ............................................................................................................................................ 26
     16.2 NTA ............................................................................................................................................... 27
     16.3 TREN ........................................................................................................................................... 28
     16.4 TPA ............................................................................................................................................... 29
     16.5 BPA ............................................................................................................................................... 30
  1.7. SOLUTION CONDITIONS ............................................................................................................... 30
  1.8. ISOTHERMAL TITRATION CALORIMETRY ..................................................................................... 31

**CHAPTER 2: ITC STUDIES OF THE INTERACTIONS OF LIGANDS WITH METAL IONS(II) ........33**
  2.1 ITC STUDIES OF THE INTERACTIONS OF LIGANDS WITH Cu\(^{2+}\) ............................................... 33
     2.1.1 N-(2-(1-methylimidazolyl)methyl)iminodiacetic Acid (DA2Im) Interaction with Cu\(^{2+}\) .. 33
     2.1.2 Nitrilotriacetic Acid (NTA) Interaction with Cu\(^{2+}\) ................................................................. 36
     2.1.3 Tris(2-aminoethyl)amine (TREN) Interaction with Cu\(^{2+}\) ...................................................... 38
     2.1.4 Tris(2-pyridylmethyl)amine (TPA) Interaction with Cu\(^{2+}\) ................................................... 41
     2.1.5 Bis(2-picoly)amine (BPA) Interaction with Cu\(^{2+}\) ................................................................. 43
  2.2 ITC STUDIES OF THE LIGANDS INTERACTIONS WITH CO\(^{2+}\) ................................................... 47
     2.2.1 Tris(2-aminoethyl)amine (TREN) Interaction with CO\(^{2+}\) .................................................... 47
     2.2.2 Tris(2-pyridylmethyl)amine (TPA) Interaction with CO\(^{2+}\) ................................................... 51
     2.2.3 Bis(2-picoly)amine (BPA) Interaction with CO\(^{2+}\) ............................................................... 58
  2.3 ITC STUDIES OF THE INTERACTIONS OF LIGANDS WITH Mn\(^{2+}\) ............................................. 63
     2.3.1 Tris(2-aminoethyl)amine (TREN) Interaction with Mn\(^{2+}\) ..................................................... 63
     2.3.2 Tris(2-pyridylmethyl)amine (TPA) Interaction with Mn\(^{2+}\) ..................................................... 67
     2.3.3 Bis(2-picoly)amine (BPA) Interaction with Mn\(^{2+}\) ............................................................... 68

**CHAPTER 3: ITC STUDIES OF THE INTERACTIONS OF POTENTIAL ENZYME ACTIVE SITE METAL ION(II) STRUCTURAL MIMETICS WITH INHIBITORS** ........................................................................... 69
  3. 8-HYDROXYQUINOLINE (8-HQ) INTERACTION WITH LIGAND-METAL ION (II) MIXTURES AS POTENTIAL ENZYME ACTIVE SITE METAL ION(II) STRUCTURAL MIMETICS ................................................. 70
     3.1.1. TREN-Cu\(^{2+}\) Interaction with 8-Hydroxyquinoline .............................................................. 71
     3.1.2 Cu(TPA) Interaction with 8-Hydroxyquinoline ...................................................................... 72
     3.1.3. Cu(BPA) Interaction with 8-Hydroxyquinoline ................................................................. 74
     3.2.1. Co\(^{2+}\)(TREN) Interaction with 8-Hydroxyquinoline ...................................................... 77
     3.2.2. Co\(^{2+}\)(TPA) Interaction with 8-Hydroxyquinoline ..................................................... 80
     3.2.3. Co\(^{2+}\)(BPA) Interaction with 8-Hydroxyquinoline ..................................................... 82
3.3.1. Mn\(^{2+}\)(TREN) interaction with 8-Hydroxyquinoline ........................................... 84
3.3.2. Mn\(^{2+}\)(TPA) interaction with 8-Hydroxyquinoline ........................................... 86
3.3.3. Mn\(^{2+}\)(BPA) interaction with 8-Hydroxyquinoline ........................................... 87

CHAPTER 4: ITC STUDIES OF THE INTERACTIONS OF METAL ION(II) WITH INHIBITOR ...... 89
4.1 Cu\(^{2+}\) interaction with 8-Hydroxyquinoline ...................................................... 91
4.2 Co\(^{2+}\) interaction with 8-Hydroxyquinoline ...................................................... 92
4.3 Mn\(^{2+}\) interaction with 8-Hydroxyquinoline ...................................................... 93

CHAPTER 5: EXPERIMENTAL METHODS FOR ISOTHERMAL TITRATION CALORIMETRY (ITC) ................................................................. 94
5.1 METHODS ........................................................................................................... 94
5.2 MATERIALS ...................................................................................................... 95
5.2.1 GENERAL CONSIDERATIONS ...................................................................... 95
5.3 SYNTHESIS OF (N-(2-(1-METHYLIMIDAZOLYL)METHYL)IMINODIACETIC ACID) DA2Im 98

CHAPTER 6: UV-VIS SPECTROSCOPY ...................................................................... 99
6.1 Methods ........................................................................................................... 99
6.2. Results and Discussion ................................................................................ 103
6.2.1 UV-Vis spectra for CuL interaction with AHA ......................................... 103

CHAPTER 7: COMPUTATIONAL METHODS ................................................................. 107
7. Computational Studies of enzyme active site metal ion structural mimetics with
metalloenzyme inhibitors ...................................................................................... 107
7.1. Computational Methods ................................................................................ 107
7.2 Optimized structure of individual compound ............................................... 111
7.3 Optimized structure of MI, ML, and ML\(_2\) complexes ................................... 115
7.4 Optimized structure of MLI complex ............................................................ 122

REFERENCES ........................................................................................................ 126

List of Tables

CHAPTER 2

Table 2.1. Thermodynamic parameters from ITC study of DA2Im interaction with Cu\(^{2+}\) 31
Table 2.2. Thermodynamic parameters from ITC study of NTA interaction with Cu\(^{2+}\) 33
Table 2.3. Thermodynamic parameters from ITC study of TREN interaction with Cu\(^{2+}\) 34
Table 2.4. Thermodynamic parameters from ITC study of TPA interaction with Cu\(^{2+}\) 38
Table 2.5. Thermodynamic parameters from ITC study of BPA interaction with Cu\(^{2+}\) 43
Table 2.6. Thermodynamic parameters from ITC study of TREN interaction with Co\(^{2+}\) 47
Table 2.7. Thermodynamic parameters from ITC study of TPA interaction with Co\(^{2+}\) 52
Table 2.8. Thermodynamic parameters from ITC study of BPA interaction with Co\(^{2+}\) 61
Table 2.9. Thermodynamic parameters from ITC study of TREN interaction with Mn\(^{2+}\) 69
Table 2.10. Thermodynamic parameters from ITC study of TPA interaction with Mn\(^{2+}\) 71
Table 2.11. Thermodynamic parameters from ITC study of BPA interaction with Mn\(^{2+}\) 74
CHAPTER 3

Table 3.1. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with TREN-Cu^{2+} 76
Table 3.2. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with TPA-Cu^{2+} 78
Table 3.3. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with different molar ratios of BPA-Cu^{2+} 83
Table 3.4. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with Co-TREN, Co-TPA, and Co-BPA 90
Table 3.5. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with Mn-TREN, Mn-TPA, and Mn-BPA 97

CHAPTER 4

Table 4.1. Thermodynamic parameters from ITC study of 8-hydroxyquinoline interaction with Cu^{2+}, Co^{2+}, and Mn^{2+} 107

CHAPTER 5

Table 5.1. Concentrations of Titrant and Titrate for Ligand-M^{2+} Studies 117
Table 5.2. Concentrations of Titrant and Titrate for Ligand-M^{2+} with inhibitor or M^{2+} only with inhibitor. 119

CHAPTER 6

Table 6.1. The concentration of each product and reactant in the equilibrium reaction. 122
Table 6.2. Binding affinity of CuL binary complex interaction with AHA studied using UV-Vis spectroscopy. 126

CHAPTER 7

Table 7.1. Total energy and the basis set used of metal ions, inhibitors, and ligands 134
Table 7.2. Total and binding energies for binary or ternary complexes 142
Table 7.3. Structural parameters of optimized Cu(8-HQ), Cu(8-HQ)_{2}, Cu(BPA), Cu(BPA)_{2}, and Zn(BPA) 143
Table 7.4. Bond lengths and binding energy of BPA-Cu^{2+}-8-HQ and BPA-Zn^{2+}-8-HQ using different basis set. 146
Table 7.5. Total Energies of BSSE correction optimized structure of BPA-Zn^{2+}-8-HQ and BPA-Cu^{2+}-8-HQ ternary complex. The basis set is BLYP/ CC-pVDZ. 147

List of Figures

CHAPTER 1

Figure 1.1. Oxidase activity of [Cu]-CPA 10
Figure 1.2. Reaction catalyzed by and active sites of the metalloenzyme studied TY (2Y9W). 18
Figure 1.3. Enantioselective epoxidation of p-chlorostyrene catalyzed by [Mn]-CA 20
Figure 1.4. The dimanganese active site of St AurF. 21
Figure 1.5. N-(2-(1-methylimidazolyl)methyl) iminodiacetic acid (DA2Im). 23
Figure 1.6. Nitrilotriacetic acid (NTA) 23
Figure 1.7. Tris(2-aminoethyl)amine (TREN). 24
Figure 1.8. Tris(2-pyridylmethyl)amine (TPA). 25
Figure 1.9. Bis(2-picolyl)amine (BPA).
Figure 1.10. Ideal shape of a binding isotherm.

CHAPTER 2

Figure 2.1. DA2Im (8.8 mM) titration into CuCl$_2$ (4 mM) in Tris buffer
Figure 2.2. DA2Im (1 mM) titration into CuCl$_2$ (0.1 mM) in Tris buffer
Figure 2.3. NTA titration into CuCl$_2$ in Tris buffer
Figure 2.4. TREN titration into CuCl$_2$ in MeOH:Tris buffer
Figure 2.5. TREN titration into CuCl$_2$ in Tris buffer
Figure 2.6. TPA titration into CuCl$_2$ in MeOH:Tris buffer
Figure 2.7. BPA titration into CuCl$_2$ in buffer and MeOH:buffer
Figure 2.8. TREN titration into CuCl$_2$ in MeOH:Tris buffer
Figure 2.9. TREN titration into CoCl$_2$ in NEM buffer pH 7.25
Figure 2.10. TREN titration into CoCl$_2$ in MeOH:NEM buffer pH 6.80
Figure 2.11. TREN titration into CoCl$_2$ in MeOH:NEM buffer pH 7.25
Figure 2.12. TPA titration into CoCl$_2$ in MeOH:NEM buffer pH 7.25
Figure 2.13. BPA titration into CoCl$_2$ in NEM buffer pH 7.25
Figure 2.14. BPA titration into CoCl$_2$ in MeOH:NEM buffer pH 7.25
Figure 2.15. BPA titration into CoCl$_2$ in NEM buffer pH 7.25
Figure 2.16. BPA titration into CoCl$_2$ in MeOH:NEM buffer pH 6.80
Figure 2.17. BPA titration into CoCl$_2$ in MeOH:NEM buffer pH 7.25
Figure 2.18. TREN titration into MnCl$_2$ in NEM buffer pH 6.80
Figure 2.19. TREN titration into MnCl$_2$ in NEM buffer pH 6.80 with subtract the control run
Figure 2.20. MnCl$_2$ titrated into TREN in NEM buffer pH 6.80
Figure 2.21. TREN titration into MnCl$_2$ in MeOH:NEM buffer pH 6.80
Figure 2.22. BPA titration into MnCl$_2$ in MeOH:NEM buffer pH 6.80

CHAPTER 3

Figure 3.1. 8-hydroxyquinoline into 1:1.1 Cu$^{2+}$:TREN
Figure 3.2. 8-hydroxyquinoline into 1:1.2 Cu$^{2+}$:TPA
Figure 3.3. 3.2 8-hydroxyquinoline into 1:1 Cu$^{2+}$:BPA
Figure 3.4. 3.2 8-hydroxyquinoline into 1:2 Cu$^{2+}$:BPA
Figure 3.5. 8-hydroxyquinoline into 1:1 Co$^{2+}$:TREN
Figure 3.6. 8-hydroxyquinoline into 1:1 Co$^{2+}$:TPA
Figure 3.7. 8-hydroxyquinoline into 1:1 Co$^{2+}$:BPA
Figure 3.8. 8-hydroxyquinoline into 1:1 Mn$^{2+}$:TREN
Figure 3.9. 8-hydroxyquinoline into 1:1 Mn$^{2+}$:TPA
Figure 3.10. 8-hydroxyquinoline into 1:1 Mn$^{2+}$:BPA

CHAPTER 4

Figure 4.1. 8-hydroxyquinoline into Cu$^{2+}$
Figure 4.2. 8-hydroxyquinoline into Co$^{2+}$
Figure 4.3. 8-hydroxyquinoline into Mn$^{2+}$

CHAPTER 6

Figure 6.1. UV-Vis spectra of TPA:Cu, TPA:Cu:AHA (1:1:1), and TPA:Cu:AHA (1:1:300)
Figure 6.2. UV-Vis spectra of BPA:Cu:AHA into MeOH:Tris buffer and Tris buffer
Figure 6.3. UV-Vis spectra of NTA:Cu:AHA into Tris buffer
Figure 6.4. UV-Vis spectra of TREN:Cu:AHA into Tris buffer
Figure 6.5. UV-Vis spectra of DA2Im:Cu:AHA into Tris buffer

CHAPTER 7

Figure 7.1. Optimized structure of AHA inhibitor.
Figure 7.2. Optimized structure of 8-HQ.
Figure 7.3. Optimized structure of NTA ligand.
Figure 7.4. Optimized structure of TPA ligand.
Figure 7.5. Optimized structure of TREN ligand.
Figure 7.6. Optimized structure of 8-HQ-Cu$^{2+}$ binary complex.
Figure 7.7 Optimized structure of 8-HQ-Cu$^{2+}$ binary complex.
Figure 7.8 Optimized structure of 8-HQ-Cu$^{2+}$-8-HQ complex.
Figure 7.9. Optimized structure of Cu$^{2+}$-DA2Im complex.
Figure 7.10. Optimized structure of TREN-Cu$^{2+}$
Figure 7.11. (model 1) Optimized structure of TPA-Cu$^{2+}$ binary complex.
Figure 7.12. (model 2) Optimized structure of TPA-Cu$^{2+}$ binary complex.
Figure 7.13. Optimized structure of BPA-Cu$^{2+}$ binary complex.
Figure 7.14. Optimized structure of BPA-Cu$^{2+}$-BPA complex.
Figure 7.15. Optimized structure of BPA-Cu$^{2+}$-BPA complex.
Figure 7.16. Optimized structure of BPA-Cu$^{2+}$-BPA complex.
Figure 7.17. Optimized structure of BPA-Zn$^{2+}$ binary complex.
Figure 7.18. Two different angles of the optimized structure of BPA-Cu$^{2+}$-8-HQ ternary complex.
Figure 7.19. Optimized structure of BPA-Cu$^{2+}$-8-HQ ternary complex.
Figure 7.20. Two different angles of the optimized structure of BPA-Zn$^{2+}$-8-HQ ternary complex.
Figure 7.21. Optimized structure (not completed) of BPA-Zn$^{2+}$-8-HQ ternary complex.
Figure 7.22. Two different angles of the BSSE correction optimized structure of BPA-Zn$^{2+}$-8-HQ ternary complex.
Figure 7.23. Optimized structure of the BSSE correction BPA-Cu$^{2+}$-8-HQ ternary complex.
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Abstract

This work aimed at understanding the interactions of 1) three physiologically relevant transition metal ions copper(II), cobalt(II) and manganese(II) with five tri- or tetra-dentate metal chelators (ligand or L) and 2) the resulting ML complexes with two known metalloenzyme inhibitors, 8-hydroxyquinoline (8-HQ) and acetohydroxamic acid (AHA). The ultimate goal of the work was to identify ligands that can form enzyme active site M$^{2+}$ structural mimetics capable of interacting with metalloenzyme active site inhibitors. The ligands studied were N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid (DA2Im), nitrilotriacetic acid (NTA), tris(2-aminoethyl)amine (TREN), tris(2-pyridylmethyl)amine (TPA), and bis(2-picolyl)amine (BPA) and they were chosen to mimic the coordination environment for the common enzyme active site transition metal ions. Isothermal titration calorimetry (ITC), UV-Vis absorbance spectroscopy and computational chemistry were used for the study. The appropriate ligand to form the copper active site structural mimetic was identified as bis(2-picolyl)amine (BPA) as BPA was found to bind Cu$^{2+}$ strongly forming a 1:1 Cu(BPA) binary complex. Upon addition of 8-HQ, Cu(BPA) formed a Cu(BPA)(8-HQ) ternary complex. Cu(TREN) and Cu(TPA) however did not form CuL(8-HQ) ternary complex but rather had the TREN or TPA displaced by 8-HQ, yielding the Cu(8-HQ)$_2$ complex. Results from this work helped us better understand the binding reactivity of Cu$^{2+}$, Co$^{2+}$ and Mn$^{2+}$ ions with the small molecule ligands and the enzyme active site inhibitors. The experiments also helped us estimate the energetic contribution of the active site metal ion to enzyme inhibitor binding. Results of this study will be useful to developing new therapeutics targeting metalloenzymes involved in diseases such as cancer.
Chapter 1: Introduction

1.1. First Row Transition Metal ion (II)

Although metals only account for 3% by mass of the human body, they play a disproportionately integral role in the body’s function. Among the physiological metal ions are the first-row transition metals, which are present at the enzyme active sites – and subsequently are key to the catalytic functions of a group of enzymes termed metalloenzymes. Metalloenzymes are involved in multiple physiological processes that may include the biosynthesis of DNA, biosynthesis of certain amino acids, breakdown of metabolites, etc. The exact nature of the association of the metal ion with the active site varies in metalloenzymes. In some metalloenzymes, the metal ion is permanently attached to the active site while in others the metal ion is a labile part of a coenzyme.

To ensure that good inhibitors for metalloenzymes are developed to treat various diseases, it is important to be aware of the basic characteristics of various transition metal ion groups and their roles in enzymes. The first-row transition metal ions in the +2 oxidation state are a common species in a variety of metalloenzymes. For instance, manganese to zinc act as metalloenzyme cofactors in the cells of almost all living beings. Moreover, it is because of the essential nature of these first-row late d-block transition metal ions, the human innate immune system has developed their immunity to invading microbial pathogens in a process termed "nutritional immunity." Emerging evidence suggests that Mn, Fe, and Zn are withheld from the pathogen in classically defined nutritional immunity, while Cu is used to kill invading microorganisms. But successful
pathogens have the tendency to develop a mechanism for the adaptive response that can be used to mitigate and reduce the effects of host control of transition metal bioavailability.

Enzymes, which are mostly proteins and act as the catalysts in the living world, have been designed by nature to catalyze a variety of reactions under controlled and exquisitely selective conditions of temperature, pressure, and pH. This attribute of enzymes have been harnessed to produce desirable results for industrial and other purposes. For the purpose of this research, we have focused on Cu\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\), some of the main first-row transition metal ions that can bind to many enzymes in the living cells. The biological roles of these metals are discussed below.

1.2. Copper Ion (II)

Copper (Cu, either as Cu\(^+\) or Cu\(^{2+}\)) is an essential trace element that acts as a cofactor important for a wide variety of physiological processes such as peptide amidation, neurotransmitter biosynthesis, cellular respiration, pigment formation, nervous system development and connective tissue strengthening. Cu also contributes to the function of the central nervous system (CNS) and is found in multiple regions of the brain including the basal ganglia, hippocampus, and cerebellum. It is found in the cell bodies of several specific types of neurons as well as in the synaptic cleft. Therefore, Cu plays an integral role in the development and nourishment of neurons and other brain cells. Multiple CNS enzymes depend on Cu for activity and they include tyrosinase, peptidyl lysine-amidating mono-oxygenase, Cu/Zn superoxide dismutase, caeruloplasmin, hephaestin, dopamine-hydroxylase, and cytochrome c oxidase.
In light of its essential role in various physiological processes, copper overloading or deficiency could lead to disease states. For example, Menkes disease and amyotrophic lateral sclerosis (ALS) both are caused by copper deficiency while Wilson’s disease is a result of copper overloading. Additionally, physiological accumulation of copper is also characteristic of multiple other diseases such as Alzheimer’s disease, Huntington’s disease, Parkinson’s disease, prion disease, and occipital horn syndrome, although it is not clear whether copper accumulation is the direct cause or consequence of the disease.\textsuperscript{7,8}

Homeostatic imbalances of copper can be caused by excessive intake or by genetic abnormality that can cause copper deficiency or abnormal accumulation.\textsuperscript{9} For healthy adults, the optimal daily dietary intake of copper is no less than 0.9 mg and no more than 10 mg, preferably within 1–2 mg.\textsuperscript{7,10} Low concentrations of copper can inhibit cell growth while abnormal storage or piling of copper in the liver can cause long term liver damage.\textsuperscript{11,5} For example, in Wilson’s disease, copper binding with ATP7B, an ATPase, is dysfunctional, which prevents copper incorporation into ceruloplasmin, a copper binding protein in plasma, therefore leading to an abnormally high accumulation of copper in the liver. If not corrected, this copper accumulation will cause permanent liver damage.\textsuperscript{9} Similarly, Menkes disease is caused by a X-linked recessive mutation in an ATPase, ATP7A, responsible for transporting copper across cell membrane for copper uptake from the blood, therefore leading to copper deficiency in the cell.\textsuperscript{9} Abnormal accumulation of copper in neurological disorders have also been shown to be caused by abnormal copper transport and aberrant copper-protein interactions. One of the consequences of such abnormality could be oxidative stress that copper exerts on cells and tissues as copper is
known to increase oxidative stress that could cause mitochondrial damage, DNA breakage, and neuronal injury.9

The toxicity of copper in part has to do with the tendency of Cu to participate in Fenton-like reactions.12 Such reactions likely exist in most forms of copper-induced cell damage. The Cu⁺ ion induces the formation of hydroxyl radical (HO•) via its reaction with hydrogen peroxide that is a product of metabolism (Eq. (1)). It can also generate superoxide anion (O₂•−) in reaction with molecular oxygen (Eq. (2)), which is the most toxicity-generating mechanism.

\[
\text{Cu}^+ + \text{H}_2\text{O}_2 \rightarrow \text{Cu}^{2+} + \text{HO}^\bullet + \text{HO}^- \quad \text{Eq. (1)}
\]

\[
\text{Cu}^+ + \text{O}_2 \rightleftharpoons \text{Cu}^{2+} + \text{O}_2^\bullet^- \quad \text{Eq. (2)}
\]

Additionally, the superoxide anion (O₂•−) generated in the second reaction will react with H₂O₂, generating HO• (Eq. (3)) in the Haber and Weiss reaction.13

\[
\text{O}_2^\bullet^- + \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + \text{HO}^\bullet + \text{HO}^- \quad \text{Eq. (3)}
\]

The HO• free radical generated in the first mechanism is highly reactive, capable of oxidizing every type of biological molecules including lipids, proteins and nucleic acids. Oxidation of these molecules will lead to loss of function.12

Even trace amount of copper can impact the proper neurodevelopment and neurological function.7 Our body has in place an elaborate mechanism to avoid the exposure of cells to free copper. Copper is therefore suggested to be used as one of the selective targets for treatments of
neurological diseases through control of copper levels using metal chelators.\textsuperscript{13} Metal chelators have been shown to be effective in bringing down copper concentration in the human blood such as in the case of copper toxicity.\textsuperscript{4} The use of metal chelators also helps treating cancer, especially in treating colon cancer.\textsuperscript{4}

To avoid the exposure of cells to free copper, multiple copper binding and transport proteins are used. To begin with, Cu is absorbed in the stomach and intestine in complex with amino acids (such as histidine) or short peptides. These Cu complexes enter the blood stream where Cu becomes bound to the serum albumin and transcuprein. Copper is then taken up and deposited into the liver where it binds to the glycoprotein caeruloplasmin, specifically at 6 - 7 moles of copper per mole of protein with very high affinity. Cu-bound caeruloplasmin is later released into the circulation where Cu\textsuperscript{2+} is reduced to Cu\textsuperscript{+} as a result of redox reactions with components such as low density lipoprotein (LDL).\textsuperscript{14,15} The majority of the circulating Cu exiting the liver is bound to caeruloplasmin with the remaining trace amount of Cu transported through the plasma protein albumin and the amino acid histidine. Careuloplasmin-bound Cu\textsuperscript{+} is then taken up by peripheral cells through multiple transmembrane transporters including Ctr1 and Ctr3. This likely occurs via careuloplasmin degradation to release the bound Cu ion due to its high affinity to the very tight binding. This means that cells invest energy to break down the copper-binding protein to avoid exposure of copper to other copper-binding molecules thereby avoiding toxicity.\textsuperscript{14}

In light of the importance of copper in physiological functions, much research has been done to understand the involvement of copper in human physiology and the processes that impact the circulation of copper in the body. According to recent research, copper deposits is
also integral to the generation of inflammations and tumor growth. The concentration of caeruloplasmin and other copper-binding components such as transcuprein is significantly higher in patients with such conditions than in healthy subjects. Tumor cells are found to readily take up copper from caeruloplasmin fractions of the plasma. It turns out that copper is a vital cofactor for tumor angiogenesis thus is crucial for tumor growth and metastasis. Therefore, any form of copper deposits in the body can be dangerous. Research has also shown that patients diagnosed with cancer of the breast, prostate, colon, lung, and brain have higher concentrations of copper in their blood stream. This makes high concentrations of copper ion a risk factor as it predisposes a person to cancer.

All organisms have developed sophisticated copper homeostasis and resistance systems to maintain the normal cellular copper supply to essential cuproenzymes while detoxifying excess copper. Recent work suggests that host-derived copper is used as an antibacterial weapon; thus both host and bacterial pathogens actively engage cellular processes to manipulate copper levels at key sites during bacterial infections. Upon entering cells, copper ions bind to different sites such as bacterial metallothionein (MymT) in M. tuberculosis, cytochrome oxidase, superoxide dismutase and copper chaperones that operate in both the periplasm and the cytoplasm, multicopper oxidases (MCOs) that oxidize Cu\(^+\) to less toxic Cu\(^{2+}\) to avoid its oxidative damage to proteins, DNA and cellular membranes.

To understand the interaction of Cu with these biological Cu binding molecules, the interaction of copper with ligands has been extensively studied for decades. Copper in biological systems can exist in either the reduced Cu\(^+\) (3d\(^{10}\)) state or the oxidized Cu\(^{2+}\) (3d\(^{9}\)) state. The reduction potential of +150 mV for Cu\(^{2+}/Cu^+\) makes Cu\(^+\) as the major oxidation state of copper in the
reducing environment of the cytosol (approximately −220 mV). Copper(I) is a soft acid while 
Cu^{2+} is a borderline acid; as such, first coordination shell ligands in copper-sensing or -transporting proteins are formed primarily by cysteine, methionine, and histidine residues. Copper(I) is relatively unique in biology in that it adopts thermodynamically stable complexes characterized with a low coordination number (n) of 2−4. In contrast, Cu^{2+} forms the higher number of coordination bonds in complexes with an n of 4−6. The resulting complexes have the tendency to exchange oxidation state and thus the coordination complexes within the biological systems.\textsuperscript{18,19}

Copper(II) ligands complexes with coordination number of 4 - 6 has many kinds of irregular coordination geometries.\textsuperscript{20} This phenomenon is caused by the “plasticity effect” as a result of the Jahn-Teller theorem regarding the structures of transition metal ion-ligand complexes which causes distortion from symmetrical octahedral geometries. The John-Taller theorem states that "A nonlinear polyatomic system in a spatially degenerate electronic state distorts spontaneously in such a way that the degeneracy is lifted and a new equilibrium structure of lower symmetry is attained".\textsuperscript{20} This means that"... stability and degeneracy are not possible simultaneously unless the molecule is a linear one ..."\textsuperscript{20}

Based on the theorem, other subtle ligand factors such as ligand constraints, steric hindrance and the strength of the ligands’ donor atoms will also affect the geometry of the coordination polyhedron around Cu^{2+}.\textsuperscript{20} For example, two closely related imidazole thioether compounds with a N\textsubscript{2}S\textsubscript{2} donor set form complexes of different geometry (pseudoctahedral or trigonal bipyramidal) for the difference of a single methyl group. One of the compounds also formed two different geometry (pseudoctahedral or trigonal bipyramidal) around Cu^{2+} depending on the
anion identity, further highlighting the effect of ligand factors.\textsuperscript{21} Given its d\textsuperscript{9} electronic configuration (t\textsuperscript{6}g\textsubscript{2} e\textsuperscript{3}g), the Jahn–Teller effect is prominent for octahedral Cu\textsuperscript{2+} complexes\textsuperscript{22} because an odd number of electrons occupy the two high energy e\textsubscript{g} orbitals. Distorting the geometry has the effect of lowering the energy of the system, although to a small extent, breaking the degeneracy of the e\textsubscript{g} set, reducing the symmetry, and leading to occupations at lower energy orbitals.\textsuperscript{23}

An example of a cuproenzyme is \textit{mushroom tyrosinase} (TY), a rate-limiting enzyme controlling the production of melanin from tyrosine. This enzyme has a dinuclear Cu\textsuperscript{2+} which are oxidized in a controlled manner during the production of melanin. As shown in (Figure 1.2), the two active-site Cu\textsuperscript{2+} ions are bound by six histidine residues. TY converts l-tyrosine to l-DOPA (L-3,4-dihydroxyphenylalanine) and subsequently l-DOPA to dopaquinone (Figure 1.2). Dopaquinone eventually forms melanin. Inhibition of TY therefore decreases melanin production and even leads to skin whitening. Developing TY inhibitors for inclusion in lotions is therefore gaining popularity in cosmetic industry.\textsuperscript{24}
Copper ion has been used as spectroscopic probe for other metalloenzyme such as carboxypeptidase A (CPA), a zinc ($d^{10}$) metalloenzyme. Such metal substitution strategies were introduced by Yamamura & Kaiser (1976). CPA exhibits peptidase, esterase, and thioester hydrolysis activities.\(^2\) When Cu\(^{2+}\) substitutes the Zn\(^{2+}\) in CPA, it yields [Cu]-CPA that no longer exhibits the peptidase and esterase activities but retains the thioester hydrolysis activity, and the unpaired electron in Cu(II) allows for more spectroscopic methods to be used.\(^3\) Spectroscopic studies have shown that Cu\(^{2+}\) coordination in CPA causes distortion of the N\(_2\)O\(_2\) tetrahedral geometry, explaining the loss of the peptidase and esterase activities, but not the ability to bind CPA inhibitors.\(^4\)

Copper ligand complexes have also been developed for diseases involving copper overloading. The pharmacological activities of such metal ligand complexes are dependent on the nature of the ligands and the metal ions. Among complexes of the first-row transition metal ions with a given ligand, copper complexes often have the highest binding efficiency, will be further discussed in the results section.

1.3. Cobalt Ion (II)
Cobalt exists primarily as Co\(^{2+}\) and Co\(^{3+}\) under biological conditions. Co\(^{2+}\), the only commonly-occurring d\(^7\) metal ion, is paramagnetic in both low or high spin, and can adopt either tetrahedral or octahedral geometry. Co\(^{3+}\), on the other hand, exhibits predominantly octahedral geometry, and therefore in low spin configuration is diamagnetic. Cobalt complexes have a variety of reported applications, ranging from the control of radical polymerisation to the catalysis of hydrogen-generation from water. There is also much current interest in the interaction of cobalt complexes with biological systems, in understanding their antioxidant behavior, anticancer activity, and potential uses as chaperones of cytotoxic agents.\(^{27}\)

Cobalt is less frequently encountered in metalloproteins than the other first-row transition metals such as manganese, iron, copper and zinc. However, as vitamin B\(_{12}\)’s central metal, it is an important cofactor in the vitamin-B\(_{12}\)-dependent enzymes involved in DNA synthesis, fatty acid and amino acid metabolism.\(^{28,29}\) Cobalt plays a key role in the normal function of the brain and nervous system, and the formation of red blood cells. In B\(_{12}\), the cobalt ion is found in the center of the tetrapyrrole ring, bonding to its four nitrogen atoms and a dimethylbenzimidazole group at the 5\(^{th}\) coordination site. The 6\(^{th}\) coordination site is occupied by one of four possible groups, a cyano group (-CN), a hydroxyl group (-OH), a methyl group (CH\(_3\)) or a 5’-deoxyadenosyl group with the latter two ligands coordinated through a Co-C\(_{sp3}\) bond.\(^{30}\)

In addition to its essential role as part of enzyme cofactor B\(_{12}\), cobalt is often used as a spectroscopically active substitute for Zn(II) in enzymes.\(^{31-35}\) Cobalt (II) has been extensively used as a spectroscopic probe in many proteins, mainly replacing zinc, but also substituting for iron, manganese and copper ions.\(^{31}\) The relatively short electronic relaxation times of high-spin
cobalt(II) makes this ion suitable as a paramagnetic probe for NMR spectroscopy. Metal binding sites in enzymes that contain catalytically active Zn(II) generally yield high-spin $S = 3/2$ Co(II) ions when substituted with cobalt, and these provide EPR spectra rich in information. The applicability of cobalt as a spectroscopic probe for the structure and function of zinc metalloenzyme active site is dependent on how close cobalt mirrors the properties of zinc.\textsuperscript{36} Co$^{2+}$ ions form 3- or 4-coordination bonds at enzyme active site with supporting groups similar to those for enzyme active site zinc. They include all O ligand groups (Asp/Glu), mixed O and N groups (Asp/Glu and His), mixed N and S groups (His and Cys) and all N groups (His).\textsuperscript{30} However, the difference in their $d$ orbital electron configuration ($d^7$ Co$^{2+}$ and $d^{10}$ Zn$^{2+}$) means that there are likely differences in the structure and affinity of these two metal ions interacting with ligands. Similar to zinc, cobalt forms octahedral, tetrahedral and trigonal bipyramidal geometries depending on the ligands.\textsuperscript{37}

Methionine aminopeptidases are ubiquitous enzymes that cleave the N-terminal methionine from newly translated polypeptide chains in both prokaryotes and eukaryotes. The enzyme from \textit{Salmonella typhimurium} is stimulated only by Co$^{2+}$, not by Mn$^{2+}$, Mg$^{2+}$, or Zn$^{2+}$. However, when purified, the enzyme does not contain significant amounts of any metal ions; enzymatically important cobalt is loosely associated and so the identity of the metal ion bound in vivo has not been firmly established. However, the enzyme from \textit{E.coli} has been shown by X-ray crystallography to contain cobalt ions. The \textit{E.coli} methionine aminopeptidase binds two Co$^{2+}$ ions in its active site.\textsuperscript{30} The enzyme has several weak absorption bands between 550 and 700 nm (d $\rightarrow$ d transitions of the d$^7$ Co$^{2+}$ ions).\textsuperscript{38} Understanding the interactions of cobalt with the active site residues, substrate and inhibitors are important for rational drug design targeting specific
cobalt metalloenzymes. In the enzyme, the two cobalt ions are coordinated by two Asp, one His, and two Glu residues, all conserved in several methionine aminopeptidases analyzed except for one of the Glu that is substituted by Gln in the Bacillus subtilis enzyme. The two cobalt ions bond to each other; each also bond to four other atoms, with one cobalt binding the ring N of the His, one O of the Glu204, one O of Glu235 and one O of Glu108. The other cobalt coordinates to four O atoms, one from Glu235, two from Asp97 and a fourth from Glu108. These Co-O and Co-N bonds occupy the equatorial bonds of the octahedral geometry except the one with the O atom of Glu235 that occupies the axial position (5th coordination) for each cobalt. The 6th coordination site at the active site is not occupied and likely bond to a solvent water molecule.

1.4. Manganese Ion (II)

Manganese (Mn) is a required enzyme cofactor in many enzymes. It plays a significant role in many biochemical processes in plants. It usually acts as an enzyme activator. It is easy to be replaced by other divalent metal cations (often Mg$^{2+}$). Therefore Mn has similar biochemical function as Mg and is involved in activating enzyme catalyzed reactions such as phosphorylation, decarboxylation, reduction and hydrolysis reactions. It affects processes such as respiration, amino acid synthesis, lignin biosynthesis and the level of hormones in plants.

Mn has a low affinity for binding ligands compared to other metal ions based on the order of the stability trend for complexes of bivalent transition metals, namely Mn < Fe < Co < Ni < Cu > Zn. Mn$^{2+}$ complexes are thermodynamically less stable than the other metal ions in the trend above because of the lack of ligand-field stabilization energy, which can be traced back to the symmetric $d^5$ electron configuration system of the Mn$^{2+}$ ion. For example, in for the
octahedral complex, the stabilization contributed by the three electrons in the \( t_{2g} \) is canceled by the two in the \( e_g \). Octahedral coordination makes the complex less stable and an unfavorable geometry for Mn\(^{2+}\).\(^{45}\)

An example of a manganese metalloenzyme is prolidase, a proline dipeptidase in humans and microorganisms and is important for recycling proline required for the production of collagen, a structural component of various tissues such as the skin. Manganese is therefore important for everyday skin health.\(^{46}\) Prolidase has a dinuclear Mn\(^{2+}\) and specifically cleaves the peptide bond between Xaa and Pro in Xaa-Pro dipeptides.\(^{30}\) Each active-site Mn\(^{2+}\) ion forms six coordination bonds thus assuming a distorted octahedral geometry. Two of the bonds are formed by an Asp and a Glu residue each of which acting as a bidentate ligand bridging the two Mn\(^{2+}\) ions. Two additional bonds are formed with a His (H) and a Glu (E) residue to one Mn\(^{2+}\) but with two Asp (D) residues to the other. The 5\(^{th}\) and 6\(^{th}\) coordination bonds on each Mn\(^{2+}\) are formed with a buffer component glycolate which acts as a bidentate chelator through its carboxyl and the hydroxyl oxygens (Figure 1.3).\(^{47}\)
In addition to its collagen production role, manganese also functions as an antioxidant in skin cells and other cell types. It helps protect skin against oxidative damage as well as damage from ultraviolet (UV) light. This is due to manganese being a redox-active metal and is found at the active site of the mitochondrial superoxide dismutase SOD2 (Mn-SOD). SOD1 in cytosol and SOD3 in extracellular matrix are the other known human isoforms of SOD which contain Cu-Zn and Fe, respectively, at the active site. Mn-SOD are also found in various bacteria. SOD catalyzes the conversion of superoxide radicals to hydrogen peroxide which can then be reduced to water by antioxidant enzymes such as catalase. Mn therefore helps protect cells against the toxicity of the superoxide free radicals.

Another example of a manganese metalloenzyme is arginase. Arginase is required in the liver for catalyzing the physiologically important final step in the urea cycle, facilitating the removal of nitrogenous waste during protein catabolism. The enzyme requires a dinuclear
manganese cluster to maintain proper function. As in prolidase, the two Mn\(^{2+}\) ions are bridged by the carboxyl groups of two Asp residues. Both Mn\(^{2+}\) also bond to additional residues including a His and an Asp residue each with one Mn\(^{2+}\) forming two bonds with the corresponding Asp. These two Mn\(^{2+}\) ions also coordinate with the substrate water molecule as its 5\(^{th}\) or 6\(^{th}\) coordination bond. As such, the two Mn\(^{2+}\) ions help orient and stabilize the water, allowing it to act as a nucleophile to attack L-arginine, hydrolyzing it into ornithine and urea.\(^{50}\)

In light of the essential functions that manganese metalloenzymes play, manganese deficiency in experimental animals are associated with biochemical and structural abnormalities and diseases. Deficient animals can be characterized by impaired insulin production, alterations in lipoprotein metabolism, an impaired oxidant defense system, and perturbations in growth factor metabolism. If the deficiency occurs during early development, there can be pronounced skeletal abnormalities and an irreversible ataxia. Several lines of evidence suggest that manganese deficiency may be a problem in some human populations.\(^{51}\)

Low dietary manganese or low levels of manganese in blood or tissue have also been associated with several chronic diseases in humans. For example, women with osteoporosis have decreased plasma or serum levels of manganese and also an enhanced plasma response to an oral dose of manganese, suggesting they may have lower manganese status than women without osteoporosis. Manganese deficiency also results in glucose intolerance similar to diabetes mellitus in some animal species.\(^{52,53}\)

Excess manganese can also pose a significant health risk. Manganese toxicity may result in multiple neurologic problems and is a well-recognized health hazard for people who inhale
manganese dust, such as welders and smelters.\textsuperscript{51,52} Unlike ingested manganese, inhaled manganese is transported directly to the brain before it can be metabolized in the liver.\textsuperscript{53} The symptoms of manganese toxicity generally appear slowly over a period of months to years. In its worst form, manganese toxicity can result in a permanent neurological disorder with symptoms similar to those of Parkinson's disease, including tremors, difficulty walking, and facial muscle spasms.\textsuperscript{54,55} Additionally, environmental or occupational inhalation of manganese can cause an inflammatory response in the lungs.\textsuperscript{56} Clinical symptoms of effects to the lung include cough, acute bronchitis, and decreased lung function.\textsuperscript{57}

1.5. Metalloenzymes (II)

Metalloenzymes are involved in an extensive range of biological processes, and their over-expression often coincides with various diseases. Metalloenzymes such as histone deacetylase (HDAC) play an integral role in the proliferation of tumor cells, angiogenesis and many other processes. Inhibiting HDAC therefore can be a mode of cancer treatment.\textsuperscript{58} The American Cancer Society estimates that in 2015 there were over 500,000 deaths in the United States that resulted from the four major cancers – colon and rectum, lung and bronchus, breast, and prostate.\textsuperscript{58} Developing new therapies to treat cancer is a major focus of the pharmaceutical industry as well as in academia, and metal-inhibitors are one potential therapeutic approach. Inhibition of metalloenzymes is often dependent on the ability of the inhibitor to bind the active site metal ion. Understanding the energetics of this active site metal ion-inhibitor interaction can aid in the design of potent and selective inhibitors.\textsuperscript{58} Current FDA approved metalloenzyme inhibitors are associated with many adverse effects such as nausea and blood disorders because the inhibitors also bind other metalloenzymes as well as other metal ion pools in the body.
Therefore identifying a way to target inhibitors to specific metalloenzymes is essential in minimizing adverse effects.\(^5\)

The interaction of metalloenzymes with inhibitors is often studied using the isolated enzyme. Such studies give information about the overall energetics of the interaction but do not allow for the extraction of the energetic contribution of individual components of the interaction – such as active site metal binding by the inhibitor. In addition, the complexity of making and using significant amounts of pure enzyme makes this approach impractical for most enzymes. On the other end of the spectrum of complexity, the energetics of the inhibitor binding to the free metal ion can be determined, but it does not equate the energetics of the inhibitor binding to the active site metal ion. A better alternative is to use an enzyme active site metal ion structural mimetic formed by a metal ion bound to a ligand consisting of the same or similar set of coordinating atoms as in the enzyme active site. In this study, we are examining the potential of three metal ions – \(\text{Cu}^{2+}\), \(\text{Co}^{2+}\), and \(\text{Mn}^{2+}\) with five different ligands to form suitable structural mimetics. Specific information about each of the five different ligands is given below. The interactions of each ligand with each of the metal ions and the interaction of the resulting metal-ligand complexes with metalloenzyme inhibitors are detailed in the following chapters.

1.6. Supporting Ligands

The ligands used in the study are DA2Im (N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid), NTA (nitrilotriacetic acid), TREN (tris(2-aminoethyl)amine), TPA (tris(2-pyridylmethyl)amine), and BPA (bis(2-picolyl)amine). In order for these ligands to be considered as a potential enzyme active site structural mimetic, they need to meet several requirements. It is imperative that the ligand chelates the metal ion (II) strongly and the resulting
complex remain intact upon binding to the inhibitor. If the inhibitor binds more strongly than the ligand for the metal ion (II), it could displace the ligand. A second requirement is that the stoichiometric ratio of the ligand (either tri- or tetra-dentate) to M\(^{2+}\) in the complex be 1:1. Note that the supporting ligand used is tri- or tetra-dentate and that Cu\(^{2+}\), Co\(^{2+}\), and Mn\(^{2+}\) each can form up to 6 coordination bonds in solution\(^{59}\). Therefore, a ML complex ensures that additional coordination sites are available on the M\(^{2+}\) ion for inhibitor interaction. However, an ML\(_2\) complex will not be suitable since no coordination site will be available to inhibitor binding. The final requirement is that the structural mimetic must be soluble at concentrations necessary for ITC study (0.1-10 mM). Buffer solutions chosen for the study include a 50 mM buffer (NEM (N-ethylmaleimide) or Tris (tris(hydroxymethyl)aminomethane)) containing 0.15 M NaCl at pH 6.80, 7.25 or 7.40 and a 60:40 by volume mixture of MeOH: buffer at pH 6.80, 7.25 or 7.40. These buffers are relatively close to biological systems when compared to pure organic solvents. The individual properties of the ligands are outlined below.

### 1.6.1 DA2Im

N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid (DA2Im) (Figure 1.5), a tertiary amine having two carboxylate groups (pK\(_{a1}\) = 2.72, pK\(_{a2}\) = 4.20) along with a single methylimidazole group (pK\(_{a3}\) = 8.09)\(^{59}\), was synthesized in Dr. Grice’s lab with his guidance.
DA2Im is a tetradebate ligand that can coordinate to transition metal ions with its carboxylic acid oxygens, tertiary amine, and imidazole nitrogen. It is designed to mimic the groups of a His and two Asp or Glu residues commonly found to bind to transition metal ions at zinc- or cobalt-containing enzymes.\textsuperscript{59}

1.6.2 NTA

Commercially available nitrilotriacetic acid (NTA) (Figure 1.6) is a tertiary amine with three acetate groups (pK\textsubscript{a1} = 1.87, pK\textsubscript{a2} = 2.53 and pK\textsubscript{a3} = 9.40)\textsuperscript{59} and thus can act as a tetradebate ligand. The acetate groups mimic Glu or Asp sides chains often seen involved in coordinating to enzyme active site metal ions.
1.6.3 TREN

Commercially available tris(2-aminoethyl)amine (TREN) (Figure 1.7) is a tetradentate ligand that binds \( M^{2+} \) with its three primary amines (\( pK_{a1} = 8.42, pK_{a2} = 9.42, pK_{a3} = 10.13 \)) and tertiary amine.\(^{59} \) Although primary amines are not commonly involved in coordinating to enzyme active site metal ions, they nonetheless represent groups that are capable of coordinating to metal ions when deprotonated and are potential candidates for creating enzyme active site metal ion structural mimetics.
1.6.4 TPA

![Diagram of TPA molecule]

**Figure 1.8** Tris(2-pyridylmethyl)amine (TPA).

Commercially available tris(2-pyridylmethyl)amine (TPA) (Figure 1.8) was explored as a potential ligand to form the enzyme active site structural mimetic. TPA is a tetradentate ligand that is a tertiary amine with three pyridylmethyl groups ($\text{pK}_{a1} = 2.55$, $\text{pK}_{a2} = 4.35$ and $\text{pK}_{a3} = 6.17$)\textsuperscript{59}, making it a potentially tetradentate ligand for chelating $M^{2+}$ through its three pyridine nitrogens and the tertiary amine nitrogen. The pyridyl nitrogens have properties close to the imidazole nitrogen of histidine residue commonly bound to enzyme active site metal ions.
1.6.5 BPA

Figure 1.9 Bis(2-picoly)amine (BPA).

BPA is a tridentate ligand that can coordinate to M$^{2+}$ with its two pyridyl nitrogens and the secondary amine. BPA (Figure 1.9) resembles TPA except that it is a secondary amine bonded to two rather than three 2-pyridylmethyl groups (pK$_{a1}$ = 1.75, pK$_{a2}$ = 2.41 and pK$_{a3}$ = 7.27). This makes BPA a tridentate ligand for chelating M$^{2+}$.

1.7. Solution Conditions

NEM was used as the buffer component for the study involving Co$^{2+}$ and Mn$^{2+}$. NEM was chosen for its pK$_{a}$ value of 7.67 and for its lack of affinity for transition metal ions. In the case of Cu$^{2+}$, due to its strong tendency to form Cu(OH)$_2$ precipitate thus its low solubility in aqueous solution, Tris buffer was used instead. Tris binds transition metal ions and therefore was able to solubilize Cu$^{2+}$ to high enough concentration necessary for the ITC study. However, the resulting affinity constants for Cu$^{2+}$ interacting with ligands were apparent (observed) constants, lower than it would be in the absence of Tris. As detailed in later sections, this would not affect the relative affinity of the five ligands for Cu$^{2+}$ nor the determination of the stoichiometry of the resulting Cu$^{2+}$ ligand complexes.
Tris, with its pK$_a$ of 8.1, is commonly used as a buffering system for the pH range of 7-9. Nevertheless, it should be noted that Tris shows copper-complexing capacity and henceforth will not only affect the apparent affinity of copper for other ligands but may also influence the stoichiometry in the complexes formed.$^{61}$ In this work, the associations of five small molecule ligands with Cu$^{2+}$, Co$^{2+}$, and Mn$^{2+}$ were examined utilizing Isothermal Titration Calorimetry (ITC) to identify ligands capable of forming binary complexes as potential structural mimetics for enzyme active site metal ion. First, the interaction of each of the five ligands with a given metal ion was examined in the buffer or the methanol: buffer mixture depending on their solubility in each.

The subsequent potential mimetics, i.e., the binary complexes as ML, were further examined in its interaction with the metal chelator and known enzyme inhibitor, 8-hydroxyquinoline (8-HQ). 8-HQ was chosen as the inhibitor molecule for its moderate metal ion affinity required for unambiguous determination of the feasibility of the binary complex as an enzyme active site structural mimetic. The thermodynamics of the binary complexes interacting with the enzyme active site inhibitor prototype, acetohydroxamic acid (AHA), was also evaluated using ITC and UV-Vis spectroscopy.

1.8. Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) is an efficient analytical method to determine the thermodynamic parameters for a given interaction and can be applied over a broad range of systems. ITC works by measuring the heat produced during a titration reaction. Upon titration of one reactant (titrant) placed in the syringe and injected in a series of small volumes (8-15 μL)
into the 1.4 mL of solution containing the other reactant (titrate), the difference in heat between the thermocouple reference cell and the sample cell is measured upon a series of injections. Since the titrant is injected in such small volumes, a large dilution factor is associated with the injection. The raw data from the experiment is plotted as the heat power (μcal/sec) versus time (minutes). The zone under each peak is calibrated and standardized to kcal/mole of titrant and plotted against the titrant: titrate molar ratio. This plot is fit with a non-direct least squares utilizing an appropriate binding model, for example, the one-set-of-sites or two-sets-of-sites models incorporated into the Origin software package\textsuperscript{62,63} Heat is either released (negative peaks) reflecting an exothermic reaction or absorbed (positive peaks) indicating an endothermic reaction. The heat of reaction is measured through the amount of power required to maintain a difference in temperature between the reference and sample cells equal to zero. With subsequent additions of titrant, the heat signal diminishes, indicating that binding is near completion. The concentrations of titrant and titrate are chosen so that only a portion of titrant binds upon each titration. This makes the final binding curve of heat generated per injection versus titrant: titrate molar ratio dependent on the molar enthalpy change for the binding response (ΔH°), the affinity consistent (K_a), and the stoichiometric proportion between the two interacting species in the subsequent complex (n). These parameters would then be able to be utilized to compute other valuable parameters, for example, the standard Gibbs free energy change (ΔG°), the equilibrium dissociation constant (K_d) and the entropy change for the reaction (ΔS°). Further information about ITC analysis is provided in the Experimental Methods in Chapter 6. \textsuperscript{59,63}

A c value is used to determine the acceptable range of concentrations to be used in a titration experiment. It is defined as the products of stoichiometry (n, ratio of the titrant to titrate in the
complex formed), equilibrium binding constant ($K_a$) and the concentration of titrate in the form of $c = n K_a C$. The optimal $c$-values is 1-1000 but the ideal range is 5-500. This ensures that the resulting binding isotherm best defines thus the most accurate extraction of a set of binding parameters including $n$, $K_a$ and $\Delta H$ (molar enthalpy change of the binding reaction). An ideal titration curve is shown in Figure 1.10 has a unique shape. Notably, to shift binding to completion, the titrant concentration is chosen to be at least ten times the titrate concentration for a 1:1 binding or 20 times the titrate concentration for a 2:1 binding.62

Figure 1.10. An S-shaped curve is the ideal shape of a binding isotherm for accurate extraction of binding parameters. The line is a curve that best fit the data and is obtained from fitting to a one set of sites binding model.62

Chapter 2: ITC Studies of the Interactions of Ligands with Metal Ions(II)

2.1 ITC Studies of the Interactions of Ligands with Cu$^{2+}$

2.1.1 N-(2-(1-methylimidazolyl)methyl)iminodiacetic Acid (DA2Im) Interaction with Cu$^{2+}$

No structure of DA2Im bound with a metal ion is reported in the literature. The interaction of DA2Im with Cu$^{2+}$ was studied in buffer but not in MeOH:buffer mixture due to the low solubility of DA2Im in the latter. Injection of DA2Im (8.8 mM) into CuCl$_2$ (0.33 mM) in 50
mM Tris buffer (0.15 M NaCl, pH 7.4) resulted in a binding isotherm that, after omitting the first six data points, fit well to a one-set-of-sites binding model (Figure 2.1). The overall binding stoichiometry was close to 2 (n = 1.84 ± 0.05) indicating two DA2Im binding each Cu$^{2+}$ ion. The resulting $K_a$ was $(3.15 \pm 0.07) \times 10^3$ M$^{-1}$. Given the fact that the first 5 peaks exhibited similar heat suggests that the first DA2Im bound Cu$^{2+}$ with very strong affinity such that all DA2Im injected bound Cu$^{2+}$. This prevented the determination of the binding constant for the first DA2Im. However, the much stronger affinity of the first DA2Im also made it possible to form a 1:1 (DA2Im:Cu$^{2+}$) binary complex without the presence of a significant percentage of a 2:1 (DA2Im:Cu$^{2+}$) complex by controlling the molar ratio of DA2Im:Cu$^{2+}$ at 1:1. To determine the $K_a$ for the first DA2Im binding, we lowered the concentration of DA2Im to 1 mM and titrated it into 0.1 mM CuCl$_2$. The resulting binding isotherms fit well to a one-set-of-sites model (Figure 2.2) and yielded a very high apparent affinity constant of $(4.35 \pm 0.06) \times 10^8$ M$^{-1}$ and an n value of 0.97 ± 0.01 DA2Im per Cu$^{2+}$ (Table 2.1), confirming our hypothesis that DA2Im and Cu(II) formed a strong Cu(DA2Im) binary complex. At this low concentration, the second binding which has an affinity constant of $(3.15 \pm 0.07) \times 10^3$ M$^{-1}$ was not observed. In summary, the Cu$^{2+}$ ion was capable of binding one DA2Im very strongly ($K_a \sim 10^8$ M$^{-1}$) and a second DA2Im weakly ($K_a \sim 10^3$ M$^{-1}$). Notably, ITC date was only able to determent molar ratio between the ligand and the metal and it does not show if the Cl$^-$ bind or not.
**Figure 2.1.** Raw data (top panel) and binding isotherm (bottom panel) for titrations of 8.8 mM DA2Im into 0.33 mM CuCl₂ in Tris buffer, pH 7.4, 25°C.
Figure 2.2. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 1 mM DA2Im into 0.1 mM CuCl₂ in Tris buffer at pH 7.4, 25 °C.

Table 2.1 Thermodynamic parameters from ITC study of DA2Im interaction with Cu²⁺ in 50 mM Tris buffer (0.150 M NaCl, pH 7.40).*

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>Kₐ (M⁻¹)</th>
<th>K_d (μM)</th>
<th>ΔG° (kcal/mol)</th>
<th>ΔH° (kcal/mol)</th>
<th>TΔS° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>High Conc.</td>
<td>1.84 ± 0.05</td>
<td>(3.15 ± 0.07) x10²</td>
<td>(3.18 ± 0.07) x10²</td>
<td>-4.8 ± 0.0</td>
<td>-4.8 ± 0.1</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td>Low Conc.</td>
<td>0.97 ± 0.01</td>
<td>(4.35 ± 0.06) x10⁸</td>
<td>2.29 ± 0.03</td>
<td>-9.74 ± 0.3</td>
<td>3.42 ± 0.17</td>
<td>13.15 ± 3.30</td>
</tr>
</tbody>
</table>

* Titrations of 8.8 mM DA2Im into 0.33 mM CuCl₂ and titrations of 1 mM DA2Im into 0.1 mM CuCl₂.
† The n value obtained at the high concentrations indicate the total number of DA2Im bound but the Kₐ obtained here was for the second DA2Im as it resulted from deleting the first six data points corresponding to the first DA2Im binding.

2.1.2 Nitrilotriacetic Acid (NTA) Interaction with Cu²⁺

The interaction of NTA with Cu²⁺ was investigated in buffer but not in MeOH:buffer because NTA was insoluble in the latter. Experiments were performed by titrating NTA into CuCl₂. The resulting binding isotherms fit well to a one-set-of-sites model (Figure 2.3) and yielded a high apparent affinity constant of (4.6 ± 0.3) x10⁶ M⁻¹ and an n value of 1.01 ± 0.03 NTA per Cu²⁺ (Table 2.2), indicating the formation of a Cu(NTA) binary complex. This made
NTA a potentially suitable ligand for creating an enzyme active site copper structural mimetic. It should be noted that NTA and Cu$^{2+}$ binding isotherm was endothermic, unlike for DA2Im for which it was exothermic, likely the result of the fact the 2-(1-methylimidazolyl)methyl group in DA2Im is replaced with an acetate group in NTA. The endothermic heat suggests loss of overall bonding in the process of NTA and Cu$^{2+}$ desolvation and subsequent formation of complex with each other. The NTA and Cu$^{2+}$ complex formation evidently was driven by entropy as a result of the entropic gain from releasing water molecules from the solvation shell of the carboxyl anions and the Cu$^{2+}$ cation.
Figure 2.3. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 1.5 mM NTA into 0.15 mM CuCl₂ in Tris buffer, pH 7.4, 25 °C.

Table 2.2. Thermodynamic parameters from ITC study of NTA interaction with Cu²⁺ in 50 mM Tris buffer (0.150 M NaCl, pH 7.40).*

<table>
<thead>
<tr>
<th>n</th>
<th>Kₐ</th>
<th>K_d</th>
<th>ΔG°</th>
<th>ΔH°</th>
<th>TΔS°</th>
</tr>
</thead>
<tbody>
<tr>
<td>(NTA/Cu²⁺)</td>
<td>(M⁻¹)</td>
<td>(μM)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
</tr>
<tr>
<td>1.01 ± 0.03</td>
<td>(4.6 ± 0.3) x 10⁶</td>
<td>0.22 ± 0.01</td>
<td>-9.09 ± 0.04</td>
<td>1.71 ± 0.02</td>
<td>10.8 ± 0.1</td>
</tr>
</tbody>
</table>

*Titration of 1.5 mM NTA into 0.15 mM Cu²⁺

2.1.3 Tris(2-aminoethyl)amine (TREN) Interaction with Cu²⁺

The interaction of commercially available tris(2-aminoethyl)amine (TREN) with Cu²⁺ has been studied by Kay et al.⁶³ They reported the crystal structure of Cu(TREN) with one 2-aminoethyl amine arm deprotonated. Another study showed a crystal structure of two Cu²⁺ bound to one TREN and two Cl⁻ with two BPh₄ as the counter ions in the formula of [Cu₂(tren)₂Cl₂](BPh₄)₂.⁶⁴ ITC experiments with TREN consisted of TREN being titrated into a
solution of copper in either 50 mM Tris buffer (0.15 M NaCl, pH 7.40) or 60:40 (by volume) MeOH: buffer (50 mM Tris, 0.15 M NaCl) mixture at pH 7.40. Titration of 0.15 or 0.5 mM TREN into 0.015 or 0.05 Cu$^{2+}$ in buffer or in MeOH: buffer, respectively demonstrated that the interaction was exothermic and binding isotherms fitted well to a one-set-of-sites binding model in both solution conditions (Figures 2.4 and 2.5). Fitting yielded an n value of 1.07 ± 0.05 TREN per Cu$^{2+}$ in buffer and 1.04 ± 0.0 TREN per Cu$^{2+}$ in methanol:buffer mixture (Table 2.3), indicating that a binary complex of Cu(TREN) has formed in either medium. This result agreed with the finding by A. Bencini et al. that copper (II) yields a mononuclear trigonal-bipyramidal complexes with TREN. The apparent binding affinity ($K_a$) of Cu$^{2+}$ for TREN was (2.3 ± 3.1) ×10$^8$ M$^{-1}$ in buffer or (4.8 ± 1.8) ×10$^7$ M$^{-1}$ in MeOH:buffer. The weaker affinity in buffer can be explained by the competition of protons for binding the tertiary and the three primary amine groups. In MeOH:buffer, the presence of methanol disfavors the protonation of the amines thus less extensive proton competition. This is consistent with stronger affinity in methanol:buffer mixture. Overall, in both media, TREN formed a strong binary complex with Cu$^{2+}$. The strong affinity makes TREN in the binary complex less prone to be displaced by the inhibitor when the complex is used for the inhibitor binding study.

**Table 2.1.** Thermodynamic parameters from ITC study of TREN interaction with Cu$^{2+}$ in either 50 mM Tris buffer (0.150 M NaCl, pH 7.40) or 60:40 (by volume) mixture of MeOH: 50 mM Tris buffer (0.150 M NaCl, pH 7.40).*

<table>
<thead>
<tr>
<th>Solvent</th>
<th>n (TREN/Cu$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G°$ (kcal/mol)</th>
<th>$\Delta H°$ (kcal/mol)</th>
<th>$T\Delta S°$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>1.1 ± 0.1</td>
<td>(4.8 ± 1.8) ×10$^7$</td>
<td>0.02 ± 0.01</td>
<td>-10.4 ± 0.2</td>
<td>-2.7 ± 0.1</td>
<td>7.7 ± 0.2</td>
</tr>
<tr>
<td>MeOH:Buffer</td>
<td>1.0 ± 0.0</td>
<td>(2.3 ± 3.1) ×10$^8$</td>
<td>0.01 ± 0.01</td>
<td>-11.0 ± 0.9</td>
<td>-3.4 ± 0.3</td>
<td>7.6 ± 0.9</td>
</tr>
</tbody>
</table>

*Titrations of (0.15 or 0.5) mM TREN into (0.015 or 0.05) Cu$^{2+}$ in buffer and MeOH:Tris buffer, respectively.
Figure 2.4. Raw data (top panel) and binding isotherm (bottom panel) for titration of 0.15 mM TREN into 0.015 mM CuCl$_2$ in Tris buffer, pH 7.4, 25 °C.
**Figure 2.5.** Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.5 mM TREN into 0.05 mM CuCl$_2$ in 60:40 (by volume) mixture of MeOH:Tris buffer, pH 7.4, 25 °C.

### 2.1.4 Tris(2-pyridylmethyl)amine (TPA) interaction with Cu$^{2+}$

TPA was found to be insoluble in Tris buffer, pH 7.4 at the concentration necessary for ITC experiments; therefore, ITC experiments were carried out in 60:40 (by volume) MeOH:Tris buffer, pH 7.4. When 0.75 mM of TPA was titrated into 0.075 mM of CuCl$_2$ the resulting data was fit to a one-set-of-sites binding model (Figure 2.6), which yielded an apparent affinity constant ($K_a$) of $(3 \pm 2) \times 10^7$ M$^{-1}$ and a stoichiometric ratio of 1.21 ± 0.03 TPA per Cu$^{2+}$, corresponding approximately to a complex of 1:1 (TPA: Cu$^{2+}$) molar ratio (Table 2.4). This stoichiometric ratio met the requirement for a 1:1 binary complex for it to serve as an enzyme active site copper structural mimetic. X-ray crystallographic studies suggest that TPA, a tripodal tetradeptate ligands, forms a square pyramidal Cu$^{2+}$ complex in the presence of Cl$^-$ ion which
forms the 5th coordination bond in the complex.66 Replacing a methylene with an ethylene group linking the central aliphatic nitrogen to the pyridyl group in TPA instead leads to the formation of a trigonal bipyramidal complex also having Cl− forming the 5th coordination bond.66 This suggests that ligand constraints is responsible for the markedly different structural behavior and also redox and electronic properties of the copper(II) complex. Regardless of the geometry, this work indicates that Cu(II) forms 1:1 complex with TPA. This is confirmed by Hirotaka Nagao et al. who also reported a Cu(II) TPA complex with the formula of [Cu(TPA)(H2O)]^{2+} at pH 7.0. Therefore, out ITC data is consistent with literature reports in the formation of a 1:1 (Cu^{2+} : TPA) complex.67

**Figure 2.6.** Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.75 mM TPA into 0.075 mM CuCl2 in 60:40 (by volume) mixture of MeOH:Tris buffer, pH 7.4, 25 °C.
Table 2.4. Thermodynamic parameters from ITC study of TPA interaction with Cu$^{2+}$ in 60:40 (by volume) mixture of MeOH:Tris buffer, pH 7.4*  

<table>
<thead>
<tr>
<th>n (TPA/Cu$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/(mol·K))</th>
<th>$T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.21 ± 0.03</td>
<td>(3.0 ± 2.0) x10^7</td>
<td>0.05 ± 0.04</td>
<td>-10.1 ± 0.5</td>
<td>-5.4 ± 0.8</td>
<td>16.0 ± 4.0</td>
<td>5.0 ± 1.0</td>
</tr>
</tbody>
</table>

*Titrations of 0.75 mM TPA into 0.075 Cu$^{2+}$

2.1.5 Bis(2-picoly)amine (BPA) Interaction with Cu$^{2+}$

Bis(2-picoly)amine (BPA) (Figure 1.9) was studied as a potential ligand to develop an enzyme active site structural mimic. BPA is a well-known tridentate ligand capable of donating three electron pairs to a cationic metal center. Its geometry and its conformational flexibility gives this ligand a strong affinity for biologically interesting Cu$^{2+}$ and Zn$^{2+}$ ions. The tridentate amine – metal chelation also leaves basic coordination positions free of counterions.\(^6^8\) Cu(II) is known to form complexes in solution in coordination numbers of 4, 5, or 6.\(^4^2\) Therefore, with a tetradebate ligand, Cu$^{2+}$ is more likely to form a 1:1 (Cu$^{2+}$:ligand) complex and the resulting CuL complex will still have two coordination sites remaining to interact with other coordinating atoms such as from a bidentate ligand, amenable to serving as an enzyme active site copper structural mimic. For a tridentate ligand, it is likely that Cu$^{2+}$ will form a 2:1 (Ligand:Cu$^{2+}$) complex, leaving no or insufficient coordination sites for inhibitor binding since the octahedral geometry preferred by Cu$^{2+}$ provide only six coordination sites, unless the second ligand binds with orders of magnitude weaker affinity than the first ligand in which case a strong 1:1 complex can be formed.

The interaction of BPA with Cu$^{2+}$ was studied in both the buffer and the MeOH:buffer mixture by titrating BPA into CuCl$_2$. Two buffers, Tris and NEM, were used in the study. ITC experiments were done by titrating BPA into CuCl$_2$ at various concentrations such as 10 mM BPA into 0.5 mM Cu$^{2+}$ or 0.3 mM BPA into 0.03 mM Cu$^{2+}$. (Figure 2.7). Binding isotherms
from experiments performed in NEM solvent systems (buffer or MeOH:buffer mixture) were fit to a one-set-of-sites binding model, while those performed in Tris solvent systems (buffer or MeOH:buffer mixture) were fit to a sequential binding model with $n$ equal to 2 (Figure 2.7). In Tris solvents, the stoichiometry of 2 is consistent with the crystal structure in the presence of $\text{BF}_4^-$ as counter ion as reported by Palaniandavar et al. \textsuperscript{21} Another crystallographic study also showed that Cu(II) bound two BPA forming $\text{Cu(BPA)}_2(\text{ClO}_4)_2 \cdot 2\text{H}_2\text{O}$.\textsuperscript{69} In Tris buffer or the MeOH:Tris buffer mixture, the ITC binding curves suggest that the two BPA molecules bound Cu$^{2+}$ with different affinity (Table 2.5). In Tris buffer, the second BPA bound Cu$^{2+}$ with 3-orders of magnitude lower affinity than the first BPA. However, in MeOH:Tris, the second BPA bound 2-orders of magnitude more weakly than the first. Weaker BPA binding has also been reported for Zn$^{2+}$ except that Zn$^{2+}$ binds the second BPA with 4-orders of magnitude lower affinity than it does the first BPA.\textsuperscript{59} This is consistent with the known difference between Cu$^{2+}$ and Zn$^{2+}$ in that Cu$^{2+}$ is capable of hexacoordination.\textsuperscript{59}

The results in the NEM solvent systems (NEM buffer or MeOH:NEM buffer mixture) differed from those in the Tris solvent systems. The binding curves suggest that only one BPA molecule bound Cu$^{2+}$ with no evidence for the presence of a second BPA. The affinity of the BPA was one-order of magnitude higher in NEM buffer than in the MeOH:NEM mixture (Table 2.5). This difference in the types of complexes formed in Tris versus NEM buffer can be attributed to the fact that much higher concentrations of BPA and Cu$^{2+}$ were used in Tris buffer given the presence of Tris competition with BPA to bind Cu$^{2+}$. In Tris the higher concentrations helped detect the weak binding of a second BPA while in NEM the lower concentrations allowed only the detection of the stronger binding of the first BPA. The difference in the metal ion ability
of NEM versus Tris explains the weaker BPA affinity in the MeOH:buffer mixture using Tris ($K_a = (1.8\pm0.2) \times 10^5\ M^{-1}$) than using NEM as buffer ($K_a = (2.73\pm0.68) \times 10^6\ M^{-1}$).

Given that the second BPA binds Cu$^{2+}$ with orders of magnitude weaker affinity than the first BPA, a strong Cu(BPA) binary complex can be formed by mixing BPA with CuCl$_2$ at 1:1 molar ratio and used to test its potential as an enzyme active site copper structural mimic, specifically in its ability to interact with a metalloenzyme inhibitor.

It should also be noted that in a given buffer systems (Tris or NEM), BPA bound Cu$^{2+}$ with lower affinity in the presence of MeOH, given that $K_a$ is $(5.0\pm2.0) \times 10^6\ M^{-1}$ in NEM buffer but $(2.73\pm0.68) \times 10^6\ M^{-1}$ in MeOH:NEM mixture or $(3.3\pm1.5) \times 10^7\ M^{-1}$ in Tris but $(1.8\pm1.0) \times 10^5\ M^{-1}$ in MeOH:buffer mixture for both the first and the second BPA (Table 2.5). This was an unexpected result since methanol lowers the pK$_b$ of nitrogeneous bases$^{70}$, therefore less deprotonation would be coupled to copper coordination in MeOH:buffer than in buffer. Another factor must be responsible for the lower affinity in the presence of methanol. One possibility is the difference in Cu$^{2+}$ ion solvation. In the presence of methanol, Cu$^{2+}$ is less solvated by water and thus the gain of entropy upon release of the solvated water when Cu$^{2+}$ binds BPA is less in the presence of methanol.
Figure 2.7. Raw data (top panel) and binding isotherm (bottom panel) for titration of a) 10 mM BPA into 0.5 mM CuCl$_2$ in Tris buffer, pH 7.4, 25 °C. b) 0.08 mM BPA into 0.004 mM CuCl$_2$ in NEM buffer, pH 6.80, 25 °C. c) 10 mM BPA into 0.5 mM CuCl$_2$ in 60:40 (by volume) mixture of MeOH:Tris buffer, pH 7.40, 25 °C. d) 0.3 mM BPA into 0.03 mM CuCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 6.80, 25 °C.
Table 2.5. Thermodynamic parameters from ITC study of BPA titration into CuCl$_2$ in buffer and

<table>
<thead>
<tr>
<th>Solvent</th>
<th>n (BPA/Cu$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$ΔG^o$ (kcal/mol)</th>
<th>$ΔH^o$ (kcal/mol)</th>
<th>TΔS$^o$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Buffer</td>
<td>Sequential Binding Sites $n=2$</td>
<td>$(3.3 \pm 1.5) \times 10^7$</td>
<td>$0.03 \pm 0.0$</td>
<td>$-10.3 \pm 0.0$</td>
<td>$(-2.38\pm0.05) \times 10^3$</td>
<td>$-7.9 \pm 0.0$</td>
</tr>
<tr>
<td>NEM Buffer</td>
<td>$1.03 \pm 0.14$</td>
<td>$(5.0 \pm 2.0) \times 10^6$</td>
<td>$0.20\pm0.06$</td>
<td>$-9.13 \pm 0.18$</td>
<td>$-6.70\pm0.05$</td>
<td>$2.4 \pm 0.2$</td>
</tr>
<tr>
<td>MeOH:Tris Buffer</td>
<td>Sequential Binding Sites $n=2$</td>
<td>$(1.8 \pm 0.2) \times 10^2$</td>
<td>$6.4 \pm 3.0$</td>
<td>$-7.1 \pm 0.3$</td>
<td>$(5.3\pm1.0) \times 10^2$</td>
<td>$-7.6 \pm 0.4$</td>
</tr>
<tr>
<td>MeOH:NEM Buffer</td>
<td>$0.85 \pm 0.06$</td>
<td>$(5.0\pm4.2) \times 10^3$</td>
<td>$(3.0\pm 2.1) \times 10^2$</td>
<td>$-5.0 \pm 0.5$</td>
<td>$(-9.0\pm4.3) \times 10^3$</td>
<td>$-4.0 \pm 0.4$</td>
</tr>
</tbody>
</table>

MeOH (60:40 by volume) mixture of MeOH:Tris buffer, pH 7.4, 25 °C.*

* From titration of 10 mM BPA into 0.5 mM Cu$^{2+}$ in Tris and MeOH:Tris buffer, 0.08 mM BPA into 0.004 mM Cu$^{2+}$ in NEM buffer, and 0.3 mM BPA into 0.03 mM Cu$^{2+}$ in MeOH:NEM buffer.

2.2 ITC Studies of the Ligands Interactions with Co$^{2+}$

2.2.1 Tris(2-aminoethyl)amine (TREN) Interaction with Co$^{2+}$

The interaction of Co$^{2+}$ with TREN to form Co$^{2+}$-TREN complex has been reported in two solutions, water and in dimethysulfoxide (DMSO), based on absorbance spectroscopy.\textsuperscript{71, 72} In both solutions, Co$^{2+}$ forms a 1:1 strong binary complex with TREN. In DMSO solution, a weak association of a second TREN has also been implicated.\textsuperscript{72} In this work, to better mimic physiological conditions, the interaction of TREN with Co$^{2+}$ was studied in buffer and a MeOH:buffer mixture at pH 6.80 and 7.25. In all four-solution conditions, the binding isotherms from titrations of TREN into CoCl$_2$ fitted well to a one-set-of-sites binding model (Figures 2.8, 2.9, 2.10, and 2.11). In all conditions except in buffer at pH 6.80, the fit yielded a stoichiometric ratio of a 1:1 binary complex Co$^{2+}$-TREN (Table 2.1). However, an n value of $0.54 \pm 0.05$ TREN per Co$^{2+}$ was obtained in buffer at pH 6.80 (Table 2.6), indicating the formation of a 1:2 (TREN:Co$^{2+}$) ternary
complex. Dicobalt (II) complexes have indeed been reported in aqueous solutions and a μ-peroxo bridge linking the two cobalt ions is known.\textsuperscript{73} An additional bridge through a μ-hydroxo has also been reported when coordination site is available.\textsuperscript{73} A direct Co\textsuperscript{2+–Co\textsuperscript{2+}} bond has also been reported for some dicobalt complexes in pure organic solvent or under anaerobic condition but none has been reported in aqueous solution. Formation of a μ-peroxo bridge in Co\textsuperscript{2+} complexes is dependent on many factors such as ligand identity, pH, and solvent polarity.\textsuperscript{72,75} The formation of cobalt a peroxo-bridge favors higher pH and more polar solvent. Therefore the ternary complex (Co\textsuperscript{2+})\textsubscript{2}-TREN in buffer at pH 6.80 likely formed as a result of a μ-peroxo bridge. A partial if not complete charge transfer from Co\textsuperscript{2+} to the dioxygen makes the binding of a dioxygen more likely to form\textsuperscript{73}, which could be due in part to the tertiary amine groups that have been reported to stabilize the lower (II) oxidation state of cobalt in both aqueous and non-aqueous solutions.\textsuperscript{73–75} Given the tendency of Co\textsuperscript{2+}-TREN complex to form μ-peroxo bridge, there was a possibility that the ITC result of a 1:1 (TREN: Co\textsuperscript{2+}) complex formed in buffer at pH 7.25 and in MeOH:buffer mixture at both pH values corresponded to a (Co\textsuperscript{2+})\textsubscript{2}(TREN)\textsubscript{2} rather than a Co\textsuperscript{2+}-TREN complex. A (Co\textsuperscript{2+})\textsubscript{2}(TREN)\textsubscript{2} complex has indeed been reported and has been shown to act as a reversible O\textsubscript{2} carrier without permanently oxidizing Co\textsuperscript{2+}.\textsuperscript{72,76} It has also been shown that the μ-peroxo bridge is favored only at higher pH.\textsuperscript{76} Since no coupled slow process was observed in the ITC binding isotherm, if μ-peroxo and μ-hydroxo bridging did occur, they likely had fast kinetics that rendered them integral to the process of Co\textsuperscript{2+} binding to TREN. On the other hand, the presence of methanol in the MeOH:buffer mixture lowers the solvent polarity and is therefore expected to disfavor the
μ-peroxo bridge formation, making a (TREN)Co$^{2+}$($μ$-O$_2$)Co$^{2+}$ (TREN) complex in the MeOH:buffer mixture less likely. Additionally, a (Co$^{2+}$)$_2$-TREN complex with two cobalt (II) coordinating to the same ligand was not observed at pH 7.25 nor in methanol:buffer at either pH indicate that the (Co$^{2+}$)$_2$TREN complex was favored by increased proton competition as was true at the lower pH and in the absence of methanol.

**Figure 2.8.** Raw data (top panel) and binding isotherm (bottom panel) for titrations of 10 mM TREN into 1 mM CoCl$_2$ in NEM buffer, pH 6.80, 25 °C.
Figure 2.9 Raw data (top panel) and binding isotherm (bottom panel) for titrations of 10 mM TREN into 1 mM CoCl₂ in NEM buffer, pH 7.25, 25 °C.
Figure 2.10 Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.4 mM TREN into 0.04 mM CoCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 6.80, 25 $^\circ$C.
Figure 2.11. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.4 mM TREN into 0.04 mM CoCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 7.25, 25 °C.

Regardless of the types of complex formed between Co$^{2+}$ and TREN, increasing pH from 6.80 to 7.25 increased Co$^{2+}$ affinity for TREN up to an order of magnitude (by 4- and 14-fold) in buffer and MeOH:buffer mixture, respectively. The different pH dependence of affinity in buffer versus in MeOH:buffer likely reflects the shift in binding mode (2:1 versus 1:1 complex) in buffer. At a given pH, the interaction was two-orders of magnitude stronger in MeOH:buffer mixture ($K_a = (2 \pm 1) \times 10^6$ M$^{-1}$ at pH 6.8 and $(3 \pm 2) \times 10^7$ M$^{-1}$ at pH 7.25) than in buffer ($K_a = (2.9 \pm 0.4) \times 10^4$ M$^{-1}$ at pH 6.80 and $(1.45 \pm 0.04) \times 10^5$ M$^{-1}$ at pH 7.25) (Table 2.6). These trends can be explained by the reduced proton competition for the amine groups at the higher pH and/or in the presence of methanol. The need of the primary and tertiary ammonium ions to deprotonate in buffer
to coordinate to Co\(^{2+}\) also explains the overall endothermic binding heat in buffer at pH 7.25. Binding of TREN to Co\(^{2+}\) in buffer at pH 7.25 was therefore entropically driven as a result of the favourable entropic contribution from desolvation of the metal ion and the ammonium ions. In buffer at pH 6.80, due to each TREN bonding two Co\(^{2+}\) ions in forming the ternary complex which more than offset the endothermic TREN deprotonation heat, the binding heat was exothermic and the reaction was enthalpically driven. These results indirectly support the claim that a different complex formed at the two pH in buffer. In the MeOH:buffer mixture, the formation of the 1:1 complex was also enthalpically driven coupled with a small favourable (pH 6.80) or unfavourable (pH 7.25) entropic contribution. This was consistent with the reduced need to deprotonate in the presence of methanol. The need to deprotonate also explained the orders of magnitude smaller conditional binding constant determined with ITC as compared with the formation constants reported in the literature (1.20×10\(^{13}\) M\(^{-1}\) in water and 1.45×10\(^{12}\) M\(^{-1}\) in DMSO) obtained via potentiometric method as the previously reported formation constants do not take into account the ligand protonation equilibria.\(^{71,72,76}\)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>pH</th>
<th>N</th>
<th>(K_d) (M(^{-1}))</th>
<th>(K_d) (μM)</th>
<th>(\Delta G^\circ) (kcal/mol)</th>
<th>(\Delta H^\circ) (kcal/mol)</th>
<th>(T\Delta S^\circ) (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NEM Buffer</td>
<td>6.8</td>
<td>0.54 ± 0.05</td>
<td>(2.9 ± 0.4) (\times) 10(^4)</td>
<td>35 ± 5</td>
<td>-6.09 ± 0.08</td>
<td>-10.6 ± 0.9</td>
<td>-4.5 ± 0.9</td>
</tr>
<tr>
<td>MeOH:NEM Buffer</td>
<td>7.2</td>
<td>1.09 ± 0.01</td>
<td>(1.45 ± 0.04) (\times) 10(^5)</td>
<td>6.9 ± 0.2</td>
<td>-7.04 ± 0.02</td>
<td>6.03 ± 0.04</td>
<td>13.07 ± 0.04</td>
</tr>
<tr>
<td>NEM Buffer</td>
<td>6.8</td>
<td>0.95 ± 0.04</td>
<td>(2 ± 1) (\times) 10(^6)</td>
<td>0.4 ± 0.2</td>
<td>-8.7 ± 0.3</td>
<td>-6.3 ± 1.0</td>
<td>2 ± 1</td>
</tr>
<tr>
<td>MeOH:NEM Buffer</td>
<td>7.2</td>
<td>1.02 ± 0.03</td>
<td>(3 ± 2) (\times) 10(^7)</td>
<td>0.04 ± 0.03</td>
<td>-10.0 ± 0.4</td>
<td>-13 ± 2</td>
<td>-3 ± 2</td>
</tr>
</tbody>
</table>

* Titrations of 10 mM TREN into 1 mM Co\(^{2+}\) in NEM, titrations of 0.4 mM TREN into 0.04 mM Co\(^{2+}\) in MeOH:NEM buffer. Both at pH(6.8 and 7.25) \(^{71,72,76}\)

Table 2.6 Thermodynamic parameters from ITC study of TREN interaction with Co\(^{2+}\) in buffer and MeOH 60:40 (by volume) mixture of MeOH:NEM buffer, pH (6.80, 7.25) 25 °C.*

In summary, ITC results indicated that Co\(^{2+}\) formed a 1:1 complex in buffer at pH 7.25 and MeOH:buffer mixture at pH 6.80 and 7.25, and a (Co\(^{2+}\))\(_2\)-TREN ternary complex in buffer at pH
6.80. Unlike Co$^{2+}$, Zn$^{2+}$ and Cu$^{2+}$ form a Zn$^{2+}$-TREN and Cu$^{2+}$-TREN binary complex in both the buffer and the methanol:buffer mixture, Zn$^{2+}$ at NEM solvent at pH 6.80 (No pH 7.25 data available)$^{59}$ and Cu$^{2+}$ at Tris solvent at pH 7.40. Despite this difference, Co$^{2+}$ bound TREN with similar affinity as Zn$^{2+}$ $^{59}$ and Cu$^{2+}$ do (Table 2.3).

2.2.2 Tris(2-pyridylmethyl)amine (TPA) Interaction with Co$^{2+}$

Based on absorbance spectroscopic studies a 1:1 binary complex Co$^{2+}$-TPA has been reported in both water and DMSO.$^{71,72}$ A Crystal structures of 1:1 (Co$^{2+}$ : TPA) binary complexes containing of various ancillary ions (including Cl$^\text{-}$) prepared in pure methanol have been reported.$^{77}$ In this research, we chose to examine the interaction of Co$^{2+}$ with TPA in the methanol:buffer (60:40 by volume) mixtures at both pH 6.80 and 7.25. The solvent mixture helped solubilize TPA while still maintaining a physiologically relevant solution condition. The resulting binding isotherms when TPA was titrated into CoCl$_2$ were fit to a one-set-of-sites binding model (Figures 2.12 and 2.13). The binary complexes were formed at either pH, as suggested by the stoichiometric ratio of 1 TPA per Co$^{2+}$ (Table 2.7). The Co$^{2+}$-TPA has been reported to be oxidized to [(TPA)Co(µ-OH)(µ-O$_2$)Co(TPA)]$^{3+}$ in solution but only upon lengthy (2-day) exposure to molecular oxygen.$^{78}$ Therefore, in comparison to TREN, TPA was less prone to bind dioxygen. In fact, tertiary aminic groups and pyridic groups have been reported to stabilize the lower (II) oxidation state of cobalt in both aqueous and non-aqueous solutions as they tend to not bind oxygen.$^{72,75,79-81}$


Figure 2.12. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.1 mM TPA into 0.01 mM CoCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 6.80, 25 °C.
Figure 2.13. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.1 mM TPA into 0.01 mM CoCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 7.25, 25 °C.

Curve fitting of the ITC binding isotherm yielded an affinity constant of \((2 \pm 1) \times 10^8\) M$^{-1}$ and \((3 \pm 2) \times 10^8\) M$^{-1}$ at pH 6.80 and 7.25, respectively (Figure 2.12 and 2.13) (Table 2.7). The lack of an effect of pH on the affinity constant was consistent with the acidic pK$_a$ values of the three pyridylmethyl nitrogens. At either pH, the three pyridylmethyl nitrogens were predominantly deprotonated prior to Co$^{2+}$ binding. However, the current $K_{\text{obs}}$ of \(2-3 \times 10^8\) M$^{-1}$ was still 3-orders of magnitude smaller than the reported formation constant (log$\beta = 11.38$ or $\beta = 2.4\times10^{11}$ M$^{-1}$)\textsuperscript{71}, indicating the effect of proton competition on metal ion binding even when the ligand was predominantly deprotonated. Conversely, the $K_{\text{obs}}$ of \(2-3 \times 10^8\) M$^{-1}$ was more than 2-orders of magnitude higher than the formation constant reported in DMSO (log$\beta = 5.77$ or $\beta = 5.89\times10^5$ M$^{-1}$)\textsuperscript{72}, highlighting the effect of solvent polarity on metal ligand affinity.
Although Co$^{2+}$ bound TREN with equal affinity as Zn$^{2+}$, it bound TPA 3-times more strongly at pH 6.8 ($K_{a, \text{Co(TPA)}} = (2 \pm 1) \times 10^8 \text{ M}^{-1}$) than Zn$^{2+}$ ($K_{a, \text{Zn(TPA)}} = (5 \pm 2) \times 10^7 \text{ M}^{-1}$; no pH 7.25 data was available for Zn$^{2+}$). Because Co$^{2+}$ has a slightly larger ionic radius (0.74 Å) than Zn$^{2+}$ (0.72 Å) and is therefore more polarizable than Zn$^{2+}$, the fact that Co$^{2+}$ exhibited a stronger affinity for TPA suggests that the covalent rather than the ionic nature of the coordination bonds predominated the interaction between Co$^{2+}$ and TPA. This effect was evidently significant for TPA, a more polarizable ligand given its three 2-pyridylmethyl groups. As described in the next section, a lesser difference was also observed for BPA, an analog of TPA that contains two 2-pyridylmethyl groups.

**Table 2.7.** Thermodynamic parameters from ITC study of TPA interaction with Co$^{2+}$ in 60:40 (by volume) mixture of MeOH:NEM buffer pH (6.80 and 7.25).*

<table>
<thead>
<tr>
<th>pH</th>
<th>$n$ (TPA/Co$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.80</td>
<td>1.02 ± 0.06</td>
<td>(2 ± 1) $\times 10^8$</td>
<td>0.006 ± 0.005</td>
<td>-11.2 ± 0.5</td>
<td>-11.8 ± 0.4</td>
<td>-0.6 ± 0.6</td>
</tr>
<tr>
<td>7.25</td>
<td>1.04 ± 0.07</td>
<td>(3 ± 2) $\times 10^8$</td>
<td>0.003 ± 0.002</td>
<td>-11.6 ± 0.4</td>
<td>-11.5 ± 0.5</td>
<td>0.1 ± 0.6</td>
</tr>
</tbody>
</table>

* Titrations of 0.1 mM TPA into 0.01 mM Co$^{2+}$ in MeOH:NEM buffer at pH (6.8 and 7.25)

In summary, Co$^{2+}$ formed a M$^{2+}$(TPA) binary complex just as Zn$^{2+}$ does in the MeOH:buffer mixture at pH 6.80 but did so with a stronger affinity due to its larger ionic radius and its different $d$ orbital electron configuration. The binding constant observed in this work is a conditional constant dependent on the protonation equilibrium and characteristic of the pH and the medium polarity, which provided a more relevant measure than the formation constant of the actual affinity of the Co$^{2+}$ ion for the ligand.
2.2.3 Bis(2-picoly)amine (BPA) Interaction with Co\(^{2+}\)

BPA was soluble in both buffer and the methanol:buffer mixture and therefore its interaction with Co\(^{2+}\) was studied in both solutions at pH 6.80 and 7.25. Binding isotherms from titrations of BPA into CoCl\(_2\) in all four solution conditions clearly revealed two types of binding events (Figure 2.14), suggesting weaker affinity for binding the second versus the first BPA. The isotherms were thus fitted to a sequential-binding-sites model with the number of BPA bound per Co\(^{2+}\) set as two (Figure 2.14). In all four solution conditions, the second BPA exhibited a two-orders of magnitude lower affinity 
\[
K_{a2, \text{buf}} = (2.0 \pm 0.3) \times 10^5 \text{ M}^{-1} \text{ at pH 6.8 and } (2.69 \pm 0.06) \times 10^5 \text{ M}^{-1} \text{ at pH 7.25};
\]
\[
K_{a2, \text{MeOH:buf}} = (2.0 \pm 0.4) \times 10^5 \text{ M}^{-1} \text{ at pH 6.8 and } (2.5 \pm 0.1) \times 10^5 \text{ M}^{-1} \text{ at pH 7.25})
\]
than the first BPA 
\[
K_{a1, \text{buf}} = (5.6 \pm 0.8) \times 10^7 \text{ M}^{-1} \text{ at pH 6.8 and } (4.6 \pm 0.6) \times 10^7 \text{ M}^{-1} \text{ at pH 7.25};
\]
\[
K_{a1, \text{MeOH:buf}} = (3.6 \pm 0.9) \times 10^7 \text{ M}^{-1} \text{ at pH 6.8 and } (7.0 \pm 0.3) \times 10^7 \text{ M}^{-1} \text{ at pH 7.25})
\]
(Table 2.8). Within error, affinity constants were independent of pH except for the first BPA in MeOH:buffer where affinity \((K_{a1})\) doubled from pH 6.80 to 7.25. The lack of a significant pH dependence of the affinity for both BPA under most solution conditions were consistent with the very acidic p\(K_a\) values of the two pyridylmethyl amines \((pK_{a1} = 1.75,\ pK_{a2} = 2.41)\) and the relatively small pH difference but inconsistent with the p\(K_a\) value of the secondary amine \((7.27)\) which is close to the pH tested. The lack of a significant effect of pH on BPA affinity likely suggests that the binding affinity was derived disproportionately from coordination of the two 2-pyridylmethyl amines. The affinity was independent of the presence of methanol at pH 6.80 but increased slightly \(((4.6 \pm 0.6) \times \)
$10^7$ vs. $(7.0 \pm 0.3) \times 10^7 \text{ M}^{-1}$) in the presence of methanol at pH 7.25. The lack of a significant effect of methanol on affinity for BPA between pH 6.80 and 7.25 was more of a property of the ligand as a similar lack of effect is reported for Zn$^{2+}$-BPA interaction as well\textsuperscript{59} while TREN binding exhibited strong methanol dependence for both metal ions.

A similar sequential binding of BPA with decreasing affinity has also been observed for Zn$^{2+}$ in the same buffer and methanol:buffer mixture at pH 6.80 (no pH 7.25 data available).\textsuperscript{59} For the first BPA binding, Co$^{2+}$ exhibited slightly higher affinity (by 1.3- and 0.5-fold in buffer and MeOH:buffer mixture, respectively) than Zn$^{2+}$ ($K_{a1, \text{buf}, \text{Co(II)}} = (5.6 \pm 0.8) \times 10^7 \text{ M}^{-1}$ and $K_{a1, \text{buf}, \text{Zn(II)}} = (2.4 \pm 0.2) \times 10^7 \text{ M}^{-1}$; $K_{a1, \text{MeOH:buf}, \text{Co(II)}} = (3.4 \pm 0.9) \times 10^7 \text{ M}^{-1}$ and $K_{a1, \text{MeOH:buf}, \text{Zn(II)}} = (2.3 \pm 0.6) \times 10^7 \text{ M}^{-1}$) (Table 2.8).\textsuperscript{59} For the second BPA binding, Co$^{2+}$ exhibited a 28- and 29-fold stronger affinity (in buffer and MeOH:buffer mixture, respectively) than Zn$^{2+}$ ($K_{a2, \text{buf}, \text{Co(II)}} = (2.0 \pm 0.3) \times 10^5 \text{ M}^{-1}$ and $K_{a2, \text{buf}, \text{Zn(II)}} = (7 \pm 3) \times 10^3 \text{ M}^{-1}$; $K_{a2, \text{MeOH:buf}, \text{Co(II)}} = (2.3 \pm 0.6) \times 10^5 \text{ M}^{-1}$ and $K_{a2, \text{MeOH:buf}, \text{Zn(II)}} = (7 \pm 7) \times 10^3 \text{ M}^{-1}$) (Table 2.8).\textsuperscript{70} Co$^{2+}$ thus exhibited a less drastic reduction in affinity for the second BPA. This affinity reduction for either metal ion can be explained by the ligand field theory in that filling electrons from the first BPA yields more energy stabilization than filling electrons from the second BPA. Given the $d$ orbital electron configurations of Co$^{2+}$ ($d^7$) and Zn$^{2+}$ ($d^{10}$), adding the sixth (for Co$^{2+}$) and the 5th (for Zn$^{2+}$) pair of ligand electrons tend to raise the energy of the complex, thus explaining the drastic difference between Co$^{2+}$ and Zn$^{2+}$ in bonding the second BPA. These results are consistent with the fact that Zn$^{2+}$ prefers to form 4- or 5- coordination tetrahedral or trigonal bipyramidal complex while Co$^{2+}$ in addition also forms 6-coordination octahedral complexes. The
Co$^{2+}$-(BPA)$_2$ and Zn$^{2+}$-(BPA)$_2$ complexes therefore differ not only in the affinity of the second BPA but likely the number of coordination bonds with the second BPA thus the geometry of the resulting complex.

No Co$^{2+}$-(BPA)$_2$ complexes have been reported in solution or in crystal form. But complexes of Co$^{2+}$-BPA plus ancillary ligands have been reported such as [Co$^{2+}$(BPA)(NO$_2$)Cl] obtained in the presence of 1:1:1:1 molar ratio of Co$^{2+}$:BPA:NO$_2$:Cl$^-$.

Given that Co$^{2+}$-(BPA)$_2$ complex was observed in the presence of excess amount of BPA and that the second BPA binds with much weaker affinity. Finally, no dioxygen binding has been report for Co$^{2+}$-BPA. There was no indication of dioxygen binding in the ITC data either.
Figure 2.14 Raw data (top panel) and binding isotherm (bottom panel) for titrations a) 4.5 mM BPA into 0.3 mM CoCl$_2$ in NEM buffer, pH 6.80. b) 1.5 mM BPA into 0.05 mM CoCl$_2$ in NEM buffer, pH 7.25. c) 1 mM BPA into 0.05 mM CoCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 6.80. d) 1 mM
BPA into 0.05 mM CoCl₂ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 7.25. All at 25 °C.

BPA had approximately equal binding affinity for the first BPA binding in NEM buffer pH 6.80 and pH 7.25 (Kₐ₁, Co(BPA) = (3.4 ± 2.6) × 10⁷ M⁻¹ and Kₐ₁, Co(BPA) = (2.9 ± 0.9) × 10⁷ M⁻¹ in NEM buffer pH 6.80 and pH 7.25, respectively). BPA had a 5-fold decrease in binding affinity for the second BPA(Co) binding in NEM buffer pH 6.80 and pH 7.25 (Kₐ₂, Co(BPA) = (3.2 ± 0.5) × 10⁴ M⁻¹ and Kₐ₂, Co(BPA) = (1.8 ± 0.1) × 10⁵ M⁻¹ in NEM buffer pH 6.80 and pH 7.25, respectively). No data is available for BPAbinding in MeOH:buffer pH 6.80, therefore, no comparison can be made based on pH changes. Co²⁺ behaved similarly as Zn²⁺ does in their interaction with BPA in NEM buffer pH 6.80. Both formed a 1:1 ML binary complex with an affinity that is the same within error (Kₐ₁, Zn(BPA) = (2.4 ± 0.2) × 10⁷ M⁻¹ and Kₐ₁, Co(BPA) = (3.4 ± 2.6) × 10⁷ M⁻¹ in NEM buffer at pH 6.80). However, the second binding affinity was 2.5 fold stronger for Co:BPA compared to Zn:BPA (Kₐ₂, Zn(BPA) = (7 ± 3) × 10³ M⁻¹ and Kₐ₂, Co(BPA) = (1.8 ± 0.1) × 10⁵ M⁻¹ in NEM buffer pH 6.80).

Table 2.8 Thermodynamic parameters from ITC study of BPA interaction with Co²⁺ in 60:40 (by volume) mixture of MeOH:NEM buffer pH (6.80 and 7.25).*

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>n</th>
<th>Kₐ₁</th>
<th>Kₐ₂</th>
<th>ΔG°</th>
<th>ΔH°</th>
<th>TΔS°</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(BPA/Co²⁺)</td>
<td>(M⁻¹)</td>
<td>(μM)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
<td>(kcal/mol)</td>
</tr>
<tr>
<td>NEM Buffer</td>
<td>6.80</td>
<td>1st BPA</td>
<td>(5.6 ± 0.8) × 10⁷</td>
<td>0.018 ± 0.002</td>
<td>-10.6 ± 0.1</td>
<td>-10.3 ± 0.1</td>
<td>0.7 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>7.25</td>
<td>2nd BPA</td>
<td>(2.0 ± 0.3) × 10⁷</td>
<td>5.1 ± 0.7</td>
<td>-7.2 ± 0.1</td>
<td>-7.8 ± 0.3</td>
<td>0.2 ± 0.3</td>
</tr>
<tr>
<td>MeOH:NEM Buffer</td>
<td>6.80</td>
<td>1st BPA</td>
<td>(4.6 ± 0.6) × 10⁷</td>
<td>0.022 ± 0.003</td>
<td>-10.45 ± 0.01</td>
<td>-8.84 ± 0.03</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>7.25</td>
<td>2nd BPA</td>
<td>(2.69 ± 0.06) × 10⁹</td>
<td>3.72 ± 0.08</td>
<td>-7.41 ± 0.01</td>
<td>-6.6 ± 0.3</td>
<td>0.1 ± 0.4</td>
</tr>
</tbody>
</table>

* Titrations of 1 mM BPA into 0.05 mM Co²⁺ in MeOH:NEM buffer at both pHs, 1.5 mM BPA into 0.05 mM Co²⁺ in NEM buffer only at both pHs. In all cases, the ligand was titrated into the CoCl₂ solution. *Two BPA molecules were bound per Co²⁺ thus two sets of binding parameters derived from a sequential binding site model are shown. All parameters were averages from at least three experiments. Kₐ and Kₐ refer to the pH-dependent conditional equilibrium association and dissociation constant, respectively, for an equilibrium including ligand deprotonation, buffer protonation and ligand coordination to Co²⁺. The values of ΔG°, ΔH and TΔS were for per mole of ligand for the overall equilibrium.
2.3 ITC Studies of the Interactions of Ligands with Mn$^{2+}$

2.3.1 Tris(2-aminoethyl)amine (TREN) interaction with Mn$^{2+}$

Complexation of TREN with Mn$^{2+}$ have been studied by Schaefer et al using X-ray crystallography.$^{83}$ Mn$^{2+}$ is found to coordinate to two TREN molecules, forming an octahedral complex.$^{83}$ In our work, the interaction of TREN with Mn$^{2+}$ was studied in MeOH: NEM buffer mixture at pH 6.80. A solution of TREN was titrated into MnCl$_2$ and a corresponding control experiment of TREN titration into MeOH:buffer mixture was also performed. The control heat which corresponded to the dilution of TREN was subtracted from the heat of TREN into MnCl$_2$ titration to remove heat not stoichiometrically correlated with the TREN-Mn$^{2+}$ binding event (Figure 2.15). The control-subtracted binding isotherms showed endothermic binding and fitted well to a one-set-of-sites binding model (Figure 2.16). The fit yielded a stoichiometric ratio of one TREN per Mn$^{2+}$, indicating the formation of a 1:1 (Mn$^{2+}$:TREN) binary complex (Table 2.9). As manganese is capable of six coordination bonds and Mn(TREN)$_2$ complex has been observed$^{84,85}$, future experiments could use higher titrant and titrate concentrations to determine if a weak second TREN binding can be observed in ITC. It should be pointed out that the Mn$^{2+}$-TREN binding heat was small, even smaller than the TREN dilution heat, greatly limiting data reliability. An alternative method such as UV-Vis can be used to confirm the binding parameters from ITC.
Figure 2.15. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 10 mM TREN into 1 mM MnCl₂ in 60:40 (by volume) mixture of MeOH:NEM buffer pH 6.80, 25 °C.
Figure 2.16. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 10 mM TREN into 1 mM MnCl$_2$ in 60:40 (by volume) mixture of MeOH:NEM buffer, pH 6.80, 25°C. after subtract the control run of 0.5 mM TREN into MeOH:NEM buffer, pH 6.80, 25°C.

When a reverse order titration was performed, i.e., when MnCl$_2$ (10 mM) was titrated into TREN (1 mM) in the same solution condition, no binding was detected (Figure 2.17). The differences could be due to different availability of titrant and titrate in the two injection orders, although this could not fully explain the lack of binding. Additional experiments are needed to resolve the discrepancy.
Figure 2.17. Overlay of binding isotherms for MnCl₂ titrated into TREN in 60:40 (by volume) mixture of MeOH: 50 mM NEM buffer (pH 6.80, at 25°C) experiment and the control experiment of MnCl₂ titration into the same buffer condition.

Table 2.9. Thermodynamic parameters of TREN interaction with Mn²⁺ in 60:40 (by volume) mixture of MeOH: NEM buffer at pH 6.80.*

<table>
<thead>
<tr>
<th>n ((\text{TREN/Mn}^{2+}))</th>
<th>(K_d) ((\text{M}^{-1}))</th>
<th>(K_d) ((\mu\text{M}))</th>
<th>(\Delta G^\circ) ((\text{kcal/mol}))</th>
<th>(\Delta H^\circ) ((\text{kcal/mol}))</th>
<th>(T\Delta S^\circ) ((\text{kcal/mol}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before subtract control heat</td>
<td>0.93</td>
<td>(1.04 \times 10^4)</td>
<td>96.2</td>
<td>-5.48</td>
<td>-0.18</td>
</tr>
<tr>
<td>After subtract control heat</td>
<td>0.78</td>
<td>(4.04 \times 10^4)</td>
<td>24.8</td>
<td>-6.28</td>
<td>0.38</td>
</tr>
</tbody>
</table>

*Titrations of 10 mM TREN into 1 mM Mn²⁺. The value was from only one experiment.

2.3.2 Tris(2-pyridylmethyl)amine (TPA) interaction with Mn²⁺

TPA was titrated into MnCl₂ in the MeOH:NEM mixture at pH 6.80. The resulting binding isotherms were fit to a one-set-of-sites binding model (Figure 2.18). Curve fitting yielded an affinity constant of \((4.6 \pm 0.3) \times 10^5 \text{ M}^{-1}\) (Figure 2.18 and Table 2.10). TPA and Mn²⁺ formed a binary complex as suggested by the stoichiometric ratio of 1 TPA per
Mn$^{2+}$ (Table 2.10). Therefore Mn$^{2+}$ behaved similarly as other two metal ions, Cu$^{2+}$ and Co$^{2+}$, in forming a binary complex with TPA but differ in the affinity, with Mn$^{2+}$ having the weakest affinity of the three metal ions.

![Figure 2.18](image)

**Figure 2.18.** Raw data (top panel) and binding isotherm (bottom panel) for titrations of 0.75 mM TPA into 0.075 mM MnCl$_2$ in 60:40 (by volume) mixture of MeOH: 50 mM NEM buffer (0.150 M NaCl, pH 6.80) at 25 °C.

**Table 2.10** Thermodynamic parameters from ITC study of TPA interaction with Mn$^{2+}$ in MeOH:NEM buffer, pH 6.80*

<table>
<thead>
<tr>
<th>n (TPA/Mn$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.97 ± 0.02</td>
<td>(4.6 ± 0.3) × 10$^5$</td>
<td>2.1 ± 0.2</td>
<td>-7.74 ± 0.04</td>
<td>-3.5 ± 0.0</td>
<td>4.21 ± 0.0</td>
</tr>
</tbody>
</table>

*Titration of 0.75 mM TPA into 0.075 mM Mn$^{2+}$.
2.3.3 Bis(2-picolyl)amine (BPA) interaction with Mn$^{2+}$

BPA was soluble in both the NEM buffer and in MeOH: NEM buffer mixture at pH 6.8. In this study we examined the interaction in the MeOH:NEM mixture only, because our immediate goal was to study the interaction of the resulting binary complex with 8-HQ which is soluble only in the MeOH:buffer mixture. When 10 mM of BPA was titrated into 1 mM MnCl$_2$ the resulting binding isotherm was fit to a one-set-of-sites binding model (Figure 2.19) which yielded an apparent affinity constant ($K_a$) of $(1.3 \pm 0.1) \times 10^4$ M$^{-1}$ and a stoichiometric ratio of 0.99 ± 0.04 BPA per Mn$^{2+}$, corresponding to a 1:1 (BPA: Mn$^{2+}$) binary complex (Table 2.11). This stoichiometric ratio met the requirement for a 1:1 binary complex for it to serve as an enzyme active site copper structural mimic. Although we did not observe the binding of a second BPA, Glerup et al. reported a [Mn(BPA)$_2$](ClO$_4$)$_2$ crystal structure showing Mn$^{2+}$ bound to two BPA molecules.$^{86}$ The absence of a second BPA binding in ITC may be explained by the different anions used, i.e. Cl$^-$ in the ITC study versus the ClO$_4^-$ in the crystallographic study. Chloride ion is known to have an affinity for divalent metal ions therefore compete with BPA for Mn$^{2+}$ binding while ClO$_4^-$ does not. Additionally, the second BPA likely binds Mn$^{2+}$ with orders of magnitude weaker affinity than the first BPA, as was true for Co$^{2+}$ and has also been reported for Zn$^{2+}$. Given that Mn$^{2+}$ bound the first BPA significantly weaker ($K_a = (1.3 \pm 0.1) \times 10^4$) than Co$^{2+}$ ($K_a = (3.6 \pm 0.9) \times 10^7$ M$^{-1}$), it is possible that the second BPA binds Mn$^{2+}$ too weakly to be detected at the concentration (1 mM Mn$^{2+}$) used in the ITC experiment.
Figure 2.19. Raw data (top panel) and binding isotherm (bottom panel) for titrations of 10 mM BPA into 1 mM MnCl$_2$ in 60:40 (by volume) mixture of MeOH: 50 mM NEM buffer (0.150 M NaCl, pH 6.80) at 25 °C.

Table 2.11 Thermodynamic parameters from ITC study of BPA interaction with Mn$^{2+}$ in 60:40 (by volume) mixture of MeOH: 50 mM NEM buffer (0.150 M NaCl, pH 6.80).*

<table>
<thead>
<tr>
<th>n (BPA/Mn$^{2+}$)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.99 ± 0.04</td>
<td>(1.3 ± 0.1) x10$^4$</td>
<td>78.5 ± 9.9</td>
<td>-5.60 ± 0.08</td>
<td>-2.54± 0.04</td>
<td>3.06 ± 0.12</td>
</tr>
</tbody>
</table>

*Titrations of 10 mM BPA into 1 mM Mn$^{2+}$

Chapter 3: ITC studies of the interactions of potential enzyme active site metal ion(II) structural mimetics with inhibitors

The first goal of this project was to examine the potential of five ligands in forming an enzyme active site metal ion (II) structural mimetics. Three of the ligands, BPA, TPA, and TREN, were selected for further ITC studies to examine their interaction with 8-HQ, given their shared solubility with 8-HQ in the MeOH: buffer mixture as well as their strong affinities for the
metal ions (II) and/or their favorable 1:1 M$^{2+}$:ligand stoichiometric ratio in the resulting complex. An ideal enzyme active site metal ion (II) structural mimetic would be formed with a ligand that has a very high affinity (K_a ≥ 10^5 M$^{-1}$) for the divalent metal ion and forms strong ML binary complex while still leaving adequate coordination sites for an inhibitor binding. The inhibitor selected for ITC study of the interaction with the enzyme active site metal ion (II) structural mimetics was 8-hydroxyquinoline (8-HQ).

3. 8-Hydroxyquinoline (8-HQ) interaction with ligand-metal ion (II) mixtures as potential enzyme active site metal ion(II) structural mimetics

ITC experiments were done to study the interaction between 8-HQ and various ML binary complexes that had been identified as potential enzyme active site metal ion(M$^{2+}$) structural mimetics in the previous section based on the ITC studies of ligand and M$^{2+}$ interactions. The three different binary systems were selected which include M(TPA), M(BPA), and M(TREN). Experiments were performed by titrating 8-HQ solution into a solution of ML complex in MeOH:buffer (60:40 by volume) mixture (buffer being 50 mM Tris or NEM, 0.15 M NaCl) at pH 6.80 or 7.40.

3.1. ITC studies of the interactions of 8-HQ with Cu$^{2+}$L Complexes

Given that all three ligands formed a strong Cu$^{2+}$L binary complex in the MeOH:Tris buffer mixture, we examined the interaction of each of the three binary complexes with 8-HQ, a known metalloenzyme active site inhibitor in the solvent mixture at pH 7.4. 8-HQ was chosen as it is a known inhibitor for copper-metalloenzymes active site$^{88}$ and it exhibits metal chelation affinities that can be accurately determined using ITC. The MeOH:Tris buffer mixture was chosen to solubilize 8-HQ and TPA which are otherwise insoluble in buffer. If a ML binary
complex is able to bind one 8-HQ without losing the bound ligand thereby forming a Cu²⁺L(8-HQ) ternary complex, it would in effect serve as an enzyme active site Cu²⁺ structural mimetic. The ΔG⁰ for the inhibitor interaction with the binary complex provides an estimation of the Gibbs free energy contribution of Cu²⁺ to the overall inhibitor enzyme active site interactions.

3.1.1. TREN-Cu²⁺ interaction with 8-Hydroxyquinoline

As stated in chapter 2, TREN was determined to bind Cu²⁺ in a 1:1 stoichiometric ratio and had a stronger affinity for Cu²⁺ in MeOH:Tris buffer than in Tris buffer. ITC experiments for 8-HQ binding to the Cu(TREN) binary complex were done with a mixture of TREN and CuCl₂ at 1.05:1 (TREN:Cu²⁺) molar ratio to ensure that no free Cu²⁺ existed and that if 8-HQ binding was indeed observed, it was binding to Cu(TREN) complex rather than free Cu²⁺. Fitting of the binding isotherm to a one set of sites model (Figure 3.1) yielded an n value of 2.68 ± 0.03 (approx. 3) 8-HQ per Cu²⁺(Table 3.1), This suggests that 8-HQ had displaced the TREN and has formed a [Cu(8-HQ)₃]⁻² complex instead of a Cu(TREN)(8-HQ) ternary complex. The binding curve also suggests that the three 8-HQ molecules bound with the same affinity to Cu²⁺ with Kₐ of (2.8 ± 0.1) x10⁴ M⁻¹. This affinity of 8-HQ for Cu²⁺-TREN was much smaller than that for free Cu²⁺ (Kₐ=(3.24 ± 2.76) x 10⁶ M⁻¹). This can be explained by the presence of TREN which competes with 8-HQ for Cu²⁺ binding. The formation of a Cu(8-HQ)₃ complex also indicates that this complex was more stable than a Cu(TREN)((8-HQ) ternary complex. The inability in forming a Cu(TREN)((8-HQ) ternary complex makes Cu(TREN) unsuitable as an enzyme active site copper ion structural mimetic.
Figure 3.1 Raw data (top panel) and binding isotherm (bottom panel) for titration of 4 mM 8-hydroxyquinoline into a solution containing 0.2 mM CuCl₂ and 0.21 mM TREN in 60:40 (by volume) mixture of MeOH: 50 mM Tris Buffer (0.150 M NaCl) at pH 7.40 at 25 °C

Table 3.1 Thermodynamic parameters from ITC study of 8-hydroxyquinoline into a solution containing CuCl₂ and TREN at 25 °C

<table>
<thead>
<tr>
<th>n</th>
<th>Kₐ (M⁻¹)</th>
<th>Kₐ (μM)</th>
<th>ΔG° (kcal/mol)</th>
<th>ΔH° (kcal/mol)</th>
<th>TΔS° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.68 ± 0.00</td>
<td>(2.8 ± 0.1) x10⁴</td>
<td>35 ± 1</td>
<td>-6.02 ± 0.02</td>
<td>-4.38 ± 0.05</td>
<td>1.7 ± 0.1</td>
</tr>
</tbody>
</table>

*Titrations of 8 mM 8-HQ into 0.2 mM CuCl₂ and 0.2 mM TREN in MeOH:Tris buffer.

3.1.2 Cu(TPA) interaction with 8-Hydroxyquinoline

In Chapter 2, TPA was determined to form a 1:1 binary complex with Cu²⁺ in MeOH:Tris buffer with a strong affinity for Cu²⁺. ITC experiments for 8-HQ binding to the Cu(TPA) binary complex were done by titrating 8-HQ into a mixture of TPA and CuCl₂ at 1.2:1 (TPA:Cu²⁺) molar ratio which was the result of injecting TPA into Cu²⁺ chapter 2 and also to ensure that no free Cu²⁺ existed and that if 8-HQ binding was indeed observed, it was binding to Cu(TPA)
complex rather than free Cu$^{2+}$. When the ITC data from the 8-HQ into Cu$^{2+}$-TPA binary complex was analyzed, the n value was determined by using sequential binding model with n of 2, i.e., two 8-HQ sequentially bind each Cu$^{2+}$ and therefore have different affinities as evidenced by the shape of the curve. The binding isotherm (Figure 3.2) showed an initial downward region followed by an upward region. The $K_{a1}$ and $K_{a2}$ values were $(2.0 \pm 1.3) \times 10^3$ and $(5.9 \pm 1.8) \times 10^4$ M$^{-1}$, respectively (Table 3.2). These observed $K_a$ values were much smaller than that for 8-HQ binding to free Cu$^{2+}$ ($(3.24 \pm 2.76) \times 10^6$ M$^{-1}$) because of the competition of TPA (Figure 3.2). Given that free Cu$^{2+}$ also bound to two 8-HQ, we believe that TPA was displaced by 8-HQ and no Cu(TPA)(8-HQ) complex was formed. Therefore, TPA is not an acceptable ligand to create an enzyme active site copper ion structural mimic.

**Figure 3.2** Raw data (top panel) and binding isotherm (bottom panel) for titration of 8 mM 8-hydroxyquinoline into a solution containing 0.2 mM CuCl$_2$ and 0.24 mM TPA in 60:40 (by volume) mixture of MeOH: 50 mM Tris Buffer (0.150 M NaCl) at pH 7.40 at 25°C
Table 3.2 Thermodynamic parameters from ITC study of 8-hydroxyquinoline into a solution containing CuCl₂ and TPA

<table>
<thead>
<tr>
<th>n</th>
<th>$K_a$ (M⁻¹)</th>
<th>$K_d$ (µM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$T\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 (sequential)</td>
<td>$(2.0 \pm 1.3) \times 10^3$</td>
<td>$(6.4 \pm 4.2) \times 10^2$</td>
<td>$-4.4 \pm 0.4$</td>
<td>$39.7 \pm 4.5$</td>
<td>$43.4 \pm 4.0$</td>
</tr>
<tr>
<td></td>
<td>$(5.9 \pm 1.1) \times 10^4$</td>
<td>$17.9 \pm 0.3$</td>
<td>$-6.5 \pm 0.2$</td>
<td>$47.5 \pm 7.9$</td>
<td>$41.0 \pm 6.8$</td>
</tr>
</tbody>
</table>

*Titrations of 8 mM 8-HQ into 0.2 mM CuCl₂ and 0.24 mM TPA in MeOH:Tris buffer.

3.1.3. Cu(BPA) interaction with 8-Hydroxyquinoline

Titrations of 8-HQ into Cu²⁺-BPA mixture were done with varying ratios of BPA to Cu²⁺. As shown in chapter 2, the BPA into Cu²⁺ titration experiments revealed two BPA molecules binding each Cu²⁺, with the first BPA binding Cu²⁺ with high affinity ($K_a = (1.8 \pm 1.0) \times 10^5$ M⁻¹) and the second BPA binding two-times weaker ($K_a = (5.0 \pm 4.2) \times 10^3$ M⁻¹) than the first BPA (Table 2.5). Although this was not an ideal mimetic system, since the second BPA binds very weakly, it was hypothesized that an inhibitor (such as 8-HQ) would be capable of displacing the second BPA in binding to Cu²⁺.

To avoid the issue of 8-HQ competing off the second BPA molecule, 1:1 binding was forced by making the Cu(BPA) complex using a 1:1 molar ratio of (BPA:Cu²⁺). The raw ITC data did not show signs of the weakly bound BPA being displaced by 8-HQ, nor did it show signs of 8-HQ binding free rather than BPA-complexed Cu²⁺ (Figure 3.3) since the n value differed from the n value obtained for the titration of 8-HQ titration into free Cu²⁺ (n = 2.0 ± 0.3) (Table 4.1, Chapter 4).

Titrations of 8-HQ into 2:1 (BPA:Cu²⁺) solutions were also performed (Figure 3.4), which yielded an n value of 1.03 ± 0.01 (Table 3.3). The decreasing n value with the increasing ratios of BPA:Cu²⁺ reflects the decreasing presence of the Cu(BPA) complex but increasing presence of the Cu(BPA)₂ complex over the 1:1 (BPA:Cu²⁺) complex. It also suggests that not all
Cu$^{2+}$ were bound with two BPA molecules at the 2:1 (BPA:Cu$^{2+}$) ratio despite twice as much BPA as Cu$^{2+}$ was present, which can be explained by the weak affinity of the second BPA molecule. As the ratio of BPA:Cu$^{2+}$ increased from 1:1 to 2:1, the $K_a$ value for 8-HQ binding also decreased, specifically by approximately 5-fold from $(2.0 \pm 0.4) \times 10^5 \text{M}^{-1}$ to $(3.9 \pm 0.3) \times 10^4 \text{M}^{-1}$. This decrease of affinity was likely due to the competition of the free BPA in the solution.

To conclude, for the BPA to be used as an potential enzyme active site copper ion structural mimic, a 1:1 ratio of BPA:Cu$^{2+}$ was necessary and sufficient for creating a stable 1:1 complex capable of coordinating to one molecule of 8-HQ.

**Figure 3.3** Raw data (top panel) and binding isotherm (bottom panel) for titration of 5 mM 8-hydroxyquinoline into a solution containing 0.5 mM CuCl$_2$ and 0.5 mM BPA (1:1) in 60:40 (by volume) mixture of MeOH: 50 mM Tris Buffer (0.150 M NaCl) at pH 7.40 at 25 °C
**Figure 3.4** Raw data (top panel) and binding isotherm (bottom panel) for titration of 10 mM 8-hydroxyquinoline into a solution containing 1 mM CuCl$_2$ and 2 mM BPA (1:2) in 60:40 (by volume) mixture of MeOH: 50 mM Tris Buffer (0.150 M NaCl) at pH 7.40 at 25 °C.

**Table 3.3.** Thermodynamic parameters from ITC studies of 8-HQ interaction with Cu$^{2+}$:BPA at 25 °C in the MeOH: Tris buffer mixture at pH 7.4.

<table>
<thead>
<tr>
<th>Ratio of (BPA/Cu$^{2+}$)</th>
<th>n</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/(mol·K))</th>
<th>T$\Delta S^\circ$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:1</td>
<td>1.18 ± 0.01</td>
<td>(2.02 ± 0.4) $\times$ 5.0 ± 0.1</td>
<td>-7.24 ± 0.01</td>
<td>-11.4 ± 0.1</td>
<td>-13.9 ± 0.6</td>
<td>-4.2 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>2:1</td>
<td>1.03 ± 0.01</td>
<td>(3.9 ± 0.3) $\times$ 10$^4$ 2.6 ± 0.2</td>
<td>-6.26 ± 0.05</td>
<td>-6.77 ± 0.05</td>
<td>-1.72 ± 0.32</td>
<td>-0.51 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

* Titrations of 10 mM 8-HQ into (0.5 mM Cu$^{2+}$ and 1 mM BPA) in Tris buffer, titrations of 5 mM 8-HQ into (0.5 mM Cu$^{2+}$ and 0.5 mM BPA) in MeOH:Tris buffer.

**3.2. ITC studies of the interactions of 8-HQ with Co$^{2+}$L Complexes**

Given that all three ligands formed a strong Co$^{2+}$L binary complex in the MeOH:buffer mixture, we examined the interaction of each of the three binary complexes with 8-HQ, a known metalloenzyme active site inhibitor in the solvent mixture at pH 6.80. The methanol:buffer mixture was chosen to solubilize 8-HQ and TPA which were otherwise insoluble in buffer. Like
copper, the interaction of 8-HQ, a known inhibitor for cobalt-metalloenzymes\textsuperscript{87}, with cobalt can be studied using ITC. Co\textsuperscript{2+} is reported to bind two molecules of 8-HQ.\textsuperscript{89} If a binary complex is able to bind one 8-HQ without losing the bound ligand thereby forming a CoL(8-HQ) ternary complex, it would in effect serve as an enzyme active site Co\textsuperscript{2+} structural mimetic. The ΔG\textdegree{} for the inhibitor interaction with the binary complex provides an estimation of the Gibbs free energy contribution of Co\textsuperscript{2+} to the overall inhibitor enzyme active site interactions.

3.2.1. Co\textsuperscript{2+}(TREN) interaction with 8-Hydroxyquinoline

A mixture of TREN and CoCl\textsubscript{2} at 1:1 (TREN:Co\textsuperscript{2+}) molar ratio was formed and titrated with 8-HQ in the methanol:buffer mixture at pH 6.80. Care was taken when choosing the exact molar ratio of TREN and Co\textsuperscript{2+} so that no excess Co\textsuperscript{2+} was present in the mixture. If residual amount of free TREN was present, it was not expected to affect the resulting binding parameters. The observed binding curve (Figure 3.5), which corresponded to 8-HQ binding to the 1:1 (TREN:Co\textsuperscript{2+}) complex, fitted well to a one-set-of-sites model. Curve fitting yielded a K\textsubscript{a,obs} of (2.0 ± 0.4) \times 10\textsuperscript{4} M\textsuperscript{-1} and an n value of 0.56 ± 0.08 8-HQ per TREN-associated Co\textsuperscript{2+} which corresponded to one 8-HQ bound to two Co\textsuperscript{2+}-TREN complexes. Given that ligand-free Co\textsuperscript{2+} binds two 8-HQ molecules\textsuperscript{89}, this result suggests that TREN did not dissociate from Co\textsuperscript{2+} upon titration of 8-HQ. Rather, two Co\textsuperscript{2+}-TREN complexes bound to one 8-HQ. In light of the tendency of Co\textsuperscript{2+} to form µ-peroxo-bridged dicobalt complexes with TREN, the current result supports the idea that the 1:1 (TREN:Co\textsuperscript{2+}) complex formed in methanol:buffer at pH 6.80 was likely Co\textsubscript{2}(TREN)\textsubscript{2}(µ-O\textsubscript{2}). Therefore the complex formed with 8-HQ was likely Co\textsubscript{2}(TREN)\textsubscript{2}(µ-8-HQ)(µ-O\textsubscript{2}). Such binding mode has also been reported for 5-pyrimidyl-tetrazolate (pmtz) in the complex of [Co\textsubscript{2}(TREN)\textsubscript{2}(µ-pmtz)(µ-O\textsubscript{2})](ClO\textsubscript{4})\textsubscript{3}.\textsuperscript{90} In the crystal structure of this complex,
the two $\text{Co}^{2+}$-TREN complexes are bridged via a peroxo plus the two neighboring tetrazolate nitrogens, forming a stable 6-membered ring. Alternatively, it was also possible that bridging of the two $\text{Co}^{2+}$-TREN complexes via the $\mu$-peroxo bridge occurred simultaneously with 8-HQ binding, although a less stable 8-membered ring would result. An alternative would be a hydroxyl group bridging the two $\text{Co}^{2+}$, forming a 7-membered ring. Two cobalt ions linked through a hydroxyl group have also been reported in proteins.\textsuperscript{91,92} The exact binding mode as well as the oxidation state of cobalt in the $(\text{Co}^{2+})_2(\text{TREN})_2(8\text{-HQ})$ complex will have to wait for further solution and crystallographic studies of the complex structure.

Figure 3.5. ITC titration raw data and binding isotherm for titrations of 10 mM 8-HQ into TREN and $\text{CoCl}_2$ mixture at 1 mM TREN and 1 mM $\text{Co}^{2+}$ in the MeOH:buffer mixture, pH 6.80, at 25 $^\circ$C.
Dicobalt centers formed upon 8-HQ binding have biological counterparts. For example, the bioremediator Glycerophosphodiesterase (GpdQ) from *Enterobacter aerogenes*, whose enzymatic activity depends on Co\(^{2+}\) or other metal ions such as Zn\(^{2+}\), recruits the second metal ion upon substrate binding to form a catalytically competent dimetal center.\(^{92}\) The crystal structures of the Co\(^{2+}\)- and Zn\(^{2+}\)-substituted GpdQ reveal that a hydroxyl group or a water molecule bridges the two metal ions.\(^{93,94}\) Product dissociation returns the enzyme to a catalytically inactive mononuclear state. Other examples of enzymes that is activated to bind a second metal ion upon substrate binding include enolase\(^{95}\) and S-adenosylmethionine synthase.\(^{96}\) The current (Co\(^{2+}\))\(_2\)(TREN)\(_2\) complex can therefore serve as a structural mimetic for dicobalt centers at such an enzyme active site.

Unlike for Co\(^{2+}\)-TREN, Zn\(^{2+}\)-TREN interaction with 8-HQ leads to displacement of TREN and formation of a Zn\(^{2+}\)-(8-HQ)\(_2\) complex.\(^{59}\) The current ITC data on Co\(^{2+}\) interacting with TREN and the binary complex interacting with 8-HQ indicate that Co\(^{2+}\) was more prone than Zn\(^{2+}\) to forming dinuclear centers upon interaction with small molecule ligand and inhibitor. The affinity constant of Co\(^{2+}\)-TREN for 8-HQ in forming the (Co\(^{2+}\))\(_2\)(TREN)\(_2\)(8-HQ) complex corresponded to a \(\Delta G^0\) value of -5.9 ± 0.1 kcal/mol 8-HQ. In light of the potential involvement of a dioxygen bridge and the uncertainty in the sequence of event in \(\mu\)-O\(_2\) and \(\mu\)-8-HQ bridge formation, this value can be taken only as a very rough estimate for the free energy contribution of a dicobalt center to the overall interaction of a dicobalt-containing active site interaction with 8-HQ.
3.2.2. Co\textsuperscript{2+}(TPA) interaction with 8-Hydroxyquinoline

A mixture of TPA and CoCl\textsubscript{2} at 1:1 (TPA:Co\textsuperscript{2+}) molar ratio was formed and titrated with 8-HQ in the methanol:buffer mixture at pH 6.80. Care was taken when choosing the exact molar ratio of TPA and Co\textsuperscript{2+} so that no excess Co\textsuperscript{2+} was present in the mixture. If residual free TPA was present, it was not expected to alter binding parameters. The observed binding curve (Figure 3.6) corresponded to 8-HQ binding to the Co\textsuperscript{2+}-TPA complex and fitted well to a one-set-of-sites model. Curve fitting yielded an n value of 1.08 ± 0.01 8-HQ per TPA-associated Co\textsuperscript{2+}, corresponding to the formation of a Co\textsuperscript{2+}-TPA-(8-HQ) ternary complex, and a K\textsubscript{a,obs} value of (1.37 ± 0.03) × 10\textsuperscript{4} M\textsuperscript{-1}. The ΔG° value of -5.64 ± 0.01 kcal per mole of 8-HQ provided an estimate of the energetic contribution of an active site Co\textsuperscript{2+} to the overall ΔG° for 8-HQ binding to a Co\textsuperscript{2+}-containing enzyme active site (Table 3.4). This result indirectly points to the absence of dioxygen binding of Co\textsuperscript{2+}-TPA complex. Unlike Co\textsuperscript{2+}-TPA, a similar Zn\textsuperscript{2+}-TPA binary complex does not bind 8-HQ.\textsuperscript{59} This difference between Co\textsuperscript{2+} and Zn\textsuperscript{2+} can be attributed to the d\textsuperscript{7} (Co\textsuperscript{2+}) and d\textsuperscript{10} (Zn\textsuperscript{2+}) electron configuration which predicts favorable and unfavorable free energy change for Co\textsuperscript{2+}-TPA and Zn\textsuperscript{2+}-TPA, respectively, in forming two additional coordination bonds to bind 8-HQ. Therefore Co\textsuperscript{2+}-TPA successfully served as an active site Co\textsuperscript{2+} mimetic while Zn\textsuperscript{2+}-TPA does not. This suggests that an active site 4-coordinated Co\textsuperscript{2+} ion can be expected to contribute to the active site interaction with a substrate or inhibitor more significantly than a 4-coordinated Zn\textsuperscript{2+} does.
Figure 3.6 ITC titration raw data and binding isotherm for titrations of 10 mM 8-HQ into TPA and CoCl₂ mixture at 1 mM TPA and 1 mM Co²⁺ in the MeOH:buffer mixture, pH 6.80, at 25 °C.
3.2.3. Co$^{2+}$(BPA) interaction with 8-Hydroxyquinoline

BPA formed a strong Co$^{2+}$-BPA binary complex. In addition, the binary complex also bound a second BPA but with 2-orders of magnitude weaker affinity. Mixing BPA and CoCl$_2$ at 1:1 (BPA :Co$^{2+}$) molar ratio at sub-milimolar concentrations helped form the Co$^{2+}$-BPA binary complex without a significant presence of the Co$^{2+}$-(BPA)$_2$ ternary complex. 8-HQ was titrated into the binary complex prepared using freshly made CoCl$_2$ solution in the methanol:buffer mixture at pH 6.80. The observed binding curve (Figure 3.7) fitted well to a one-set-of-sites model, which yielded an n value of 0.94 ± 0.10 8-HQ per BPA-bound Co$^{2+}$, indicating the formation of a Co$^{2+}$-BPA-8-HQ ternary complex. The affinity constant of 8-HQ for Co$^{2+}$-BPA was \((2.0 \pm 0.7) \times 10^6\) M$^{-1}$ and the corresponding $\Delta G^\circ$ value was $-8.6 \pm 0.2$ kcal per mole of 8-HQ (Table 3.4). This $\Delta G^\circ$ value provided an estimate for the energetic contribution of an active site Co$^{2+}$ to the overall $\Delta G^\circ$ of 8-HQ binding to a Co$^{2+}$-containing enzyme active site.

Surprisingly, Co$^{2+}$-BPA bound 8-HQ over 100-fold more strongly than Zn$^{2+}$-BPA does.$^{59}$ This corresponded to 2.9 kcal/mol additional energy stabilization for Co$^{2+}$-BPA over Zn$^{2+}$-BPA as the metal ion forms its 4$^{th}$ and 5$^{th}$ coordination bonds upon 8-HQ binding. This large difference can be attributed to the different $d$ orbital electron configuration as discussed above for Co$^{2+}$-TPA. Given this difference, an enzyme with its active site Zn$^{2+}$ replaced by Co$^{2+}$, assuming with no coupled structural changes, can be expected to bind its substrate or inhibitor 100-fold more strongly than the zinc enzyme. Additionally, Co$^{2+}$-BPA bound 8-HQ nearly two-orders of magnitude more strongly than Co$^{2+}$-TPA did. This suggests that a Co$^{2+}$ ion that is three-coordinated to the enzyme active site residues will contribute over 100-fold more significantly to the substrate or inhibitor affinity for the active site
than a 4-coordinated active site Co$^{2+}$ assuming there is no structural difference between the two scenarios.

**Figure 3.7** ITC titration raw data and binding isotherm for titrations of 3 mM 8-HQ into BPA and CoCl$_2$ at 0.3 mM BPA and 0.3 mM mM Co$^{2+}$ in the MeOH:buffer mixture, pH 6.80, at 25 °C.

**Table 3.4** Thermodynamic parameters from ITC studies of 8-HQ interaction with Co$^{2+}$:Ligand at 25 °C in the MeOH:NEM buffer mixture at pH 6.80.

<table>
<thead>
<tr>
<th>Co(Ligand) (in calorimeter cell)</th>
<th>n (8-HQ : CoL)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H^\circ$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/(mol·K))</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Co(TREN)]$^{3+}$</td>
<td>0.56 ± 0.08</td>
<td>(2.0 ± 0.4) × 10$^4$</td>
<td>50 ± 10</td>
<td>-5.9 ± 0.1</td>
<td>-11.5 ± 1.1</td>
<td>-5.6 ± 1.0</td>
</tr>
<tr>
<td>[Co(TPA)]$^{3+}$</td>
<td>1.08 ± 0.01</td>
<td>(1.37 ± 0.03) × 10$^4$</td>
<td>73 ± 2</td>
<td>-5.64 ± 0.01</td>
<td>-10.32 ± 0.06</td>
<td>-4.67 ± 0.06</td>
</tr>
<tr>
<td>[Co(BPA)]$^{3+}$</td>
<td>0.94 ± 0.10</td>
<td>(2.0 ± 0.7) × 10$^6$</td>
<td>0.5 ± 2</td>
<td>-8.6 ± 0.2</td>
<td>-24.2 ± 0.8</td>
<td>-15.6 ± 0.7</td>
</tr>
</tbody>
</table>

Data were obtained from titrations of 8-HQ into a mixture of a ligand (TREN or TPA or BPA) and CoCl$_2$ (at 1:1 molar ratio of Ligand: Co$^{2+}$) in the MeOH:buffer (60:40 by volume) mixture at pH 6.80. The values of $\Delta G^\circ$, $\Delta H^\circ$ and $\Delta S^\circ$ were for per mol of Co$^{2+}$ for an overall equilibrium of ligand-bound Co$^{2+}$ coordinating to 8-HQ coupled to any deprotonation and protonation reactions.
3.3. ITC studies of the interactions of 8-HQ with MnL

Given that all three ligands formed a strong Mn$^{2+}$L binary complex in the methanol:buffer mixture, we examined the interaction of each of the three binary complexes with 8-HQ, a known metalloenzyme active site inhibitor in the solvent mixture at pH 6.80. The methanol:buffer mixture was chosen to solubilize 8-HQ and TPA which are otherwise insoluble in buffer. Mn$^{2+}$ is reported to bind two molecules of 8-HQ in the presence of excess 8-HQ. If a binary complex is able to bind one 8-HQ without losing the bound ligand thus forming a Mn$^{2+}$L(8-HQ) ternary complex, it would in effect serve as an enzyme active site Mn$^{2+}$ structural mimic. The $\Delta G^\circ$ for the inhibitor interaction with the binary complex provides an estimation of the Gibbs free energy contribution of Mn$^{2+}$ to the overall inhibitor enzyme active site interactions.

3.3.1. Mn$^{2+}$(TREN) interaction with 8-Hydroxyquinoline

As determined previously TREN and MnCl$_2$ formed a Mn(TREN) binary complex. Therefore, a mixture of 1:1 (TREN:Mn$^{2+}$) in molar ratio was titrated with 8-HQ in the MeOH:buffer mixture at pH 6.80. The binding isotherm was fit to a one set of sites model (Figure 3.8), which yielded an n value of 1.53 ± 0.08 and a $K_a$ value (5.98 ± 0.03) × 10$^3$ M$^{-1}$ (Table 3.5). This result point to the formation of a Mn(8-HQ)$_2$ rather than a Mn(TREN)(8-HQ) ternary complex. This suggests that Mn(8-HQ)$_2$ complex was more stable than the Mn(TREN)(8-HQ) complex. The same was observed for the corresponding complexes for Cu$^{2+}$ and Zn$^{2+}$ but not Co$^{2+}$.
As reported in Section 3.2.1, Co(TREN) interaction with 8-HQ yielded a complex of 8-HQ bonding to two copies of Co(TREN). Such a complex likely was facilitated by a µ-peroxo linkage therefore the complex was likely Co₂(TREN)₂(µ-8-HQ)(µ-O₂). TREN in Co(TREN) was therefore not displaced by 8-HQ, unlike for the other three metal ions which formed M(8-HQ)₂ complex instead.⁵⁹ To conclude, the inability of forming a Mn(TREN)(8-HQ) ternary complex makes Mn(TREN) unsuitable as an enzyme active site manganese ion structural mimic.

Figure 3.8 ITC titration raw data and binding isotherm for titrations of 4 mM 8-HQ into 0.2 MnCl₂ and 0.2 TREN in the MeOH:NEM buffer mixture, pH 6.80, at 25 °C.
3.3.2. Mn$^{2+}$(TPA) interaction with 8-Hydroxyquinoline

In Chapter 2, TPA was determined to form a Mn(TPA) binary complex with a strong affinity in MeOH:Tris mixture. ITC experiments for 8-HQ binding to the Mn$^{2+}$(TPA) binary complex were done with a mixture of TPA and MnCl$_2$ at 1:1 TPA:Mn$^{2+}$ molar ratio. The observed binding curve (Figure 3.9) fitted well to a one-set-of-sites model, which yielded an n value of 1.00 ± 0.05 8-HQ per TPA-bound Mn$^{2+}$, indicating the formation of a Mn$^{2+}$(TPA)(8-HQ) ternary complex. The affinity constant of 8-HQ for Mn(TPA) was $(1.3 ± 0.1) \times 10^4$ M$^{-1}$ and the corresponding $\Delta G^\circ$ value was -5.6 ± 0.3 kcal per mole of 8-HQ (Table 3.5). This $\Delta G^\circ$ value provided an estimate for the energetic contribution of an active site Mn$^{2+}$ to the overall $\Delta G^\circ$ for 8-HQ binding to a Mn$^{2+}$-containing enzyme active site.

![Figure 3.9 ITC titration raw data and binding isotherm for titrations of 10 mM 8-HQ into 1 mM MnCl$_2$ with 1 mM TPA in the MeOH:NEM buffer mixture, pH 6.80, at 25 °C.](image)

86
3.3.3. Mn$^{2+}$(BPA) interaction with 8-Hydroxyquinoline

BPA was shown to form a Mn(BPA) binary complex in MeOH:Tris buffer mixture with a $K_a$ of $(1.3 \pm 0.1) \times 10^4 \text{M}^{-1}$. A mixture of BPA and Mn$^{2+}$ at 1:1 molar ratio was used to form a Mn(BPA) binary complex and titrated with 8-HQ. The observed binding curve (Figure 3.10) fitted well to a one-set-of-sites model, which yielded an $n$ value of $0.79 \pm 0.03$ 8-HQ per BPA-bound Mn$^{2+}$ (Table 3.5), indicating the formation of approximately a Mn$^{2+}$(BPA)(8-HQ) ternary complex. The corresponding calculated $\Delta G^\circ$ value was $-5.3 \pm 0.2$ kcal per mole of 8-HQ (Table 3.5). Note that only one experiment was performed and the standard deviation here was derived from curve fitting. This $\Delta G^\circ$ value provided a rough estimate for the energetic contribution of an active site Mn$^{2+}$ to the overall $\Delta G^\circ$ of 8-HQ binding to a Mn$^{2+}$-containing enzyme active site. The affinity constant of 8-HQ binding Mn(BPA) was $(7.4 \pm 0.1) \times 10^3 \text{M}^{-1}$, weaker than that of 8-HQ binding to Mn$^{2+}$(TPA) ($K_a = (1.3 \pm 0.3) \times 10^4 \text{M}^{-1}$) (Table 3.5). This relative affinity was unexpected since the Mn$^{2+}$ in Mn(BPA) has three coordination bonds while in Mn(TPA) it has four coordination bonds. Given the fact that Mn$^{2+}$ ($d^5$) prefers to form octahedral complexes$^{97}$, electrons from 8-HQ would be filling the lower energy orbitals in the case of Mn$^{2+}$(BPA) than in Mn(TPA). Alternatively, BPA-bound Mn$^{2+}$ has a higher net positive charge than the TPA-bound Mn$^{2+}$ therefore is expected to bind 8-HQ more strongly than Mn(TPA). Further experiments are needed to obtain more reliable data before a rationale is provided for the observed difference.
Figure 3.10 ITC titration raw data and binding isotherm for titrations of 10 mM 8-HQ into 1 mM MnCl₂ and 1 mM BPA in the MeOH:NEM buffer mixture, pH 6.80, at 25 °C.

Table 3.5 Thermodynamic parameters from ITC studies of 8-HQ interaction with Mn²⁺:Ligand at 25 °C in the MeOH:buffer mixture at pH 6.80.

<table>
<thead>
<tr>
<th>Mn(Ligand) (in calorimeter cell)</th>
<th>n</th>
<th>Kₐ (M⁻¹)</th>
<th>K₅ (µM)</th>
<th>ΔG° (kcal/mol)</th>
<th>ΔH° (kcal/mol)</th>
<th>TΔS° (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Mn(TREN)]²⁺</td>
<td>1.53 ± 0.08</td>
<td>(5.98 ± 0.03) × 10⁻¹</td>
<td>(1.8 ± 0.01) × 10²</td>
<td>-5.2 ± 0.3</td>
<td>-0.77 ± 0.004</td>
<td>4.40 ± 0.02</td>
</tr>
<tr>
<td>[Mn(TPA)]²⁺</td>
<td>1.00 ± 0.05</td>
<td>(1.3 ± 0.1) × 10⁴</td>
<td>(7.4 ± 0.4) × 10⁻¹</td>
<td>-5.6 ± 0.3</td>
<td>-0.50 ± 0.003</td>
<td>5.10 ± 0.03</td>
</tr>
<tr>
<td>[Mn(BPA)]²⁺</td>
<td>0.79 ± 0.03</td>
<td>(7.4 ± 0.1) × 10²</td>
<td>(1.5 ± 0.5) × 10²</td>
<td>-5.3 ± 0.2</td>
<td>-1.5 ± 0.1</td>
<td>3.7 ± 0.2</td>
</tr>
</tbody>
</table>

Data were obtained from titrations of 8-HQ into a mixture of a ligand (TREN or TPA or BPA) and MnCl₂ (at 1:1 molar ratio of Ligand: Mn²⁺) in the MeOH:buffer (60:40 by volume) mixture at pH 6.80. The values of ΔG°, ΔH° and TΔS° were for per mole of Mn²⁺ for an overall equilibrium of ligand-bound Mn²⁺ coordinating to 8-HQ coupled to any deprotonation and protonation reactions. The value of TREN and TPA experiment only from one experiment.
Chapter 4: ITC studies of the interactions of metal ion(II) with inhibitor

4.1 ITC studies of the interactions of 8-HQ with metal ion(II)

Complexes of the first row transition metal ions with 8-HQ have been reported and their stability constants follow the Irving-Williams series (Zn < Cu > Co > Fe > Mn) in that copper complexes are the most stable while manganese complexes are the least stable. Both 2:1 and 1:1 complexes of the divalent transition metal ions with 8-HQ have been reported based on crystallography, the former in the presence of excess 8-HQ and the latter in the presence of excess metal ion. A crystal structure of Cu(8-HQ)$_2$(H$_2$O)$_2$ with a distorted octahedral geometry was reported by N. Okabe and H. Saishu.

To understand the interaction of 8-HQ with the three transition metal ions in the absence of the ligands being investigated, ITC experiments of 8-HQ titration into the chloride salt of each of the metal ions, Cu$^{2+}$, Co$^{2+}$ and Mn$^{2+}$ were performed in the MeOH:Tris buffer mixture at pH 7.40, MeOH:NEM buffer mixture at pH 7.25, and MeOH:NEM buffer mixture at pH 6.80, respectively. In all three solutions, 8-HQ was titrated into the metal ion solution and therefore the 8-HQ was in excess at the end of the titration. The resulting binding isotherms were fit to a one-set-of-sites binding model for Cu$^{2+}$ and Co$^{2+}$ (Figures 4.1 and 4.2, respectively) and a sequential binding model for Mn$^{2+}$ (Figures 4.3). The binding curves suggested that the two 8-HQ molecules bound with the same affinity to a given metal ion (Cu$^{2+}$ and Co$^{2+}$) (Table 4.1), however with a different affinity in the Mn$^{2+}$ case (Table 4.1). Indeed, the affinity of 8-HQ was the highest for Cu$^{2+}$ ($K_{a, Cu(8-HQ)2} = (3.2 \pm 0.3) \times 10^6$ M$^{-1}$), the lowest for Mn$^{2+}$ ($K_{a1,Mn(8-HQ)} = (1.0$
± 0.2) \times 10^5 \text{M}^{-1} \text{ and } K_{\text{a2}, \text{Mn(8-HQ)}} = (1.3 \pm 0.1) \times 10^4 \text{M}^{-1} \text{ and intermediate for Co}^{2+} (K_{\text{a}, \text{Co(8-HQ)}2} = (1.3 \pm 0.3) \times 10^6 \text{M}^{-1})). \text{ Therefore, our ITC data confirms the relative stability of 8-HQ complexes between Cu}^{2+}, \text{ Co}^{2+}, \text{ and Mn}^{2+} \text{ in their affinity with 8-HQ as stated in the Irving-Williams series. It should be noted that the data for the three metal ions were obtained at somewhat different pH (7.4 or 7.25 or 6.8) with different buffer (Tris in the case of Cu}^{2+} \text{ which would underestimates affinity between metal ion and ligand; NEM for Co}^{2+} \text{ and Mn}^{2+}), \text{ the general trend for the stability of the 8-HQ complexes still held true.}

\text{In MeOH:Tris buffer, Cu}^{2+} \text{ bound two copies of the metalloenzyme inhibitor 8-HQ with a high apparent affinity (K_a) of (3.2 \pm 0.3) \times 10^6 \text{M}^{-1}. Clearly, as presented in chapter 2, 8-HQ bound Cu}^{2+} \text{ 3-orders of magnitude more strongly than the second BPA did (5.0 \pm 4.2) \times 10^3 \text{M}^{-1}. This suggests that an inhibitor with high affinity for Cu}^{2+} \text{ such as 8-HQ would be able to displace the weak BPA or possibly both BPA, resulting in the formation of a Cu(8-HQ)}_2 \text{ complex.}
4.1.1 Cu\textsuperscript{2+} interaction with 8-Hydroxyquinoline

**Figure 4.1** Raw data (top panel) and binding isotherm (bottom panel) for titration of 1 mM 8-Hydroxyquinoline into a solution containing 0.05 mM CuCl\textsubscript{2} in MeOH:Tris buffer mixture at pH 7.40 at 25 °C
4.1.2 Co$^{2+}$ interaction with 8-Hydroxyquinoline

Figure 4.2 Raw data (top panel) and binding isotherm (bottom panel) for titration of 10 mM 8-HQ into a solution containing 0.5 mM CoCl$_2$ in MeOH:NEM buffer mixture at pH 7.25 at 25 °C
4.1.3 Mn$^{2+}$ interaction with 8-Hydroxyquinoline

**Figure 4.3** Raw data (top panel) and binding isotherm (bottom panel) for titration of 2 mM 8-Hydroxyquinoline into a solution containing 0.1 mM MnCl$_2$ in MeOH:NEM buffer mixture at pH 6.80 at 25 °C

**Table 4.1** Thermodynamic parameters from ITC studies of 8-HQ interaction with Cu$^{2+}$, Co$^{2+}$ or Mn$^{2+}$ at 25 °C in the MeOH:buffer mixture at pH 7.40, 7.25 and 6.80, respectively.

<table>
<thead>
<tr>
<th>Metal</th>
<th>n (8-HQ : M)</th>
<th>$K_a$ (M$^{-1}$)</th>
<th>$K_d$ (μM)</th>
<th>$\Delta G^\circ$ (kcal/mol)</th>
<th>$\Delta H$ (kcal/mol)</th>
<th>$\Delta S^\circ$ (cal/(mol·K))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$</td>
<td>2.0 ± 0.3</td>
<td>(3.2 ± 0.3) $\times 10^6$</td>
<td>5.7 ± 0.5</td>
<td>-8.7 ± 0.6</td>
<td>-8.3 ± 2.1</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Co$^{2+}$</td>
<td>1.95 ± 0.04</td>
<td>(1.3 ± 0.3) $\times 10^6$</td>
<td>0.8 ± 0.2</td>
<td>-8.3 ± 0.2</td>
<td>-10.5 ± 0.2</td>
<td>-7.5 ± 0.9</td>
</tr>
<tr>
<td>Mn$^{2+}$</td>
<td>sequential binding model n=2</td>
<td>(1.0 ± 0.2) $\times 10^6$</td>
<td>9.7 ± 0.1</td>
<td>-6.8 ± 0.1</td>
<td>-2.8 ± 1.0</td>
<td>13.3 ± 0.6</td>
</tr>
</tbody>
</table>

Data were obtained from titrations of 8-HQ into a solution of CuCl$_2$, CoCl$_2$, and MnCl$_2$ in the MeOH:buffer (60:40 by volume) mixture.
Chapter 5: Experimental methods for Isothermal titration calorimetry (ITC)

5.1 Methods

ITC experiments were performed on a MicroCal VP-ITC unit (GE Healthcare) at 25 °C. The injection syringe and calorimeter reaction cell were allowed to soak in detergent before each experiment (10% Contrad detergent, 0.1 M EDTA). Then, rinse the syringe (30 mL), the reaction and reference cells thoroughly used Milli-Q water, and the syringe was dried with air. Degassed the sample before loading which prepared in buffer using a ThermoVac degassing station (Malvern Instruments). Samples in the present of methanol was not degassed to avoid differential evaporation of organic versus aqueous solvent thus significant change of solution composition. Always filled the reference cell of the calorimeter with the buffer that was used to prepare the samples.

Analysis of calorimetric data was performed using Origin 7.2 software that came with the instrument. The baseline for the raw data (µcal versus second) was manually adjusted. The integration of the area under each peak yields the heat associated with each injection, which is automatically normalized to per mole of titrant. This normalized heat as a function of titrant:titrant molar ratio gives rise to the binding isotherm. The heat generated or absorbed during the binding interaction is directly related to the fraction of ligand bound. If a significant control heat for the dilution of the titrant was observed, it was subtracted from the binding isotherm. The binding isotherm was then fit to either a one-set-of-sites or two-sets-of-sites binding model provided by the manufacturer in the Origin software. The fit was iterated to achieve the lowest Chi-squared value. In some instances, it was necessary to subtract a constant value corresponding to the plateau heat of a binding isotherm which is conventionally thought to
be a better control than a control experiment of injecting the titrant into a solution minus the titrante (often the buffer). A nonlinear least square approach (Levenberg-Marquardt algorithm) is then used to determine the thermodynamic parameters for the interaction studied: n, $K_a$, $\Delta H^\circ$, and $\Delta S^\circ$. The equilibrium association constant was used to calculate $\Delta G^\circ$ for the reaction (Equation 1).

$$\Delta G^\circ = -RT \times \ln(K_a)$$  \hspace{1cm} (1)

The $\Delta H^\circ$ determined from curve fitting and the calculated $\Delta G^\circ$ values were used to calculate $T\Delta S^\circ$ using Equation (2).

$$T\Delta S^\circ = \Delta H^\circ - \Delta G^\circ$$  \hspace{1cm} (2)

The dissociation constant ($K_d$) was calculated from the $K_a$ using Equation (3).

$$K_d = \frac{1}{K_a}$$  \hspace{1cm} (3)

The parameters obtained from repeat runs were averaged and error propagation was done for the parameters of each interaction studied.

5.2. Materials

5.2.1. General Considerations

Nitrilotriacetic acid (NTA, >99%), tris(2-pyridylmethyl)amine (TPA, >98.0%), tris(2- aminoethyl)amine (TREN, >96%) and bis(2-pyridylmethyl)amine (BPA, >98.0%) were purchased from TCI. Acetohydroxamic acid (AHA, >98%), N-ethylmorpholine (NEM, ≥ 99%) and methanol (MeOH, >99.9%) were from Acros Organics. 8-HQ was from Sigma Aldrich. N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid (DA2Im) was synthesized according to the literature. All Copper experiment solutions –except for BPA experiments were done in both Tris and NEM buffer- were prepared gravimetrically in 50 mM Tris buffer containing 0.15 M
NaCl (hereafter referred to as Tris buffer) or a 60:40 (by volume) mixture of MeOH:Tris buffer (hereafter referred to as MeOH: Tris buffer mixture) using Milli-Q water (≥18.2 MΩ, Milli-Q integral water purification system, Millipore), to a final pH of 7.40 ± 0.02. All cobalt and manganese experiment solutions were prepared gravimetrically in 50 mM NEM buffer containing 0.15 M NaCl (hereafter referred to as NEM buffer) or a 60:40 (by volume) mixture of MeOH:NEM buffer (hereafter referred to as MeOH: NEM buffer mixture) using Milli-Q water (≥18.2 MΩ, Milli-Q integral water purification system, Millipore), to a final pH of (6.80 or 7.25) ± 0.02. Buffer solution was chosen to ensure sufficient metal ion(II) solubility for the study using isothermal titration calorimetry.

5.2.2 Metal ion (II) interacting with different ligands

DA2Im, NTA, BPA, TPA and Tren titration experiments with M^{2+} were performed in four different solvent conditions: NEM buffer at pH (6.80 or 7.25), MeOH: NEM buffer mixture at pH (6.80 or 7.25), Tris buffer at pH 7.40 or MeOH: Tris buffer mixture at pH 7.40. Some ligands are only soluble in one solvent system and thus were not able to be studied in both solvent systems; specifically, NTA was insoluble in MeOH: (Tris or NEM) buffer mixture, and TPA was insoluble in NEM or Tris buffer. The NEM or Tris buffer were prepared in a 1 L Nalgene bottle using Milli-Q water (≥18 MΩ, Milli-Q integral water purification system, Millipore) with 0.150 M NaCl and adjusted the pH as needed. The MeOH: (Tris or NEM) buffer mixture solvent were prepared by mixing 30 mL of MeOH with 20 mL of (NEM or Tris) buffer in a 50 mL conical tube and the pH was readjusted to within ± 0.02 of pH 6.80 or 7.40 by adding 1 M NaOH. Solutions of each five different ligands and M^{2+} (M^{2+} = copper, cobalt, or manganese) chloride were prepared gravimetrically and the final solution was adjusted to within
± 0.02 units of the desired pH.

Studies of M²⁺ interaction with five ligands, NTA, DA2Im, TREN, TPA and BPA, were performed with ligand as the titrant contained in the injection syringe. The reference cell was filled with the corresponding solvent system used to prepare the M²⁺ and ligand solutions. The titrant was titrated into the reaction cell in 10 μL aliquots over 20 seconds for a total of 28 injections. The syringe was set to stir at 307 rpm for the duration of the experiment. Between each injection, 360-600 seconds elapse to ensure adequate time for the signal to return to the baseline. A minimum of two but often three or more experiments were conducted for each ligand to ensure reproducibility.

Table 5.1 Concentrations of Titrant and Titrate for Ligand-M²⁺ Studies

<table>
<thead>
<tr>
<th>Titrant</th>
<th>Titrant Concentration (mM)</th>
<th>M²⁺</th>
<th>Titrant (M²⁺) Concentration (mM)</th>
<th>Solvent System (buffer*)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>DA2Im</td>
<td>8.8</td>
<td>Cu²⁺</td>
<td>0.33</td>
<td>Tris</td>
<td>7.40</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td></td>
<td>0.1</td>
<td>Tris</td>
<td></td>
</tr>
<tr>
<td>NTA</td>
<td>1.5</td>
<td></td>
<td>0.15</td>
<td>Tris</td>
<td>7.40</td>
</tr>
<tr>
<td></td>
<td>0.15</td>
<td></td>
<td>0.015</td>
<td>Tris</td>
<td></td>
</tr>
<tr>
<td>TREN</td>
<td>0.5</td>
<td>Co³⁺</td>
<td>0.05</td>
<td>MeOH:Tris</td>
<td>6.80 and 7.25</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>1</td>
<td>NEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td></td>
<td>0.04</td>
<td>MeOH: NEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>Mn³⁺</td>
<td>0.05</td>
<td>MeOH: NEM</td>
<td>6.80</td>
</tr>
<tr>
<td>TPA</td>
<td>0.75</td>
<td>Cu²⁺</td>
<td>0.075</td>
<td>MeOH: Tris</td>
<td>7.40</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>Co³⁺</td>
<td>0.01</td>
<td>NEM</td>
<td>6.80 and 7.25</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td></td>
<td>0.01</td>
<td>MeOH: NEM</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.75</td>
<td>Mn³⁺</td>
<td>0.075</td>
<td>MeOH: NEM</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.5</td>
<td>Tris</td>
<td>7.40</td>
</tr>
<tr>
<td>BPA</td>
<td>0.08</td>
<td>Cu²⁺</td>
<td>0.004</td>
<td>NEM</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>0.3</td>
<td></td>
<td>0.03</td>
<td>MeOH: NEM</td>
<td>6.80</td>
</tr>
<tr>
<td></td>
<td>4.5</td>
<td>Co³⁺</td>
<td>0.3</td>
<td>NEM</td>
<td>6.80 and 7.25</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td></td>
<td>0.05</td>
<td>MeOH: NEM</td>
<td>6.80</td>
</tr>
</tbody>
</table>

* Buffer Tris, MeOH:Tris, NEM, and MeOH: NEM correspond to 50 mM Tris buffer (0.150 M NaCl), 60:40 (by volume) mixture of MeOH: 50 mM Tris buffer (0.150 M NaCl, pH 7.40), 50 mM NEM buffer (0.150 M NaCl), and 60:40 (by volume) mixture of MeOH: 50 mM NEM buffer (0.150 M NaCl), respectively.

Data analysis was performed using Origin 7.2 software as described above. The resulting binding isotherms were fit to an iterative, one-set-of-sites binding model or sequential binding model (MicroCal, LLC). The binding parameters of the replicate experiments were averaged and error
propagation was performed.

5.2.3 ML Interaction with 8-Hydroxyquinoline (8-HQ) and acetohydroxamic acid (AHA)

ITC experiments were performed to examine the interaction of AHA and 8-HQ, two known metalloenzyme active site inhibitors, with the ML complexes (studied in Chapter 2) acting as potential enzyme active site metal ion structural mimetics. AHA experiments were performed in Tris buffer due to its aqueous solubility. 8-HQ was insoluble in aqueous solution and therefore was studied in the MeOH:buffer (NEM or Tris) (60:40) mixture. Experiments with AHA were unsuccessful because its low affinity for the ML required the use of high AHA concentrations which in turn was associated with large AHA dilution heat, making it impossible to extract accurate binding parameters. Instead, UV-Vis absorbance spectroscopy was performed to obtain binding constant.

AHA and 8-HQ solutions were prepared gravimetrically and the final solution pH was adjusted to within ± 0.02 units of the desired pH. Each ligand and metal ion (II) solutions were prepared gravimetrically. The solutions of the ligand and the metal ion were mixed in the appropriate ratio to yield the desired final concentration and the final pH was adjusted to within ± 0.02 units of the desired value. For example, 1.5-mL of CuCl₂ at 1 mM was mixed with 1.5 mL of BPA at 1 mM to yield 3-mL of a Cu(BPA) complex at 0.5 mM each of Cu²⁺ and BPA.

ITC experiments were performed by titrating a solution of inhibitor (8-HQ or AHA) into a solution of ML, the latter acting as an enzyme active site structural mimic, or into a solution of M²⁺. Experiments with 8-HQ were done in MeOH:(NEM or Tris) buffer mixture at pH 6.80 or 7.25 when NEM was used or at pH 7.40 when Tris was used.
Table 5.2 Concentrations of Titrant (8-HQ) and Titrate for ML or M\(^{2+}\) with inhibitor.

<table>
<thead>
<tr>
<th>8-HQ Concentration (mM)</th>
<th>Titrate</th>
<th>Titrate Concentration (mM)</th>
<th>Solvent System</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>2:1 BPA:Cu(^{2+})</td>
<td>1</td>
<td>MeOH:Tris</td>
<td>7.40</td>
</tr>
<tr>
<td>5</td>
<td>1:1 BPA:Cu(^{2+})</td>
<td>0.5</td>
<td>MeOH:Tris</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1:1 BPA:Co(^{2+})</td>
<td>0.3</td>
<td>MeOH:NEM</td>
<td>6.80 and 7.25</td>
</tr>
<tr>
<td>10</td>
<td>1:1 Tren: Co(^{2+})</td>
<td>1</td>
<td>MeOH:NEM</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1:1 TPA: Co(^{2+})</td>
<td>1</td>
<td>MeOH:NEM</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>1:1 BPA:Mn(^{2+})</td>
<td>1</td>
<td>MeOH:NEM</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1:1 Tren:Mn(^{2+})</td>
<td>0.2</td>
<td>MeOH:NEM</td>
<td>6.80</td>
</tr>
<tr>
<td>10</td>
<td>1:1 TPA:Mn(^{2+})</td>
<td>1</td>
<td>MeOH:NEM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Cu(^{2+})</td>
<td>0.05</td>
<td>MeOH:Tris</td>
<td>7.40</td>
</tr>
<tr>
<td>10</td>
<td>Co(^{2+})</td>
<td>0.5</td>
<td>MeOH:NEM</td>
<td>7.25</td>
</tr>
<tr>
<td>2</td>
<td>Mn(^{2+})</td>
<td>0.1</td>
<td>MeOH:NEM</td>
<td>6.80</td>
</tr>
</tbody>
</table>

* The buffers contain 50 mM NEM or Tris, 0.150 M NaCl. The MeOH:buffer mixture corresponds to 60:40 (by volume) of MeOH over NEM or Tris buffer. Tris was used for copper only.

Data analysis was performed using Origin 7.2 software as described above. The resulting binding isotherms were fit with an iterative, one-set-of-sites binding model or sequential binding model (MicroCal, LLC). The binding parameters from the replicate experiments were averaged and error propagation was performed.

5.3. Synthesis of (N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid) DA2Im

Synthesis of N-(2-(1-methylimidazolyl)methyl)iminodiacetic acid (DA2Im) was accomplished in two steps.\(^{99,100}\) Diethyl iminodiacetate (1.80 mL, 10.3 mmol) and 1-methyl-2-imidazolecarboxaldehyde (1.031 g, 9.4 mmol) were combined in DCE (1,2-Dichloroethane). A reductive amination was achieved with sodium triacetoxyborohydride (2.976 g, 14.0 mmol) under N\(_2\). The reaction was allowed to proceed overnight under stirring. The reaction was then quenched with saturated sodium bicarbonate, extracted three times with ethyl acetate, and dried with sodium sulfate. The product was purified by column chromatography (silica gel, 100% ethyl acetate) to yield a light yellow oil. The oil was dissolved in 25 mL of 2 N HCl and refluxed overnight. The product was concentrated in vacuo and dried in a vacuum oven to remove any
residual water. Removal of solvent yielded 1.172 g of a flaky, off-white solid (38% yield). \textsuperscript{1}H NMR (300 MHz, D\textsubscript{2}O): \( \delta \) 7.25-7.32 (d, 2H), 4.2-4.4 (s, 2H), 3.8-3.9 (s, 3H), 3.6-3.8 (s, 4H). \textsuperscript{99}

In an effort to avoid generation of chlorinated waste, the synthesis was also attempted using ethyl acetate as the solvent rather than DCE. This synthesis was unsuccessful as various side products were generated and too difficult to purify. \textsuperscript{99}

**Chapter 6: UV-Vis Spectroscopy**

**6.1. Methods**

UV-Vis absorbance spectroscopy was used as a second method to study the interaction, specifically, the equilibrium binding constant \( (Ka) \) for the metalloenzyme inhibitor AHA with metal ligand binary complexes (ML).

**6.2. Formulae for Calculating \( Ka \)**

In light of the poor ITC data, and therefore inconclusive results, for acetohydroxamic acid (AHA) as an inhibitor (I) binding to the Cu\textsuperscript{2+}L (CuL) complexes, we turned to UV-Vis absorbance spectroscopy to determine the binding constant, \( Ka \). The equilibrium between the binary complex ML, the inhibitor (I) and the ternary complex MLI is shown below

\[
ML + I \rightleftharpoons MLI \quad (\text{Equation 6.1})
\]

The equilibrium constant, \( Ka \), can be expressed as follows:

\[
Ka = \frac{[MLI]}{[ML][I]} \quad (\text{Equation 6.2})
\]

To determine the equilibrium concentrations of the reactants and the products needed for the calculation of \( Ka \), UV-Vis absorbance spectra were recorded for the following samples: 1) the
ML binary complex (formed at 1:1 molar ratio of M:L); 2) the MLI ternary complex (formed at 1:1:300 molar ratio of M:L:I); 3) a mixture of ML and MLI complexes (formed at 1:1:1 molar ratio of M:L:I). At the concentrations of M and L used ( [M] = [L]; 0.5 mM for all but 1 mM when L was TREN), a 1:1 M:L mixture consisted predominantly of the ML binary complex; a mixture of M, L and I at the 1:1:300 molar ratio consisted predominantly of the MLI ternary complex; a mixture of M, L and I at 1:1:1 molar ratio consisted of both ML and MLI.

To derive an equation for calculating the $K_a$, the Beer’s Law (6.3 Equation) relating absorbance (A) to concentration (c) of the absorbing molecule was used.

$$ A = \varepsilon bc $$  \hspace{1cm} \text{(Equation 6.3)}

where $\varepsilon$ is the molar absorption coefficient (M$^{-1}$cm$^{-1}$); $b$ is the length of the path traversed by the light (which is 1 cm for all samples).

Assuming that $C_0$ is the initial concentration of ML before the addition of I. to a final molar ratio of 1:1:1 (M:L:I) which moves the equilibrium to the product side. Assuming the new equilibrium concentration of ML to be $x$, the equilibrium concentration of I and MLI would be $x$ and $C_0 - x$, respectively.

**Table 6.1** The concentration of each product and reactant in the equilibrium reaction.

<table>
<thead>
<tr>
<th></th>
<th>ML</th>
<th>I</th>
<th>MLI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial concentration (1:1:1 M:L:I)</td>
<td>$C_0$</td>
<td>$C_0$</td>
<td>0</td>
</tr>
<tr>
<td>Changes after reaching equilibrium upon addition of I to ML binary complex</td>
<td>$x - C_0$</td>
<td>$x - C_0$</td>
<td>$C_0 - x$</td>
</tr>
<tr>
<td>Equilibrium concentration</td>
<td>$x$</td>
<td>$x$</td>
<td>$C_0 - x$</td>
</tr>
</tbody>
</table>

At a given wavelength, the absorbance of ML (formed at 1:1 molar ratio of M:L) is labeled as $A_1$; the absorbance of MLI (formed at the molar ratio of 1:1:300 of M:L:I) is labeled
as $A$; the absorbance of the mixture of ML and MLI formed at the molar ratio of 1:1:1 of M:L:I is labeled as $A_0$. According to the Beer’s Law, the following equations can be written for the ML and the MLI complexes:

\[ A\ _{-1}(1:1) = \varepsilon\ _{-1} b\ C_\circ \]  
\[ \varepsilon\ _{-1} = \frac{A\ _{-1}}{b\ C_\circ} \]  
\[ A\ (1:1:300) = \varepsilon\ b\ C_\circ \]  
\[ \varepsilon = \frac{A\ }{b\ C_\circ} \]  
\[ A^\circ\ (1:1:1) = \varepsilon\_1 b x + \varepsilon b (C_\circ - x) \]  
\[ A^\circ\ (1:1:1) = \varepsilon^\circ b C = \varepsilon\ _{-1} b x + \varepsilon b (C_\circ - x) \]

(Equation 6.7) is the absorbance for the mixture of ML and MLI formed upon addition of one molar equivalent of I into the ML (i.e., 1:1:1 M:L:I). It is based on the principle that the absorbance of a mixture at any wavelength is equal to the sum of the absorbance of each component at that wavelength.

By substituting $\varepsilon\_1$ and $\varepsilon$ in (Equation 6.7) with the expressions in (Equation 6.4b and 6.5b), the following equation for $A_0$ is obtained

\[ A\ = \frac{A\ _{-1}}{b\ C_\circ} b x + \frac{A}{b\ C_\circ} b (C_\circ - x) = \frac{A\ _{-1}}{C_\circ} x + \frac{A}{C_\circ} (C_\circ - x) = \frac{A\ _{-1}}{C_\circ} x + A - \frac{A}{C_\circ} x = \frac{A\ _{-1} - A}{C_\circ} x + A \]

(Equation 6.8)
\[ A \circ -A = \frac{A_{-1} - A}{C_0} \times \]  

(Equation 6.9)

Therefore, \[ x = \frac{A \circ -A}{A_{-1} - A} \times C_0 \]  

(Equation 6.10)

where the value \( A_o \) is the absorbance observed when a molar equivalent of I was added to the binary complex ML formed at 1:1 molar ratio of M:L. This absorbance is the total absorbance of the ML and MLI present;

Substituting the expression of x into the equation for \( K_a \), we have the following:

\[
K_a = \frac{C_o - x}{x \times x} = \frac{C_o - \frac{A \circ - A}{A_{-1} - A} \times C_o}{\left(\frac{A \circ - A}{A_{-1} - A}\right)^2 C_o} = \frac{C_o \left(\frac{1}{A \circ - A} - A_{-1}\right)}{\left(\frac{A \circ - A}{A_{-1} - A}\right)^2 C_o}
\]

(Equation 6.11)

\[
= \frac{(A \circ - A_{-1})}{(A - A_{-1})^2 C_o} \times (A - A_{-1}) = \frac{(A_{-1} - A \circ) (A_{-1} - A)}{(A - A_{-1})^2 C_o}
\]

(Equation 6.12)

Equation 6.12 was used to calculate the binding constant \( K_a \) of AHA for each of the five CuL binary complexes.

UV-Vis absorbance experiments for DA2Im and NTA were performed in the Tris buffer but not MeOH:Tris buffer as the two ligands are not soluble in the presence of MeOH. Experiments for TREN was performed in the Tris buffer only and BPA was performed in both solvent systems. Experiments for TPA were performed in MeOH:Tris buffer mixture only as it was not soluble in buffer alone.

Three wavelengths were chosen within the spectral region that yielded the most absorbance difference between the ML and MLI (M= Cu\(^{2+}\), I=AHA, and L= DA2Im, NTA, TREN, BPA, or TPA). The absorbance difference at each of the three wavelengths were used to calculate the \( K_a \) using (Equation 6.12) and the resulting three \( K_a \) values were averaged. It should be noted that
none of the five ligands studied showed absorbance within the chosen region of wavelength. Analysis of the data was performed using Origin Lab software.

6.2. Results and Discussion

6.2.1 UV-Vis spectra for CuL interaction with AHA

Due to the weak affinity of AHA for CuL binary complexes, its interaction was not able to be determined accurately using ITC. Instead, UV-Vis spectroscopy was used to examine this interaction. As explained in section 6.1.1, absorbance readings of three samples of a CuL complex with and without AHA were prepared and their absorbance spectra were recorded. Specifically, the samples include the CuL binary complex, a 1:1:1 (Cu^{2+}:L:AHA) molar ratio mixture and a 1:1:300 (Cu:L:AHA) molar ratio mixture. The 1:1:300 sample corresponded to CuL(AHA) which corresponded to all CuL complexes fully bound with AHA, the binding constant of AHA for CuL was calculated using absorbance readings at several wavelengths. All the reading was taken after the baseline correction at lab temperature.

In general, the absorbance of the CuL binary complex at the chosen wavelengths decreased with the addition of AHA except for Cu(TREN) for which absorbance increased upon addition of AHA. For $K_a$ calculation, three wavelengths were chosen within a spectral region chosen for the highest absorbance difference between CuL and CuL(AHA) and the lowest or no absorbance from Cu^{2+} and the ligand alone. The range of the wavelength thus selected for all ligands was 280-400 nm. The binding constant ($K_a$) of AHA for each of the CuL complexes were on the order of $10^1$–$10^2$ M$^{-1}$ (Table 4.2),
Table 6.2 Binding affinity of CuL binary complex with AHA as derived from UV-Vis absorbance spectroscopy.

<table>
<thead>
<tr>
<th>ML</th>
<th>Solvent</th>
<th>C (M)</th>
<th>K_a (M⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu(DA2Im)</td>
<td>Tris buffer</td>
<td>0.500 × 10⁻³</td>
<td>(3.3 ± 0.2) × 10²</td>
</tr>
<tr>
<td>Cu(NTA)</td>
<td>MeOH:Tris buffer</td>
<td>1.00 × 10⁻³</td>
<td>(2.3 ± 0.4) × 10²</td>
</tr>
<tr>
<td>Cu(TREN)</td>
<td>MeOH:Tris buffer</td>
<td>1.00 × 10⁻³</td>
<td>(3.3 ± 0.2) × 10²</td>
</tr>
<tr>
<td>Cu(BPA)</td>
<td>MeOH:Tris buffer</td>
<td>1.00 × 10⁻³</td>
<td>(2.3 ± 0.4) × 10²</td>
</tr>
<tr>
<td>Cu(TPA)</td>
<td>MeOH:Tris buffer</td>
<td>1.00 × 10⁻³</td>
<td>(2.3 ± 0.4) × 10²</td>
</tr>
</tbody>
</table>

*UV-Vis absorbance readings were taken after adding 0.3 (g) AHA. A standard deviations of one-sigma error bars were used form the average of three measurements.

The experiments were done in Tris buffer and/or MeOH:Tris buffer mixture depending on the solubility of the ligand. Data for Cu(DA2Im) and Cu(NTA) were obtained in Tris buffer only while data for Cu(TPA) were obtained in MeOH:Tris buffer only.

Cu(NTA) and Cu(DA2Im) were found to bind AHA with slightly different affinity, with K_a being (2.49 ± 0.07) × 10² M⁻¹ for Cu(NTA) and (3.3 ± 0.2) × 10² M⁻¹ for Cu(DA2Im). DA2Im and NTA differ only in the fourth coordinating atom which was a carboxyl oxygen in NTA but an imidazole nitrogen in DA2Im. An imidazole nitrogen in general binds metal ions more strongly than a carboxyl oxygen, which would predict Cu(DA2Im) to have a weaker affinity for an additional ligand, AHA in this case. However, the additional negative charge on the 3rd carboxyl of NTA would likely exert a repulsive effect on the deprotonated AHA. These two opposing effects seemed to have mostly cancelled out, resulting in only slightly different affinity for the two ML complexes for AHA.

The affinity of Cu(TREN) for AHA in Tris buffer was found to be an order of magnitude weaker than that of Cu(DA2Im) and Cu(NTA). This was clearly due to the stronger affinity of Cu²⁺ for TREN than for DA2Im and NTA (Figs. 6.1, 6.2, and 6.3).

Between Cu(TPA) and Cu(BPA), Cu(BPA) bound AHA approximately twice more strongly than...
Cu(TPA) did. Again, this was consistent with TPA, a tetradentate ligand, binding Cu$^{2+}$ significantly more strongly than BPA, a tridentate ligand that shares three identical coordination groups with TPA.

Although the presence of methanol had an effect on the affinity of Cu(TREN) for AHA, it did not alter the affinity of Cu(BPA) for AHA. This was consistent with the pyridyl nitrogens in BPA being fully deprotonated in either the buffer or the MeOH:buffer mixture prior to metal binding, therefore experiencing no effect on the difference in proton competition in the presence and absence of methanol.

In summary, the affinity constant obtained from the UV-Vis method of AHA for the five CuL binary complexes followed a trend that was consistent with the relative affinity of the five ligands for Cu$^{2+}$ as determined in the ITC experiments in Chapter 3.

![Figure 6.1](image-url)  
**Figure 6.1** UV-Vis spectra of Cu:DA2Im:AHA in Tris buffer at pH 7.40 with Cu$^{2+}$:DA2Im at 0.5 mM and AHA at 1 and 300 mM. The spectrum in black was for Cu:DA2Im (1:1), the one in red was for Cu:DA2Im:AHA (1:1:1), and the one in blue was for Cu:DA2Im:AHA with the additional of 300 mg of AHA. The chosen wavelengths are 290, 295, and 300 nm. A standard deviations of one-sigma error bars were used form the average of three measurements.
Figure 6.2 UV-Vis spectra of Cu:NTA:AHA in Tris buffer at pH 7.40 with Cu$^{2+}$:NTA at 0.5 mM and AHA at 1 and 300 mM. Spectrum in black shows the absorbance of Cu:NTA, spectrum in red shows the absorbance of Cu:NTA:AHA (1:1:1), and the spectrum blue shows the absorbance of Cu:NTA:AHA with the additional of 300 mg of AHA. The chosen wavelengths were 285, 290, and 295 nm. A standard deviations of one-sigma error bars were used form the average of three measurements.

Figure 6.3 UV-Vis spectra of Cu:TREN:AHA into Tris buffer at pH 7.40 with Cu$^{2+}$:TREN at 0.5 mM and AHA at 1 and 300 mM. Spectrum in black shows the absorbance of Cu:TREN, the spectrum in red shows the absorbance of Cu:TREN:AHA (1:1:1), and the spectrum in blue shows the absorbance of Cu:TREN:AHA with the additional 300 mg of AHA. The chosen wavelengths were 380, 390, and 400 nm. A standard deviations of one-sigma error bars were used form the average of three measurements.
Figure 6.4 UV-Vis spectra of Cu:BPA:AHA into Tris buffer at pH 7.40 with Cu$^{2+}$:BPA at 0.5 mM and AHA at 1 and 300 mM. Spectrum in black shows the absorbance of Cu:BPA, the spectrum in red shows the absorbance of Cu:BPA:AHA (1:1:1), and the spectrum in blue shows the absorbance of Cu:BPA:AHA with the additional 300 mg of AHA. The chosen wavelengths were 285, 290, and 295 nm. A standard deviations of one-sigma error bars were used from the average of three measurements.

Figure 6.6 UV-Vis spectra of Cu:BPA:AHA into MeOH:Tris buffer at pH 7.40 with Cu$^{2+}$:BPA at 0.5 mM and AHA at 1 and 300 mM. Spectrum in black shows the absorbance of Cu:BPA, the spectrum in red shows the absorbance of Cu:BPA:AHA (1:1:1), and the spectrum in blue shows the absorbance of Cu:BPA:AHA with the additional 300 mg of AHA. The chosen wavelengths were 285, 290, and 295 nm. A standard deviations of one-sigma error bars were used from the average of three measurements.
Figure 6.7 UV-Vis spectra of Cu:TPA:AHA into MeOH:Tris buffer at pH 7.40 with Cu$^{2+}$:TPA at 1 mM and AHA at 1 and 300 mM. Spectrum in black shows the absorbance of Cu:TPA, the spectrum in red shows the absorbance of Cu:TPA:AHA (1:1:1), and the spectrum in blue shows the absorbance of Cu:TPA:AHA with the additional 300 mg of AHA. The chosen wavelengths were 305, 315, and 325 nm. A standard deviations of one-sigma error bars were used form the average of three measurements.

Chapter 7: Computational Methods

7. Computational Studies of enzyme active site metal ion structural mimetics with metalloenzyme inhibitors

7.1. Computational Methods

There has been growing interest in transition metals (TM) and their complexes in computational chemistry, not only because of the very important roles these elements play in modern chemistry, but also due to the known difficulties associated with their theoretical treatments. The biggest problem associated with the calculation of TM-containing systems is the near degeneracy stemming from electrons partially occupying the d orbitals. Recently, several studies have been conducted to evaluate the performance of different density functional theory (DFT) methods for predicting several molecular properties and studying reactions involving TMs. Additionally, several studies have been carried out to improve the performance of DFT methods. However, in order to obtain very accurate and reliable calculations for the TM-
containing systems, one must implement multi-reference methods such as Multi-Reference Configuration Interaction (MRCI). Unfortunately, these kinds of methods are usually very expensive to run. Therefore, as the interest in carrying out calculations for large systems (such as proteins) keeps growing, it becomes increasingly desirable to identify computational methods that are less expensive than multi-reference methods but still able to achieve good performance. DFT is a promising choice because it is able to efficiently predict the atomic and molecular properties for a variety of systems. DFT has great advantage over the Hartree-Fock (HF) method in describing electron correlation effects and has favorable scaling properties with respect to molecular size when compared to post-Hartree-Fock methods. As a result, DFT is a widely used computational approach for studying large TM-containing compounds and shows significant promise as an ab initio method that can be used to investigate large macromolecules such as proteins and DNA.

In this study, ab initio calculations were performed for the interactions of Cu$^{2+}$ with ligands and CuL binary complexes with 8-HQ using the Gaussian 2009 program package. All geometry optimizations, frequency calculations, and BSSE-corrected complexation energy calculations were conducted at the HF/3-21g and BLYP/ CC-pVDZ levels of theory. Complexation energies were calculated by subtracting the individually optimized and calculated energies of the CuL complex and inhibitors from the energy of the optimized complex. Basis set effects were explored using the larger cc-PVDZ basis set.

First, we examined the total energy for all five ligands. Second, we determined the binding affinity of the binary complexes by subtracting the total energy of the ligand and the metal ion (Cu$^{2+}$ or Zn$^{2+}$) from the total energy of the binary complexes. Finally, we obtained the binding affinity of the inhibitor 8-HQ for the binary complexes. All calculations were done by using HF/ 3-21G or BLYP/ CC-pVDZ basis set and the geometry was shown in the Figures bellow.

**7.2 Optimized structure of individual compound**

Computational studies of copper ion (II), zinc ion (II), and all five ligands were performed by using two different calculations with two different basis sets: HF/3-21G and BLYP/ CC-pVDZ (Figure 7.1-7.5). All these compounds were optimized and their energies were
determined (Table 7.1). Structural optimization of the ligand and the metal ion allows more accurate estimation of the energy state. The computational data concludes that using larger basis sets allow more accurate the compound more stable, i.e. the energy is more negative.

**Figure 7.1** Optimized structure of the inhibitor AHA as calculated in gas phase via HF level of theory with a 3-21G basis set.

**Figure 7.2** Optimized structure of the inhibitor 8-HQ as calculated in gas phase via BLYP level of theory with a 3-21G basis set.
Figure 7.3 Optimized structure of the ligand NTA as calculated in gas phase via HF level of theory with a 3-21G basis set.

Figure 7.4 Optimized structure of TREN as calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Figure 7.5 Optimized structure of the ligand TPA as calculated in gas phase via HF level of theory with a 3-21G basis set.

Table 7.1 Total energy and the basis set used for the metal ions, inhibitors, and ligands.

<table>
<thead>
<tr>
<th>Metal Ions</th>
<th>Basis Set</th>
<th>Total Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu$^{2+}$</td>
<td>HF/3-21G</td>
<td>-2.05 × 10⁶</td>
</tr>
<tr>
<td>Cu$^{2+}$</td>
<td>BLYP/ CC-pVDZ</td>
<td>-2.06 × 10⁶</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>HF/3-21G</td>
<td>-1.11 × 10⁶</td>
</tr>
<tr>
<td>Zn$^{2+}$</td>
<td>BLYP/ CC-pVDZ</td>
<td>-1.12 × 10⁶</td>
</tr>
<tr>
<td>Inhibitors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AHA</td>
<td>HF/3-21G</td>
<td>-1.76 × 10⁵</td>
</tr>
<tr>
<td>8-HQ</td>
<td>HF/3-21G</td>
<td>-2.96 × 10⁵</td>
</tr>
<tr>
<td>8-HQ</td>
<td>BLYP/3-21G.</td>
<td>-2.98 × 10⁵</td>
</tr>
<tr>
<td>8-HQ</td>
<td>BLYP/ CC-pVDZ</td>
<td>-2.99 × 10⁵</td>
</tr>
<tr>
<td>Ligands</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TPA</td>
<td>HF/ 3-21G</td>
<td>-5.68 × 10⁵</td>
</tr>
<tr>
<td>DA2Im</td>
<td>HF/ 3-21G</td>
<td>-5.06 × 10⁵</td>
</tr>
<tr>
<td>NTA</td>
<td>HF/3-21G.</td>
<td>-4.59 × 10⁵</td>
</tr>
<tr>
<td>BPA</td>
<td>BLYP/ CC-pVDZ</td>
<td>-3.95 × 10⁵</td>
</tr>
<tr>
<td>TREN</td>
<td>BLYP/ CC-pVDZ</td>
<td>-2.87 × 10⁵</td>
</tr>
</tbody>
</table>
7.3 Optimized structure of MI, ML, and ML₂ complexes

Computational studies of copper (II) complexes with the five ligands and the inhibitor 8-HQ, and zinc (II) complexes with TPA and BPA were performed by using two different calculations with different basis sets: HF/3-21G and BLYP/ CC-pVDZ. The structures of the compounds were optimized (Figures 7.6-7.15) and the calculated Gibbs free energies for each species are listed (Tables 7.1 and 7.2). The binding energies, i.e., the Gibbs free energies for the formation of the binary (ML) and the tertiary complexes (MLI), are calculated by subtracting the energies of the reactants from the energy of the binary or ternary complex (Table 7.2). The computational data concludes that using the larger basis set (BLYP), which better approximates the wave function, yielded more negative binding energy thus more stable complexes.

The binding energies for Cu(DA2Im) and Zn(TPA) were found to be positive, indicating that these complexes were unstable in the gas phase. The rest of the binary complexes studied include Cu(NTA), Cu(TREN), Cu(BPA), Cu(TPA), Cu(8-HQ) and Zn(BPA) and they were found to be stable in the gas phase. The ternary complexes studied include Cu(8-HQ)₂, Cu(DA2Im)₂ and Cu(BPA)₂ and they were also stable (Table 7.2). Other isomers for Cu(8-HQ)₂ may be more stable and more work is needed.

Formation of the Cu(DA2Im)₂ complex had a negative energy, suggesting that adding a second DA2Im to Cu(DA2Im) helped stabilize the complex. Similarly, adding a second BPA to Cu(BPA) made it slightly more stable. In addition, Cu(BPA) was more stable than Zn(BPA).
Also, Cu(NTA) was more stable than Cu(DA2Im). Cu(TREN) binary complex was the most stable complex out of the five CuL complexes.

Some of the trends in binding energy detailed above were consistent with the trend observed in solution that was determined using ITC. For example, DA2Im and BPA both formed CuL and CuL₂ complexes in solution, consistent with the findings as calculated in the gas phase that adding a second L stabilized the complex. In other cases, the trend observed in gas phase did not agree with the trend obtained in solution. For example, in solution, DA2Im bound Cu²⁺ more strongly than NTA did. However, in gas phase, Cu(NTA) was stable while Cu(DA2Im) was not.

To conclude, the low basis set and the gas phase used for the computational study indicate that the values obtained in computational study cannot be directly correlated with the ITC data in solution. Further calculations will need to be carried out by using solvation models to better mimic ion or group solvation and protonation state in order to provide a better comparison to results from the ITC studies.

![Figure 7.6](image.png)

**Figure 7.6** Optimized structure of 8-HQ-Cu²⁺ binary complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Figure 7.7 Optimized structure of 8-HQ-Cu$^{2+}$ binary complex. The complex calculated in gas phase via BLYP level of theory with a 3-21G basis set.

Figure 7.8 Optimized structure of 8-HQ-Cu$^{2+}$-8-HQ complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Figure 7.9 Optimized structure of Cu$^{2+}$-DA2Im complex. The complex calculated in gas phase via HF level of theory with a 3-21G basis set.

Figure 7.10 Optimized structure of NTA-Cu$^{2+}$ binary complex was calculated in gas phase via HF level of theory with a 3-21G basis set.
Figure 7.11 Optimized structure of TREN-Cu$^{2+}$. The binary complex was calculated in gas phase via HF level of theory with a 3-21G basis set.

Figure 7.12 Optimized structure of TPA-Cu$^{2+}$ binary complex. The binary complex was calculated in gas phase via (HF or BLYP) level of theory with a (3-21G or CC-pVDZ) basis set, respectively.
Figure 7.13 Optimized structure of BPA-Cu$^{2+}$ binary complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.

Figure 7.14 Optimized structure (not completed) of BPA-Cu$^{2+}$-BPA complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.

Figure 7.15 Optimized structure of BPA-Zn$^{2+}$ binary complex. The complex calculated in gas phase via BLYP level of theory with a 3-21G basis set.

Table 7.2 Total and binding energies for binary or ternary complexes

<table>
<thead>
<tr>
<th>Complexes</th>
<th>Basis Set</th>
<th>Total Energy</th>
<th>Binding Energy</th>
</tr>
</thead>
</table>

119
<table>
<thead>
<tr>
<th>Basis Set</th>
<th>Complex</th>
<th>Bond Lengths (Å)</th>
<th>(kcal/mol)</th>
<th>(kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-HQ-Cu^{2+}</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.33 × 10^6</td>
<td>-1.03 × 10^6</td>
<td></td>
</tr>
<tr>
<td>8-HQ-Cu^{2+}-8-HQ</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.63 × 10^6</td>
<td>-1.03 × 10^6</td>
<td></td>
</tr>
<tr>
<td>DA2Im-Cu^{2+}</td>
<td>HF/3-21G.</td>
<td>1.53 × 10^6</td>
<td>1.09 × 10^3</td>
<td></td>
</tr>
<tr>
<td>DA2Im-Cu^{2+}-DA2Im</td>
<td>HF/3-21G.</td>
<td>2.03 × 10^6</td>
<td>-4.91 × 10^3</td>
<td></td>
</tr>
<tr>
<td>NTA-Cu^{2+}</td>
<td>HF/3-21G.</td>
<td>1.48 × 10^6</td>
<td>-1.91 × 10^3</td>
<td></td>
</tr>
<tr>
<td>TREN-Cu^{2+}</td>
<td>HF/3-21G.</td>
<td>1.32 × 10^6</td>
<td>-1.03 × 10^6</td>
<td></td>
</tr>
<tr>
<td>TPA-Cu^{2+}</td>
<td>HF/3-21G.</td>
<td>1.59 × 10^6</td>
<td>-9.12 × 10^2</td>
<td></td>
</tr>
<tr>
<td>TPA-Cu^{2+}</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.60 × 10^6</td>
<td>-1.03 × 10^6</td>
<td></td>
</tr>
<tr>
<td>TPA-Zn^{2+}</td>
<td>HF/3-21G.</td>
<td>1.68 × 10^6</td>
<td>1.88 × 10^3</td>
<td></td>
</tr>
<tr>
<td>BPA-Cu^{2+}</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.42 × 10^6</td>
<td>-1.04 × 10^6</td>
<td></td>
</tr>
<tr>
<td>BPA-Cu^{2+}-BPA</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.80 × 10^6</td>
<td>-1.05 × 10^6</td>
<td></td>
</tr>
<tr>
<td>BPA-Zn^{2+}</td>
<td>BLYP/ CC-pVDZ</td>
<td>1.51 × 10^6</td>
<td>-9.17 × 10^4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.3** Bond lengths of the optimized structure of Cu^{2+}-8-HQ, Cu^{2+}-(8-HQ)\_2, Cu^{2+}-BPA, Cu^{2+}-(BPA)\_2, and Zn^{2+}-BPA using different basis set.

The bond lengths obtained for CuL and CuL\_2 complexes for other ligands are listed in (Table 7.4). The data indicate that using different basis set yielded different bond lengths thus binding energy and results from the higher basis set are expected to be more accurate. Comparing bond lengths for Cu(8-HQ) to that of Cu(8-HQ)\_2 obtained using the higher basis set indicate that the two 8-HQ molecules have identical coordination bond lengths suggesting identical binding affinity. This was consistent with the ITC result that the two 8-HQ bound Cu^{2+} with the same K\_a value. In contrast, comparing the bond lengths of Cu(BPA) to Cu(BPA)\_2, it was clear that the second BPA had longer coordination bond lengths than the first BPA, suggesting
that the second BPA bound Cu$^{2+}$ with weaker affinity. This was indeed confirmed by the ITC data.

7.4 Optimized structure of MLI complex

Calculations were performed on the interaction between Cu(BPA) and Zn(BPA) compounds with 8-HQ (Table 7.4). All of these compounds were optimized and their binding energies were determined. The computational data concludes that using larger basis set yields a more negative binding energy, i.e., more stable compound.

![Figure 7.16](image1.png)

**Figure 7.16** Optimized structure of Cu(BPA)(8-HQ) ternary complex. The complex calculated in gas phase via BLYP level of theory with a 3-21G basis set.

![Figure 7.17](image2.png)

**Figure 7.17** Optimized structure of Cu(BPA)(8-HQ) ternary complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Figure 7.18 Optimized structure of Zn(BPA)(8-HQ) ternary complex. The complex calculated in gas phase via BLYP level of theory with a 3-21G basis set.

Figure 7.19 Optimized structure (not completed) of Zn(BPA)(8-HQ) ternary complex. The complex calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Table 7.4. Bond lengths and binding energy of Cu(BPA)(8-HQ) and Zn(BPA)(8-HQ) using different basis set.

<table>
<thead>
<tr>
<th>Basis Set</th>
<th>Complex</th>
<th>Bond Lengths (Å)</th>
<th>Binding Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N₁-M²⁺</td>
<td>N₂-M²⁺</td>
</tr>
<tr>
<td>BLYP/3-21G</td>
<td>Cu(BPA)(8-HQ)</td>
<td>2.01</td>
<td>1.94</td>
</tr>
<tr>
<td>BLYP/CC-pVDZ</td>
<td></td>
<td>2.15</td>
<td>2.07</td>
</tr>
<tr>
<td>BLYP/3-21G</td>
<td>Zn(BPA)(8-HQ)</td>
<td>2.20</td>
<td>1.98</td>
</tr>
<tr>
<td>BLYP/CC-pVDZ</td>
<td></td>
<td>2.26</td>
<td>2.19</td>
</tr>
</tbody>
</table>

*Numbers correspond to numbering in Figure 7.(18, 19, 20, and 21)

Figure 7.20 Optimized structure of the BSSE correction optimized structure of Zn(BPA)(8-HQ) ternary complex. The complexes shown were calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.
Figure 7.21 Optimized structure of the BSSE correction of Cu(BPA)(8-HQ) ternary complex. The complexes shown were calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.

Table 7.5 Total Energies of BSSE correction optimized structure of BPA-Zn$^{2+}$-8-HQ and BPA-Cu$^{2+}$-8-HQ ternary complex. The complexes shown were calculated in gas phase via BLYP level of theory with a CC-pVDZ basis set.

<table>
<thead>
<tr>
<th>complex</th>
<th>Binding Energy (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zn(BPA)(8-HQ)</td>
<td>-1.50 × 10^6</td>
</tr>
<tr>
<td>Cu(BPA)(8-HQ)</td>
<td>-1.42 × 10^6</td>
</tr>
</tbody>
</table>

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