

ABSTRACT

Thermodynamic Investigation of Copper Binding to the Amyloid-Beta (A β) Peptide

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Alzheimer's disease is a fatal and neurodegenerative disease with as many as 5.3 million Americans living with the disease. One of the characteristic hallmarks of Alzheimer's is the amyloid plaques that build up around the neurons. The aggregated amyloid- β (A β) peptide is one of the main components of the amyloid plaques found in individuals with Alzheimer's disease. Interestingly, high concentrations of metals (copper, zinc and iron) were found inside these amyloid plaques. The A β peptide can be either 40 or 42 residues in length but studies have shown the metal binding site is within the first 16 residues. Copper interactions with A β have been studied extensively, but there is no consensus on the coordinating ligands and binding affinity of metal to the peptide. The residues suspected in binding are the N-terminus (N-terminal amine or aspartate group), and the histidines in positions 6, 13, and 14.

The purpose of this research is to study the thermodynamics of copper binding to the A β peptide using isothermal titration calorimetry (ITC). Our goal is to understand the thermodynamics of copper binding to A β and give insight into the residues suspected in binding.

We have studied copper binding to the shorter A β 16 and A β 28 along with Ac-A β 16 and Ac-A β 28 to determine the thermodynamic contribution from the N-terminus. We have observed a thermodynamic difference between the acetylated and nonacetylated forms of the peptide. We have also studied the thermodynamic contributions of the three histidine residues by studying copper binding to A β 28(H6A), A β 28(H13A), and A β 28(H14A). These studies provide information about the metal binding site of A β . Understanding copper coordination and affinity to A β may give insight regarding metal chelators that can be used for drug therapy.

**Thermodynamic Investigation of Copper Binding to the Amyloid-Beta (A β)
Peptide**

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Dedication

This thesis is dedicated to Shirley Edmonson Hurley. Alzheimer's disease has been slowly stealing Shirley's life and consciousness over the past ten years. In the spring of 2010, her neurons were in such distress that she could not function without constant supervision from her family and husband of 53 years. She cannot recognize her children or husband and is forced to be content with the new life Alzheimer's has given her. I did not have the pleasure of knowing Shirley earlier, but I do know she has lived an incredible life. Individuals with Alzheimer's are on a sinister voyage into the unknown. When they arrive at their destination, they lose their identity, humility, and memories to a disease that gradually creeps up on them. They can no longer remember what year it is or how to make coffee. They have trouble remembering how to dress themselves or how many minutes are in an hour. This devastating disease robs you from remembering your loved ones or how to love. Your memories make the person you are today – what are you without them?

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Table of Contents

List of Figures.....	i
List of Tables.....	ii
List of Abbreviations.....	iii
Chapter 1: Alzheimer's Disease.....	1
1.1 Significance of Disease and Involvement of the Amyloid- β Peptide.....	1
1.2 Metal Involvement in Alzheimer's Pathology and the A β Peptide.....	4
i. Copper Binding to the High Affinity A β Site.....	6
ii. Copper Binding to the Low Affinity A β Site.....	8
iii. A β Affinity for Copper.....	9
1.3 Therapeutic Strategies.....	10
Chapter 2: Thermodynamic Principles and Project Design.....	12
2.1 Thermodynamics of Metal Binding to Peptides.....	12
2.2 Isothermal Titration Calorimetry (ITC).....	16
i. Analyzing ITC Data.....	18
ii. Curve Fitting using a One Set of Sites Model.....	19

iii.	Obtaining Thermodynamic Parameters.....	21
iv.	Issues Regarding ITC Experiments.....	22
2.3	Project Design.....	24
i.	A β Peptides.....	24
ii.	Buffer.....	26
2.4	Significance of Project.....	27
2.5	Project Objectives.....	28
 Chapter 3: Characterization of Copper and A β Interactions using Isothermal Titration Calorimetry.....		
3.1	Sample Preparation.....	29
3.2	Isothermal Titration Calorimetry Parameters.....	30
3.3	Characterization of Copper Binding to A β 16.....	33
i.	Experimental.....	33
ii.	Results.....	33
iii.	Discussion.....	36
3.4	Characterization of Copper Binding to Ac-A β 16.....	38
i.	Experimental.....	38

ii. Results.....	38
iii. Discussion.....	41
3.5 Characterization of Copper Binding to A β 28.....	43
i. Experimental.....	43
ii. Results.....	43
iii. Discussion.....	46
3.6 Characterization of Copper Binding to Ac-A β 28.....	48
i. Experimental.....	48
ii. Results.....	48
iii. Discussion.....	51
3.7 Characterization of Copper Binding to A β 28(H6A).....	53
i. Experimental.....	53
ii. Results.....	53
iii. Discussion.....	56
3.8 Characterization of Copper Binding to A β 28(H13A).....	58
i. Experimental.....	58
ii. Results.....	58

iii. Discussion.....	61
3.9 Characterization of Copper Binding to A β 28(H14A).....	63
i. Experimental.....	63
ii. Results.....	63
iii. Discussion.....	66
3.10 Conclusions.....	69
3.11 Future Direction.....	71
References.....	73
Appendix A (additional titration data).....	75
Appendix B.....	88

List of Figures

Figure 1.1. Diagram depicting the amyloid hypothesis.....	2
Figure 1.2. The A β peptide sequences of varying length that are studied.	5
Figure 1.3. The two most likely coordination sites of Cu ²⁺ bound to the A β peptide at the high affinity binding site at physiological pH.....	8
Figure 2.1. View of an isothermal titration calorimeter.....	17
Figure 2.2 Raw ITC data (top plot) and the binding isotherm (bottom plot).....	21
Figure 2.3. Chemical structure of ACES.....	26
Figure 3.1. ITC titration of 2.1 mM copper into 20 mM ACES.....	32
Figure 3.2. ITC titration of 2.1 mM copper into 136 μ M A β 16.....	35
Figure 3.3. ITC titration of 2.1 mM copper into 136 μ M Ac-A β 16.....	40
Figure 3.4. ITC titration of 2.1 mM copper into 113 μ M A β 28.....	45
Figure 3.5. ITC titration of 2.1 mM copper into 143 μ M Ac-A β 28.....	50
Figure 3.6. ITC titration of 2.1 mM copper into 140 μ M A β 28(H6A).....	55
Figure 3.7. ITC titration of 2.1 mM copper into 140 μ M A β 28(H13A).....	60
Figure 3.8. ITC titration of 2.1 mM copper into 140 μ M A β 28(H14A).....	65

List of Tables

Table 2.1. Equilibria associated with total heat observed in an ITC reaction involving the titration of metal into peptide.....	23
Table 3.1. The thermodynamics of copper binding to A β 16.....	37
Table 3.2. Comparison between the thermodynamics of copper binding to A β 16 and Ac-A β 16.....	42
Table 3.3 Comparison between the thermodynamics of copper binding to A β 28 and A β 16.....	47
Table 3.4. Comparison between the thermodynamics of copper binding to A β 28 and Ac-A β 28.....	52
Table 3.5 Comparison between the thermodynamics of copper binding to A β 28 and A β 28(H6A).....	57
Table 3.6. Comparison between the thermodynamics of copper binding to A β 28 and A β 28(H13A).....	62
Table 3.7. Comparison between the thermodynamics of copper binding to A β 28 and A β 28(H14A).....	67
Table 3.8. Comparison between the thermodynamics of copper binding to A β 28 and A β 28 mutants.....	68

List of Abbreviations

A β : Amyloid-beta

ACES: *N*-(2-Acetamido)-2-aminoethanesulfonic acid

AD: Alzheimer's Disease

APP: Amyloid Precursor Protein

CD: Circular Dichroism

CSF: Cerebrospinal Fluid

CQ: Clioquinol

DP: Differential Power

EDTA: Ethylenediaminetetraacetate

EPR: Electron Paramagnetic Resonance

ESI-MS: Electrospray ionization Mass-spectrometry

ΔG : Change in Gibbs free energy

ΔH : Change in enthalpy

HEPES: 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethansulfonic acid

HSAB: Hard-soft acid-base

ITC: Isothermal Titration Calorimetry

K: Binding constant

MD: Molecular Dynamics

NMR: Nuclear Magnetic Resonance

PIXE: Microparticle-induced X-ray Emission

RP-HPLC: Reverse-phase High-pressure Liquid Chromatography

ROS: Reactive Oxygen Species

ΔS : Change in entropy

UV-Vis : Ultraviolet-visible

Chapter 1: Alzheimer's Disease

Chapter 1.1. Significance of Disease and Involvement of the Amyloid- β Peptide

Alzheimer's disease (AD) is a fatal and progressive neurodegenerative disease characterized by plaques and tangles. It affects more than 35 million people worldwide. It is the most common form of dementia and accounts for 60 to 80 percent of all cases and now affects as many as 5.3 million people living in the United States.¹ During the early onset of the disease, the most common symptom is short-term memory loss. As the disease progresses, memory impairment increases with other symptoms such as mood swings, deterioration of language skills, and other behavioral and functional disturbances. Age is the biggest known factor with incidence occurring from one in ten over 65 to half of those that are over 85.¹ With the rapid growth of the elderly population, the cost for caring has totaled more than \$148 billion from Medicare, Medicaid, and businesses alone.¹ Without advances in therapy, the number of cases in the United States is expected to rise to 13.2 million by 2050.¹ Currently there is no treatment to prevent Alzheimer's disease; only drugs that can delay symptoms.

The brain of an individual with AD suffers from nerve cell death and massive tissue loss. The cerebral cortex, which is responsible for memory, consciousness, awareness, thought and language, begins to shrink. The area with the greatest loss is the hippocampus which is the area of the brain that is responsible for the formation of new memories. In addition to shrinkage, ventricles begin to fill up with increased amounts of cerebrospinal fluid (CSF) – an inflammation response within the brain.

The leading theory for why AD occurs is based on the amyloid hypothesis depicted in **Figure 1.1.**² The amyloid hypothesis states that the production and accumulation of the amyloid- β ($A\beta$) peptide initiates a pathological process that results in neurodegeneration.² It is hypothesized once $A\beta$ accumulates, neurofibrillary tangles (hyperphosphorylation of the protein tau) begin to form. Tau is thought to provide structure and support for the nerve cell, however in AD, it begins to deteriorate and twist into tangles. It has been shown that $A\beta$ triggers the tau pathology, but the steps connecting tau to $A\beta$ are currently unknown. In addition to tangles, oxidation stress is believed to play an important role that leads to degeneration. The $A\beta$ peptide is likely to mediate redox reactions because it can bind copper, zinc and iron. Metal and $A\beta$ interactions will be discussed in further detail later. It is still unknown whether the accumulation of $A\beta$ is a cause or effect of oxidative damage.

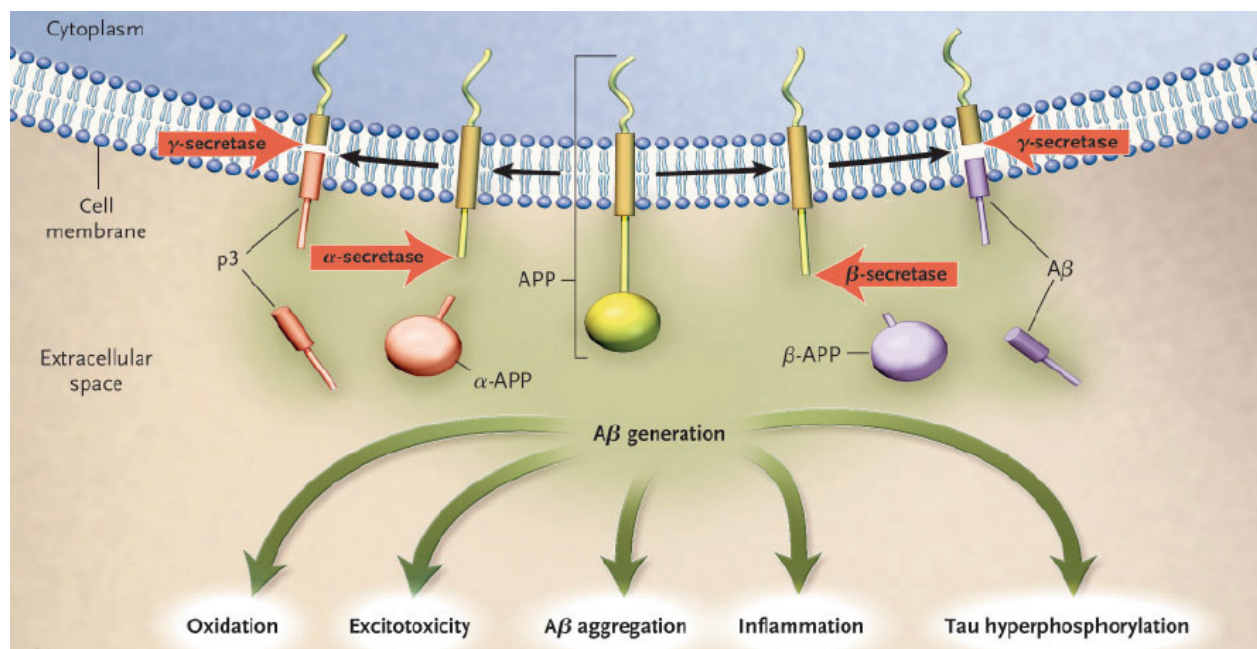


Figure 1.1. Diagram depicting the amyloid hypothesis.²

Furthermore, excitotoxicity (damage caused by glutamate, influx of Ca^{2+} , and other enzymes) and inflammation are other side effects that lead to neuronal death. Many different inflammation pathways begin to take place once $\text{A}\beta$ forms. Microglia cells, the central nervous systems' main system of immune defense, can be found clustered around amyloid plaques. It was suggested very recently the amyloid-beta peptide has antimicrobial activity against relevant microorganisms.³ This is the first evidence that $\text{A}\beta$ may have a functional role in the inflammatory response of the immune system.

The amyloid plaques, found in the gray matter of the brain, consist of the aggregated amyloid- β peptide (40 or 42 residues) which results from the cleavage of the amyloid precursor protein (APP), a transmembrane protein. APP (770 amino acids) is cleaved by β -secretase (BACE-1) which exposes the C-terminal end of the protein. This smaller 99-amino acid portion is then cleaved by γ -secretase allowing $\text{A}\beta$ to be released. The secretases dictate which forms of $\text{A}\beta$ will be produced. The segment can be between 39-42 residues while the majority is either 40 or 42 residues in length. While $\text{A}\beta_{40}$ is produced in larger quantities, it is the longer peptide that is more prone to aggregation and has been linked to early-onset of Alzheimer's.⁴

$\text{A}\beta$ is found in the CSF of normal individuals. Once $\text{A}\beta$ monomers are produced, they can self-associate to form oligomers that will eventually increase in size to become toxic fibrils. Currently it is speculated that the oligomer forms of $\text{A}\beta$ are more toxic than the aggregated fibrils, however it is still uncertain whether $\text{A}\beta$ causes AD or forms from AD complications.⁵ These amyloid fibrils, rich in β -sheets, are deposited around the neurons causing synaptic dysfunction and ultimately neuronal cell death. Interestingly, the density of the fibrillar $\text{A}\beta$ is not correlated to the severity of dementia in Alzheimer's patients.

Chapter 1.2. Metal Involvement in Alzheimer's Pathology and the A β Peptide

As previously mentioned, A β is from the cleavage of a larger protein, APP. APP is a ubiquitously expressed protein believed to moderate copper homeostasis mostly due to the fact that it can bind copper and zinc ions. Additionally, the secretases that process APP also interact with metal ions. This demonstrates that metal ions may have a direct interaction with APP processing. Furthermore, transition metals take part in the generation of reactive oxygen species (ROS) that can cause oxidative damage throughout the cell. The main source of ROS comes from the reaction of molecular oxygen with redox-active metals like copper and iron. Excess metals have been shown to induce A β precipitation *in vitro*.⁶

High concentrations of metals such as copper (390 μ M), zinc (1055 μ M) and iron (940 μ M) were found inside the amyloid plaques compared to the normal age-matched neuropil (copper (70 μ M), zinc (350 μ M) and iron (340 μ M)) using microparticle-induced x-ray emission (PIXE).⁷ Copper and zinc have been found to co-localize inside the amyloid- β deposits but iron does not.⁸ Iron is usually bound to ferritin, an iron storage protein, during plaque association. Many studies have shown that iron homeostasis does not have a direct effect on AD so it will not be discussed or studied here.⁹ Also, zinc binding studies will not be studied due its ability to aggregate A β over a wide range of conditions.⁶

DAEFRHDSGYEVHHQK₁₆

DAEFRHDSGYEVHHQKLVFFAEDVGSNK₂₈

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV₄₀

DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA₄₂

Figure 1.2. The A β peptide sequences of varying length that are studied.

It has been proposed that the metal binding site of A β lies within the first 16 amino acids.^{10,11} **Figure 1.2** shows the different A β peptides that are typically studied. Low temperature electron paramagnetic resonance (EPR) studies show identical EPR signatures for A β ₄₀, A β ₂₈, and A β ₁₆ coordinated to copper.¹⁰ The truncated peptide, A β ₁₆, is studied as a model for A β due to its decreased ability to aggregate or form fibrils. The A β ₂₈ peptide is also studied because it will aggregate and form fibril formation but at a much slower rate than the native A β ₄₀ or A β ₄₂.¹²

EPR, circular dichroism (CD), and isothermal titration calorimetry (ITC) studies show there are two equivalents of copper binding.^{13,14,15} In the past, there were discrepancies that displayed only one copper coordinating to the A β . It is rationalized that there is one high affinity binding site and one low affinity binding site. However, it is unlikely the second binding site is physiologically relevant since the concentration of copper is less than that of A β *in vivo*.

i. Copper Binding to the High Affinity A β Site

EPR and potentiometric studies have shown that two copper-A β species exist around physiological pH (between 6 and 8).¹¹ The transition between these two species (I and II) has been reported at pH 8 ± 1 .^{11,14,15,19} Most studies concern species I (dominates around pH 7.4) at the high affinity binding site, therefore this will be the one discussed here. The identities of the coordinating ligands at the high affinity binding site are still controversial. Hard-soft acid-base theory, discussed in Chapter 2, is a good indicator of what ligands will bind to the copper ion. It is known that copper interactions with peptides are pH dependent. Possible nitrogen ligands are histidines and the N-terminal amine due to their physiological pK_a: ~6.5 and ~8, respectively. Backbone amides (pK_a >15), lysine (~10.5) and arginine (~12.5) are also possible but due to their high pK_a, it is less unlikely. Possible oxygen ligands are aspartate (pK_a = 4.1), glutamate (4.1), and the C-terminus (3.1). At the high affinity binding site, Raman and EPR spectroscopy have shown a type II Cu consisting of three nitrogens and one oxygen (3N1O).^{10,14} However, EPR signature is sensitive to distortions in geometry and a 2N2O or 4N geometry cannot be ruled out.

Aggregation studies, NMR and CD data have supported copper coordination with three histidines at positions 6, 13 and 14.^{10,14} Rats, lacking His13 of A β , do not develop AD and copper induced aggregation has not been detected.^{16,17} The lack of His13 may account for decreased aggregation of ratA β which suggests that His13 plays an important role in copper coordination to human A β . EPR studies on mutated A β 28(H6A), A β 28(H13A) and A β 28(H14A) at physiological pH are similar to the EPR signal of A β 28 indicating copper is coordinating three nitrogens and one oxygen ligands.¹⁴ According to solid-state NMR studies, His13 and His14 are

in the region where β -sheets begin to form.¹⁰ Both histidines cannot be coordinated to Cu^{2+} because this would disrupt fibril formation therefore it is believed that only one of these residues coordinates to Cu^{2+} per peptide.¹⁸

The identity of the fourth ligand remains unresolved. EPR studies on $\text{A}\beta_{16}$ with the deletion of the N-terminal residue, aspartate-1, has shown copper is not bound in the same environment as the unmodified peptide.¹⁸ This indicates the N-terminus or the carboxylate of the Asp-1 may interact with the copper ion. CD studies have implicated the N-terminal amine as a ligand. This is based on the evidence that the CD signature for acetylated $\text{A}\beta_{28}$ (Ac- $\text{A}\beta_{28}$) was overwhelmingly different compared to that of the unblocked $\text{A}\beta_{28}$ upon copper addition.¹⁴ The support for all three histidines primarily comes from NMR experiments while the N-terminal amine and two histidines are supported by EPR experiments. It can be seen that copper can bind in several different modes making it difficult to discern the most populated binding mode.

As mentioned previously, EPR studies of Cu- $\text{A}\beta$ have primarily shown 3N1O coordination. The two favored possibilities for oxygen ligands have been Asp-1 and Tyr-10. However, the majority of evidence shows that tyrosine is not bound to copper due to the fact that no charge transfer band is observed using UV-Visible (UV-Vis) spectroscopy. The strongest evidence for Asp-1 being a ligand is the EPR study of the mutated aspartic acid to asparagine.¹⁹ The EPR spectrum of the mutated peptide shows a distinct signature compared to that of the native sequence.

Due to the discrepancies that exist for the possible copper ligands, it is proposed there are two most likely copper coordination sites that exist in equilibrium (**Figure 1.3**).¹² This is believed to be due to the fact that during EPR experiments the Cu- $\text{A}\beta$ solution is frozen while

other experiments occur at room temperature. Therefore it is suggested that two Cu-A β complexes exist at room temperature.

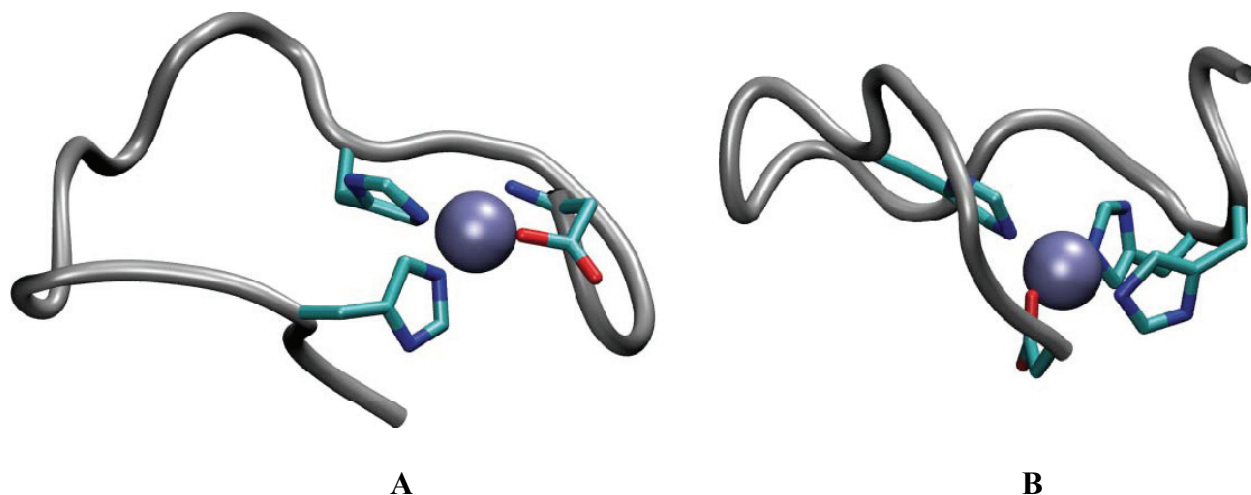


Figure 1.3. The two most likely coordination sites of Cu²⁺ bound to the A β peptide at the high affinity binding site at physiological pH. **A.** Shows copper coordination with His6 and His13/His14, the N-terminal amine, and the COO⁻ of Asp-1. **B.** Shows copper coordination with His6, His13 and His14 and the COO⁻ of Asp-1.¹²

ii. Copper Binding to the Low Affinity A β Site

Not many studies focus on the low affinity copper binding site of A β . EPR studies indicate a 2N2O or 3N1O coordination mode.^{5,14} Possible ligands may be histidines not involved at the high affinity binding site, additional carboxylates from aspartates and glutamates, and deprotonated amides. Furthermore, buffer or water could be part of the copper coordination site. An EPR study shows after the addition of a second equivalent of copper, the EPR signature 1) remained the same inferring the sites of the copper ions are independent and 2) lacked

broadening or silencing due to proximity of two copper ions. From this it can be concluded the copper ions are distant from each other.²⁰

iii. A β Affinity for Copper

The reported affinities of copper binding to the amyloid- β peptide at the high affinity site vary greatly between 10^4 to 10^9 M⁻¹. Guilloreau and coworkers have shown two equivalents of copper binding to A β 16 and report a 5×10^4 M⁻¹ apparent binding constant using isothermal titration calorimetry (ITC).¹⁵ However, also using ITC, Hatcher and coworkers have described a 1:1 stoichiometry with a 1.5×10^9 binding constant for A β 16 using glycine as a competing ligand (CuGly₂) with 2-(4-(2-Hydroxyethyl)-1-piperazinyl)ethanesulfonic acid (HEPES) buffer.²¹ Hatcher and coworkers have taken into account the copper-glycine equilibria. The discrepancies between these results may be due to the delivery of Cu(II) to the A β since buffer can interact with copper thus producing competition between peptide and buffer. Guilloreau and coworkers did not take into account the pH difference between the cell and syringe thus producing a huge signal for buffer ionization.

Many agree the discrepancies between experiments studying copper binding to the A β peptide are due to the ability of A β 16/28 to behave differently when reaction conditions, such as buffers, pH and metal concentration vary. This illuminates the fact these measurements are difficult to obtain and to accurately process the data.

Chapter 1.3 Therapeutic Strategies

There is no cure for AD, only drug therapies that can slow down the progression of the symptoms. There has been a substantial amount of data showing that A β plays a vital role in the initiation of Alzheimer's. Therefore focusing on drug therapies that alter the production of A β is very promising. In theory, inhibiting β -secretase (BACE-1) and γ -secretase can modify the amounts of A β . No successful targets of the γ -secretase involved in the APP process have reached clinical trials. Several BACE-1 targeting drugs have shown promising results, however, the drugs that do inhibit BACE-1 are too large to cross the blood-brain barrier. Other drugs have aimed to reduce the amount of aggregated A β . ColostrininTM has been shown to reduce A β cytotoxicity.^{22,9}

In addition to aggregation, metals also play a role in oxidative stress. The methionine at position 35 is thought to play a role in the reduction of metal ions.¹² A β reduces Cu²⁺ and Fe³⁺ and produces H₂O₂. The reduced metals, Cu⁺ and Fe²⁺, undergo Fenton-like chemistry:



Eq. 1.1

This produces hydroxyl radicals that cause oxidative damage to DNA, proteins, lipids carbohydrates, etc. A strategy that is being focused on is metal chelation therapy. Clioquinol (CQ), a retired compound with antibiotic properties, is a Cu/Zn chelator that has been shown to reduce A β deposition by 49%.²³ CQ does not decrease metal levels but has been shown to block production of H₂O₂ by A β *in vitro*. This may be due to its ability to selectively bind to the A β -

metal complex. Unfortunately phase II/III clinical trials were stalled due to difficulties in preventing contamination during large scale synthesis.²⁴

In contrast, triene (TETA), a high affinity Cu/Zn chelator used for treatment in Wilson's disease, was not shown to inhibit amyloid formation.²³ This is predictable because Wilson's disease occurs due to an overaccumulation of copper within the cell, whereas with AD, it is extracellular. This suggests removal of metals is not as straightforward as using a chelator to retrieve metal ions. Copper affinity for other essential proteins and enzymes must be taken into consideration. Regardless, the metal-binding site of A β is of great interest.

Chapter 2: Thermodynamic Principles and Project Design

Chapter 2.1 Thermodynamics of Metal Binding to Peptides

In living systems, metal ions are controlled and tightly regulated by metal ion homeostasis. Metal ions can be coordinated to ligands from proteins, enzymes, lipids, nucleic acids, vitamins and other biological components. The selectivity of ligands depends on the type of ligand and the coordination geometry that the metal adopts. The type of ligand depends on hard-soft acid-base (HSAB) theory, a theory that is a qualitative tool for predicting chemical stability. This theory states that hard metal ions are more likely to form stable bonds with hard ligands while soft metals are more likely to form stable bonds with soft ligands. Hard metals and ligands are smaller and less polarizable while soft metals and ligands are usually larger and more polarizable. Therefore, hard metal ions and ligands have a small ionic radius and a high charge where as soft metal ions and ligands have a large ionic radius and low charge.

HSAB theory can give information regarding metal-ligand stability, also known as the binding constant. The interactions between a metal (M) and a ligand (L) can be described by:



Eq.2.1

The binding constant, denoted as K, can be expressed as:

$$K = \frac{[ML]}{[M][L]}$$

Eq. 2.1

Therefore, a large K represents strong metal-ligand interactions while a small K represents weak interactions. Identification of the K value allows for the calculation of Gibbs free binding energy, ΔG . A larger K value will give a more negative ΔG value. ΔG can be found by using this thermodynamic relationship:

$$\Delta G = - RT \ln K$$

Eq. 2.2

This binding energy can give information about the energetics of the reaction. For example, a negative ΔG indicates the reaction is spontaneous while a positive ΔG indicates a non-spontaneous reaction. Therefore, the more negative the ΔG , the more favorable the reaction is. ΔG depends on the binding constant but also the changes of enthalpy (ΔH) and entropy (ΔS). Gibbs free energy can be related to enthalpy and entropy through this thermodynamic relationship:

$$\Delta G = \Delta H - T\Delta S$$

Eq. 2.3

The enthalpy of a reaction, ΔH , is the heat that is released or absorbed during a chemical reaction. It represents the amount of heat associated with the breaking (positive values) and

forming (negative values) of bonds. Positive ΔH values indicate the reaction is endothermic while negative ΔH values indicated the reaction is exothermic. Enthalpy reflects peptide-peptide and peptide-ligand bonding interactions along with information regarding peptide-solvent and ligand-solvent interactions. These interactions may include hydrogen bonding, electrostatic and van der Waals interactions. Enthalpy can be determined experimentally by use of a calorimeter.

The entropy of a reaction, ΔS , can represent the amount of disorder of the system. However, entropy is better described as a dependence on the dispersion of energy of a system. This means that if more energy is dispersed then the system increases in entropy. It can also be true that if less energy is dispersed then the system decreases in entropy. Hydrophobic interactions contribute to ΔS through solvent entropy. Proteins fold in such a way that hydrophobic groups fold into the protein and release water (solvent) molecules. This contributes to an increase in entropy. In spite of this, when a ligand binds to a protein, and the protein reorganizes itself, a loss in entropy can occur. Entropy also reflects changes in conformational degrees of freedom.²⁵

Chapter 2.2 Isothermal Titration Calorimetry (ITC)

ITC is a sensitive technique that can give detailed information such as a binding constant (K), stoichiometry (n), Gibbs energy (ΔG), enthalpy (ΔH), and entropy (ΔS) in one experiment. This technique has allowed for a deep understanding of biological systems and plays an important role in molecular and drug design.²⁶ ITC is an advantageous and popular tool because it can potentially reveal thermodynamics of any biomolecular reaction.

The calorimeter (**Figure 2.1**) measures changes of heat of a chemical reaction between two species in solution of known concentration. The calorimeter has a reference cell and reaction cell, connected through a thermocouple, and enclosed by an adiabatic jacket. A syringe, used for injecting and mixing, is placed inside the reaction cell. The cells are made of Hastelloy® Alloy C 276 which consists of several corrosion-resistant metal alloys. The experiment is controlled entirely by the computer. The user must input stirring speed, concentrations, temperature, the number of injections and the injection volumes. Once the experiment is complete, the user can model data using Origin® software to calculate the thermodynamic parameters.

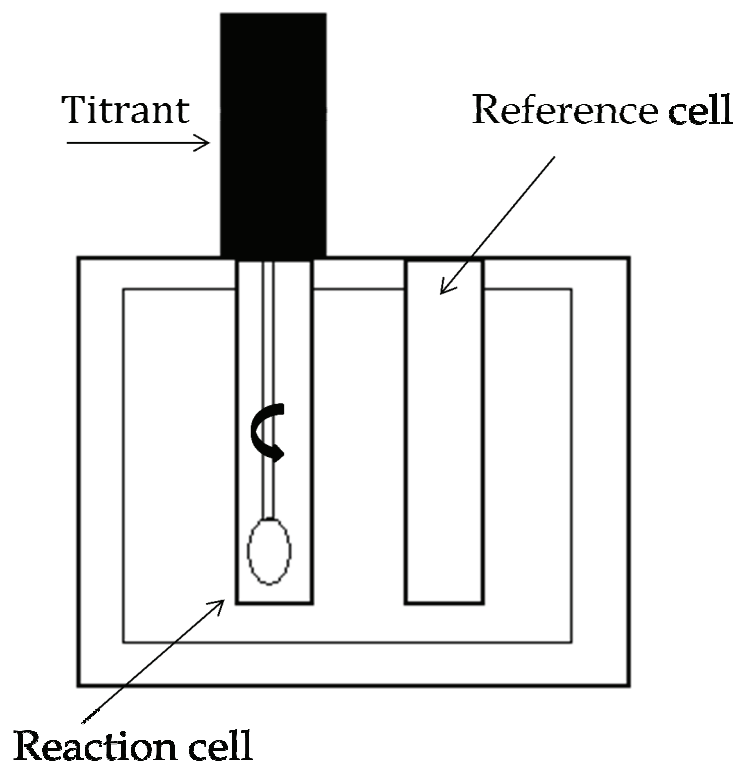


Figure 2.1. View of an isothermal titration calorimeter.

During an experiment, a macromolecule is placed into the reaction cell and the syringe injects the titrant (ligand) into the cell while stirring to ensure mixing. Known aliquots of the ligand are titrated into the cell. ITC works by measuring the amount of power ($\mu\text{cal}/\text{sec}$) it takes to keep the reaction at a constant temperature between the reference and reaction cell. The differential power (DP) used to maintain temperature equilibrium, “feedback” power, is calibrated electrically by the user. Either heat can be absorbed or released depending on if the reaction is endothermic or exothermic, respectively. After each injection, the reaction goes to equilibrium so that no heat is being produced. Over time, after each subsequent injection, less

heat is produced and eventually the macromolecule becomes saturated. The only observable heat signal is due to the heat of dilution.

i. Analyzing ITC Data

Once data is collected, it is analyzed using the Origin® software provided by MicroCal. The heat of each injection is determined by integrating the power with respect to time. This data can be fit to a model that is provided by Origin using non-linear least-squares analysis. The simplest type of model to describe the data is a one set of sites model. A one set of sites model describes any number of sites on the macromolecule if they all have the same K and ΔH . Once a model is chosen, Origin will make an initial guess on the baseline, integration, and thermodynamic parameters by floating n , K , and ΔH until chi-square is minimized. Chi-square provides the goodness of fit between measured data and calculated data.

The following fitting parameters are considered: K , n , V_0 , $[M]_t$, $[M]$, $[X]_t$, $[X]$, and Θ . K is the binding constant found from the slope, n is the number of sites found from the inflection point, V_0 is the active cell volume, $[M]_t$ and $[M]$ are the concentration of bulk and free macromolecule, $[X]_t$ and $[X]$ are the concentration of bulk and free ligand, and Θ is the fraction of sites occupied by the ligand.

The mathematical expression for the amount of heat released, $\Delta Q(i)$, during the i^{th} injection is

$$\Delta Q(i) = Q(i) + \frac{dV_i}{V_0} \left[\frac{Q(i) + Q(i-1)}{2} \right] - Q(i-1)$$

Eq. 2.4

where V_i is the injection volume. The heat that was generated from the displaced volume is added to the heat being generated from the i injection followed by the deletion of heat from the previous injection.

Other types of models used to describe ITC data are the two sets of sites and sequential models. The two sets of sites model describes ligands binding to two independent sites on the macromolecule. A sequential binding model describes a ligand binding to the first site and additional ligands binding to additional sites on the macromolecule. This model cannot distinguish between sites.

ii. Curve Fitting using a One Set of Sites Model

In the following copper and A β ITC studies, a one set of sites model was chosen to represent the data.²⁷ In order to consider the heat associated with each injection for a one set of sites model, the equilibrium constant (K) associated with a one set of sites is defined as:

$$K = \frac{\Theta}{(1 - \Theta)[X]}$$

Eq. 2.5

where $[X]$ is the free ligand in solution and Θ is the fraction of sites occupied by $[X]$. The bulk concentration of the ligand, X_t , can be written as a function of $[X]$ and M_t as described by

$$X_t = [X] + n\Theta M_t$$

Eq. 2.6

After combining Eq. 2.5 and Eq. 2.6 gives the following equation:

$$\Theta^2 - \Theta \left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right] + \frac{X_t}{nM_t} = 0$$

Eq. 2.7

The total heat content, Q , contained in V_0 (determined relative to zero for the uncomplexed species) at fractional saturation Θ is described by

$$Q = n\Theta M_t \Delta H V_0$$

Eq. 2.8

Solving Eq. 2.7 for Θ and substituting the value into Eq. 2.8 allows for the equation for $Q_{(i)}$:

$$Q_{(i)} = \frac{nM_t \Delta H V_0}{2} \left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} \right)^2 - \frac{4X_t}{nM_t}} \right]$$

Eq. 2.9

Once the heat for each injection is calculated, an initial guess is made on n , K , and ΔH . The data undergoes nonlinear least squares curve fitting using a Levenberg-Marquardt algorithm. Chi-square value must be minimized by performing iterations until chi-square is no longer decreasing.

iii. Obtaining Thermodynamic Parameters

After chi-square minimization, one can obtain thermodynamic information based on the fitted curve. **Figure 2.2** represents a typical ITC isotherm with exothermic heat events. The top plot shows the exothermic raw data while the bottom plot shows the binding isotherm. The binding constant, K , comes from the slope of the line. The stoichiometry, n , is indicated in the inflection of the slope. This gives information about how many ligands are binding to the macromolecule. The ΔH is calculated from the difference between the initial and final heat events of the reaction.

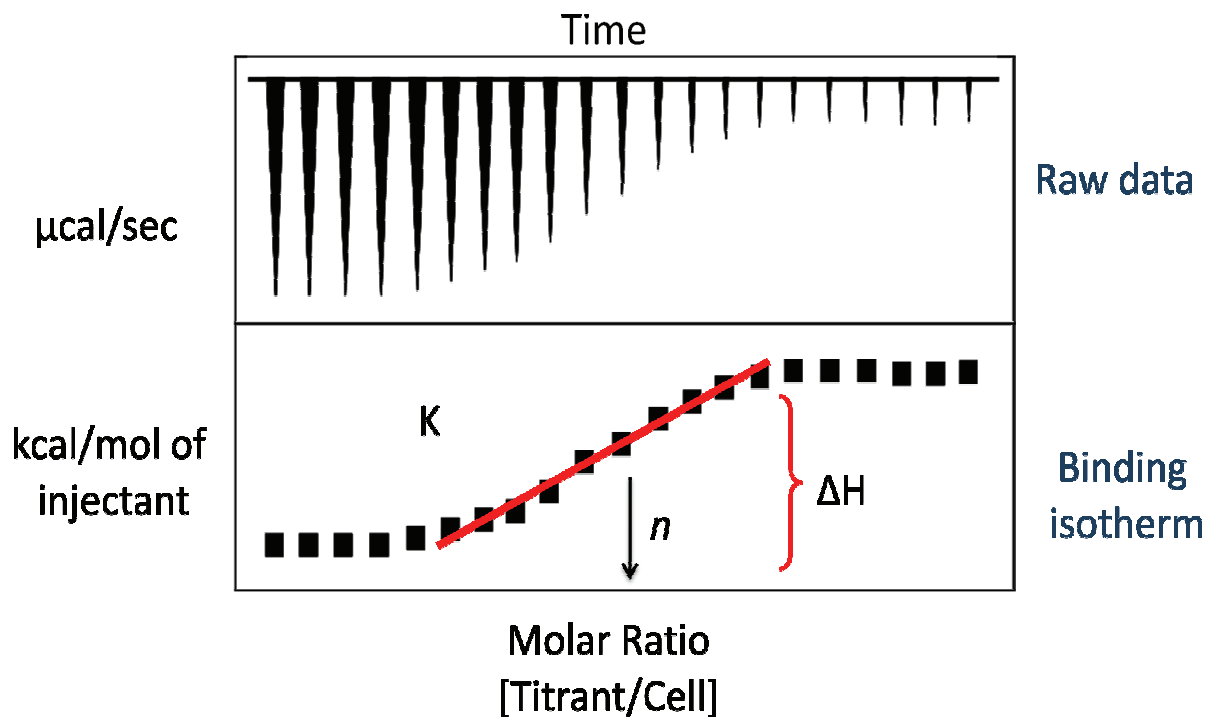


Figure 2.2 Raw ITC data (top plot) and the binding isotherm (bottom plot).

iv. Issues Regarding ITC Experiments

Studying metal binding to biomolecules using ITC may create additional issues due to complex equilibria. The calorimeter measures the total heat evolved during a chemical reaction. It is possible to subtract the background heat, or heat of dilution, but not all equilibria are taken into consideration. When considering interactions of metal ions, each equilibrium event has a heat associated with that reaction, examples of which are shown in **Table 2.1**. Additional heats can be from redox, precipitation or hydrolysis reactions. Redox chemistry and precipitation can be avoided if the ITC is in a glove box (anaerobic environment) and a stabilizing ligand (chelator) is used, respectively. Since metal ions compete with protons from proteins, metals can displace protons causing additional heat to be evolved. The sum of all the individual heats is the apparent ΔH or referred to as ΔH_{ITC} . In future discussions of enthalpy, ΔH_{ITC} will be referred to as ΔH because separate equilibria will not be considered throughout here. Similarly, the binding constant, K_{ITC} , reported is the product of the individual K 's for each equilibrium. K_{ITC} will be discussed as the apparent K within here.

<u>Equilibria</u>	<u>Enthalpy</u>	<u>Binding Constant</u>
1. Metal-Buffer \leftrightarrow Metal ⁿ⁺ + Buffer	ΔH_1	K_1
2. Buffer + H ⁺ \leftrightarrow Buffer-H	ΔH_2	K_2
3. Peptide-H \leftrightarrow Peptide + H ⁺	ΔH_3	K_3
4. Metal ⁿ⁺ + Peptide \leftrightarrow Metal-Peptide	ΔH_4	K_4

$$\Delta H_{ITC} = \Delta H_1 + \Delta H_2 + \Delta H_3 + \Delta H_4$$

$$K_{ITC} = K_1 \times K_2 \times K_3 \times K_4$$

Table 2.1. Equilibria associated with total heat observed in an ITC reaction involving the titration of metal into peptide.

Chapter 2.3 Project Design

i. A β Peptides

The following studies focus on copper binding to various fragments of the A β peptide and the A β peptide with several point mutations. The native forms of the A β peptide, A β 40 and A β 42, are prone to self-aggregation. Fortunately, the metal binding site of A β is within the first 16 residues. A β 16 and A β 28 peptides are studied more frequently because they are shorter and less likely to aggregate. This is because the peptides lack the C-terminal portion. Truncated peptides are generally used in metal-binding studies to probe the metal site. These shorter peptides are more soluble and easier to manage than the full length versions.

The truncated peptides, A β 16 and A β 28, and their acetylated counterparts were studied using ITC (**Scheme 1**). Since there are discrepancies whether the N-terminal amine or carboxylate of aspartic acid is a copper ligand, the acetylated N-terminal peptides (Ac-A β 16 and Ac-A β 28) have been studied. It is suspected that blocking or hindering a possible binding site may alter the thermodynamics. The A β 16 and A β 28 peptides and their acetylated counterparts are shown:

A β 16: DAEFRHDSGYEVHHQK

Ac-A β 16: Ac-DAEFRHDSGYEVHHQK

A β 28: DAEFRHDSGYEVHHQKLVFFAEDVGSNK

Ac-A β 28: Ac-DAEFRHDSGYEVHHQKLVFFAEDVGSNK

Scheme 1.

In addition to these peptides, A β 28 mutants were also studied (**Scheme 2**). The three histidine residues at positions 6, 13, and 14 are suspected to bind copper. In order to study the thermodynamic contribution of each histidine, the residues were substituted with the non-binding amino acid, alanine. The mutated peptides that were studied are shown below:

A β 28(H6A): DAEFRADSGYEVHHQKLVFFAEDVGSNK

A β 28(H13A): DAEFRHDSGYEVAHQKLVFFAEDVGSNK

A β 28(H14A): DAEFRHDSGYEVHAQKLVFFAEDVGSNK

Scheme 2.

All of the peptides studied had the C-terminal carboxylate group amidated. This modification occurred during peptide synthesis and it was not important to consider since the C-terminal portion of the peptide is not involved in metal binding.

ii. Buffer

In this study, we have aimed to provide accurate reaction thermodynamics by limiting the species in solution. It is important to find a ligand that is a stronger chelator than the buffer itself. In this case, we have utilized a buffer also acting as a chelator. Therefore *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) has been investigated as a buffer. It is known that ACES displays an affinity for copper ($\log\beta = 7.77$) at 25°C with the ratio of Cu:ACES being 1:2.³⁰ However, the buffer independent value for copper affinity to A β 16 reported within here is greater ($\log\beta = 9.26$). The buffer independent value is further discussed in Chapter 3.3. This makes it ideal for use as a weak chelating buffer. There have been no metal binding to A β studies using ACES as a chelator published to date.

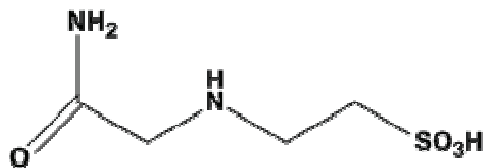


Figure 2.3. Chemical structure of ACES.

Chapter 2.4 Significance of Project

The significance of our research lies with the fact that the aggregation of A β seems to be facilitated by abnormal interactions of copper, zinc, and iron ions. Currently the research includes trying to understand metal interactions with the A β peptide. It was found in human post mortem brains that metal-induced aggregation can be reversed by copper and zinc chelators.³ Clinical trials using the copper/zinc chelator clioquinol has shown decreased A β deposition however the mode of action has yet to be confirmed.²⁰

In order to effectively develop a metal chelator for drug therapy, the affinity for metal ions towards A β must be established. Isothermal titration calorimetry (ITC) can provide a thermodynamic profile for copper binding and allow for an enhanced understanding of how copper is interacting with the A β peptide. ITC can offer additional insights about binding energetics including enthalpic and entropic contributions. This information will allow us to model what is occurring *in vivo* between copper and peptide. Knowing the thermodynamics of these interactions is vital because if a metal chelator is to be designed for drug therapy in Alzheimer's disease, the reaction thermodynamics must be well understood. Only ITC can give a complete thermodynamic profile on the reaction.

Chapter 2.5 Project Objectives

The A β peptide is the major component of aggregated plaques found in Alzheimer's disease. According to the amyloid hypothesis, oxidative stress plays an important role leading to the degeneration of neurons. Transition metals, such as copper, can generate reactive oxygen species (ROS) that cause havoc throughout the cell. A high concentration of copper has been found inside these amyloid plaques.

Our goals were to investigate the thermodynamics of copper binding to the A β 28 and A β 16 peptides that are most commonly used in metal binding studies. While A β 16 contains the metal binding site it is the longer peptide, A β 28, which contains a hydrophobic region that resembles the native A β 40. We have obtained the thermodynamics of copper binding to the Ac-A β 16 and Ac-A β 28 peptides. Probing the thermodynamic contribution from acetylation of the N-terminus has given insight into the importance of that ligand. In addition, we would like to probe the other ligands proposed in copper binding. We have obtained the thermodynamic contribution of the histidines in positions 6, 13, and 14. We have shown there are thermodynamics differences between the mutant peptides and the wild-type peptide. This information has provided insight into the copper coordination site of A β .

Finally, a comparison of our thermodynamic data obtained by ITC will be compared to spectroscopic data on the peptides we have studied. To the best of our knowledge, no ITC studies have been performed on Ac-A β 16, A β 28, Ac-A β 28, A β 28(H6A), A β 28(H13A), and A β 28(H14A).

Chapter 3: Characterization of Copper and A β Interactions using Isothermal Titration Calorimetry

3.1 Sample Preparation

Synthetic A β peptides were purchased from the W.M. Keck Facility at Yale University, New Haven, CT. The following peptides were received as pure peptides that did not need further purification: A β 16, Ac-A β 16, A β 28 and Ac-A β 28. The peptides, A β 28(H6A), A β 28(H13A), and A β 28(H14A) were crude peptides also purchased from the W.M. Keck Facility. The crude peptides were purified using RP-HPLC. All peptides were confirmed using ESI-MS at the W.M. Keck Facility. All of the peptides studied had the C-terminal carboxylate group amidated. Lyophilized peptide was dissolved in 18 M Ω H₂O. All 18 M Ω H₂O was passed through a Chelex-100 column to remove transition metals in water. The concentrations of each peptide were determined by UV-Vis spectroscopy using the A β extinction coefficient, 1410 M⁻¹cm⁻¹ at 280 nm.

Peptide and copper solutions were buffered in 20 mM *N*-(2-Acetamido)-2-aminoethanesulfonic acid (ACES) with an ionic strength of 100 mM NaCl. The pH of each solution was adjusted to 7.4 using small aliquots of 100 mM hydrochloric acid (HCl) and 100 mM sodium hydroxide (NaOH). The copper solution was atomic spectroscopy grade purchased from Sigma-Aldrich. A 31.5 mM copper stock was prepared with 1% (v/v) HCl. All other reagents were purchased from Sigma-Aldrich.

3.2 Isothermal Titration Calorimetry Parameters

Before each experiment, the ITC underwent standard cleaning procedures. Between experiments of different peptides, the loading syringe and cell were soaked with 0.25 M EDTA for up to an hour to ensure removal of copper. All A β and metal solutions were made with 18 M Ω H₂O. All A β and copper solutions were syringe-filtered with a 0.2 μ m filter to avoid any large particles that would cause heat disruptions during ITC reactions. A β and copper solutions were degassed prior to ITC experiments. The reaction cell and the automatic syringe were washed with 1.5 mL of a 20 mM ACES(100 mM NaCl, pH 7.4) buffer three times prior to loading the cell with peptide solutions. The reaction cell was loaded with 1.46 mL of peptide and the loading syringe was loaded with 281.41 μ L of 2.1 mM copper (20 mM ACES, 100 mM NaCl, pH 7.4). The syringe solution was purged and refilled once in order to dislodge any bubbles that may have accumulated inside the syringe.

The ITC experiments were carried out using a 2.1 mM copper solution that was titrated into various A β peptides. The concentration of A β ranged from 113-143 μ M. The exact concentrations for each peptide can be found within the respective experimental section of this chapter. All ITC experiments were carried out at 37°C with 35 total injections of titrant. The ITC reference power was set to 20 μ cal/sec with an initial delay of 60 seconds. The stirring speed was set to 307 rpm for sufficient mixing time of copper and A β solutions. ITC injection parameters can be found in the experimental section for individual peptides.

Titration of background heat, or heat of dilution, from copper into ACES were performed using ITC under similar conditions. A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 20 mM ACES (100 mM NaCl, pH 7.4) is shown in **Figure 3.1**. The heat of dilution,

-45.9 cal/mole, was subtracted from the copper and A β titrations. This value was found by averaging the last thirteen points of integrated heat values. The background heat was found from an average of two ITC reactions. The other ITC plot can be found in **Appendix A**. All ITC data was processed using Origin® software provided by MicroCal using a one set of sites model. An initial guess of thermodynamic parameters was provided by Origin followed by chi-square minimization. The first injection point was deleted for the data analysis.

It is important to note only one binding site of copper has been observed with the following ITC studies although studies have indicated the binding of an additional copper ion. It is possible the second binding site cannot be observed due to ACES competing with A β for the metal. Therefore data was fit to a one set of sites model.

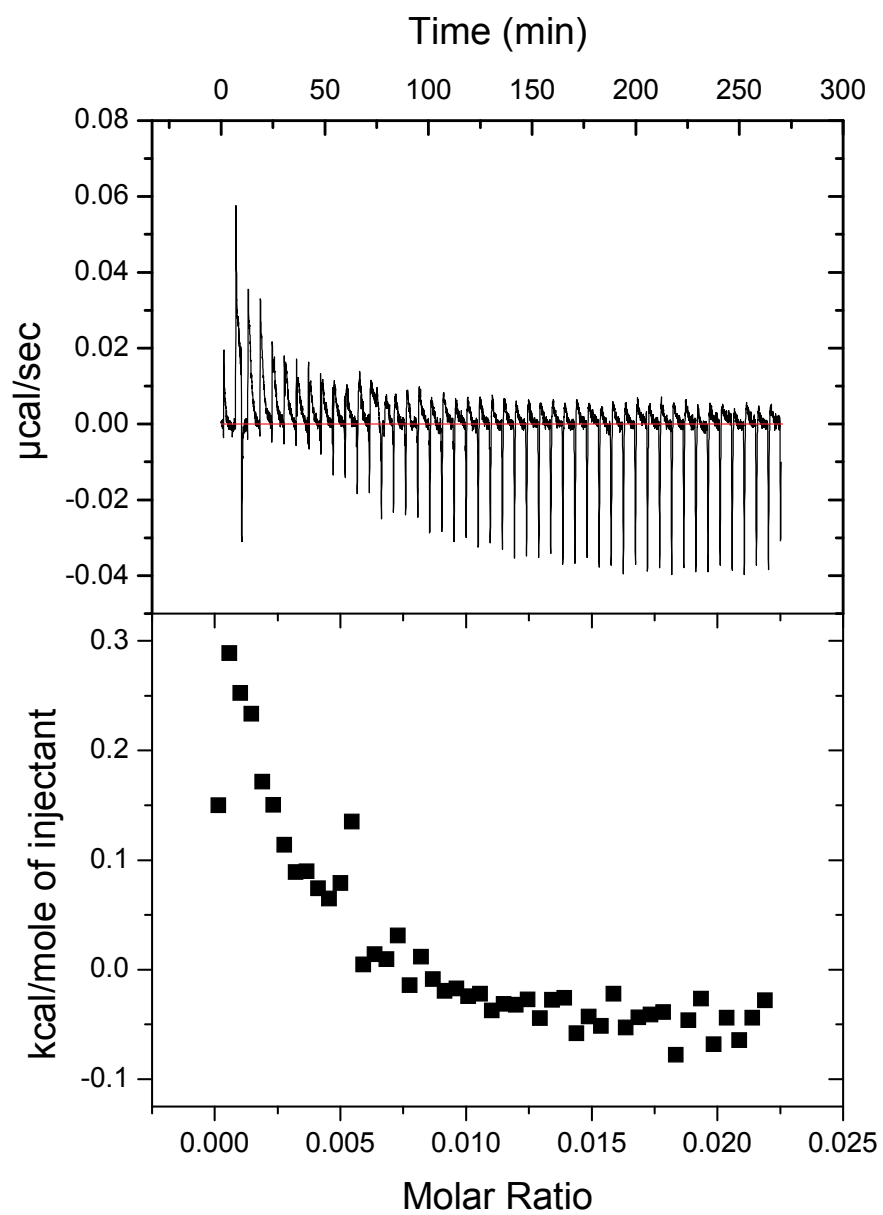


Figure 3.1. ITC titration of 2.1 mM copper into 20 mM ACES (100 mM NaCl, pH 7.4 at 37°C).

The heat of dilution for this ITC reaction was found to be -45.96 cal/mole of injectant.

3.3 Characterization of Copper Binding to A β 16

i. Experimental

In a 10 mL volumetric flask, 19.74 mg of pure, lyophilized peptide was dissolved in 18M Ω H₂O. The stock concentration was determined to be 0.681 mM using UV-Vis spectroscopy. A volume of 0.5 mL A β 16 was added to microcentrifuge tubes, flash frozen with liquid N₂ and kept in a freezer until ready for use. To make 5 mL of a 140 μ M solution, 1.03 mL of stock peptide was needed, 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual A β 16 concentration was 136 μ M A β 16.

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 350s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 350s spacing, and a 2s filter period. The ITC figure presented in **Figure 3.2** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other two ITC plots can be found in **Appendix A** along with the fitting parameters for those titrations.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 136 μ M A β 16 is shown in **Figure 3.2**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately 1. The results were an average of three titration experiments. Fitting the data to a “single set of sites model” provides the following data: $n = 1.11 (\pm 0.09)$, $K = 7.77 (\pm 1.8) \times 10^4 \text{ M}^{-1}$, and $-2.37 (\pm$

0.19) kcal/mol. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-6.88 (\pm 0.17)$ kcal/mol and $4.51 (\pm 0.06)$ kcal/mol, respectively.

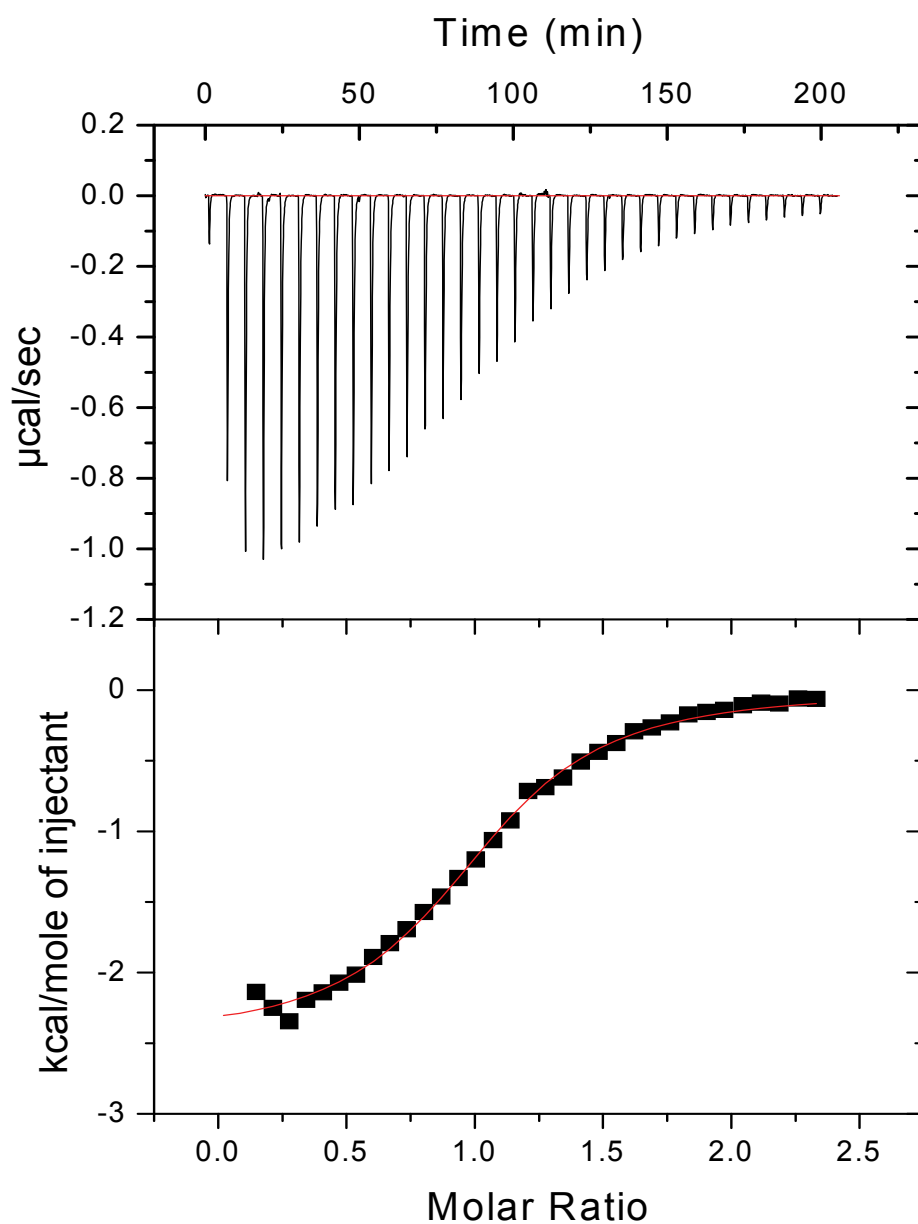


Figure 3.2. ITC titration of 2.1 mM copper into 136 μM A β 16 (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 1.03$, $K = 9.93 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.47 \text{ kcal/mole}$, $\Delta G = -7.08 \text{ kcal/mole}$, and $T\Delta S = 4.61 \text{ kcal/mole}$.

iii. Discussion

The thermodynamics established for copper binding to A β 16 can be found in **Table 3.1**. As mentioned previously, the results were an average of three titration experiments. The stoichiometry, n , is 1.05 which signifies one copper ion is binding per A β 16 molecule. The apparent binding constant, K , was found to be $7.77 (\pm 1.8) \times 10^4 \text{ M}^{-1}$. The binding constant for copper and A β interactions can be expressed as:

$$K = \frac{[A\beta - Cu(II)]}{[Cu(II)][A\beta]}$$

Eq. 3.1

Gibbs free energy, ΔG , was obtained from **Equation 2.2** and found to be $-6.88 (\pm 0.17)$ kcal/mol. This value gives information regarding the energetics of the reaction. The negative ΔG indicates an energetically favorable process. The enthalpy change of the reaction, ΔH , was found to be $-2.37 (\pm 0.19)$ kcal/mol. This value was negative indicating the reaction is exothermic and enthalpically favored. The enthalpy of the reaction reflects the energy changes associated with bonds breaking and forming. Entropy, ΔS , can represent the amount of disorder of a system but really describes the dependence on the dispersion of energy in the system. A positive entropy ($T\Delta S$) was obtained from **Equation 2.3** and found to be $4.51 (\pm 0.06)$ kcal/mol which indicates the reaction is entropically favorable as well.

Currently the most accepted study in the literature is the ITC study by Hatcher and coworkers who utilized glycine as a copper chelator. They fail to report an apparent binding constant obtained by Origin but do report a binding constant of $2.9 (\pm 0.2) \times 10^9 \text{ M}^{-1}$ when incorporating Cu-Gly₂ equilibrium. Our binding constant for Cu-A β is lower because we do not

account for ACES and copper equilibrium. However, when the buffer independent Cu-A β binding constant is calculated, an affinity of $1.85 \times 10^9 \text{ M}^{-1}$ is calculated for our system (**Appendix B**). This agrees with the value reported by Hatcher and coworkers.²¹ This assumes that the dominate complex for Cu:ACES is 1:2. Clearly, the buffer plays a role in determination of the Cu-A β binding affinity. As mentioned previously, these discrepancies illuminate the fact that copper and A β binding studies are difficult to accurately describe the data. See end of **Appendix B** for derivation.

	<u>Aβ16</u>
K/M ⁻¹	7.77 (\pm 1.8) x 10 ⁴
Δ H/kcal · mole ⁻¹	-2.37 (\pm 0.19)
Δ G/kcal · mole ⁻¹	-6.88 (\pm 0.17)
T Δ S/kcal · mole ⁻¹	4.51 (\pm 0.06)

Table 3.1. The thermodynamics of copper binding to A β 16 established by ITC using a one set of sites model.

3.4 Characterization of Copper Binding to Ac-A β 16

i. Experimental

In a 10 mL volumetric flask, 20.23 mg of pure, lyophilized peptide was dissolved in 18 M Ω H₂O. The stock concentration was determined to be 0.681 mM using UV-Vis spectroscopy. A volume of 0.5 mL Ac-A β 16 was added to microcentrifuge tubes, flash frozen with liquid N₂ and kept in a freezer until ready for use. To make 5 mL of a 140 μ M solution, 1.03 mL of stock peptide was needed, 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 136 μ M Ac-A β 16.

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 450s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 450s spacing, and a 2s filter period. Ac-A β 16 titrations had to be concatenated to ensure peptide was saturated with copper. The representative results were an average of two titration experiments. The ITC figure presented in **Figure 3.3** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other ITC plots can be found in **Appendix A** along with the fitting parameters for that titration.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 136 μ M Ac-A β 16 is shown in **Figure 3.3**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately

1. The results were an average of three titration experiments. Fitting the data in to a “single set of sites model” provides the following data: $n = 0.88 (\pm 0.12)$, $K = 1.37 (\pm 0.95) \times 10^4 \text{ M}^{-1}$, and $\Delta H = -1.40 (\pm 0.67) \text{ kcal/mol}$. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-5.66 (\pm 0.53) \text{ kcal/mol}$ and $4.26 (\pm 1.20) \text{ kcal/mol}$, respectively. Note: The n value for **Figure 3.3** had to be fixed to $n = 1.00$ due to Origin not being able to resolve n in the fitting procedure.

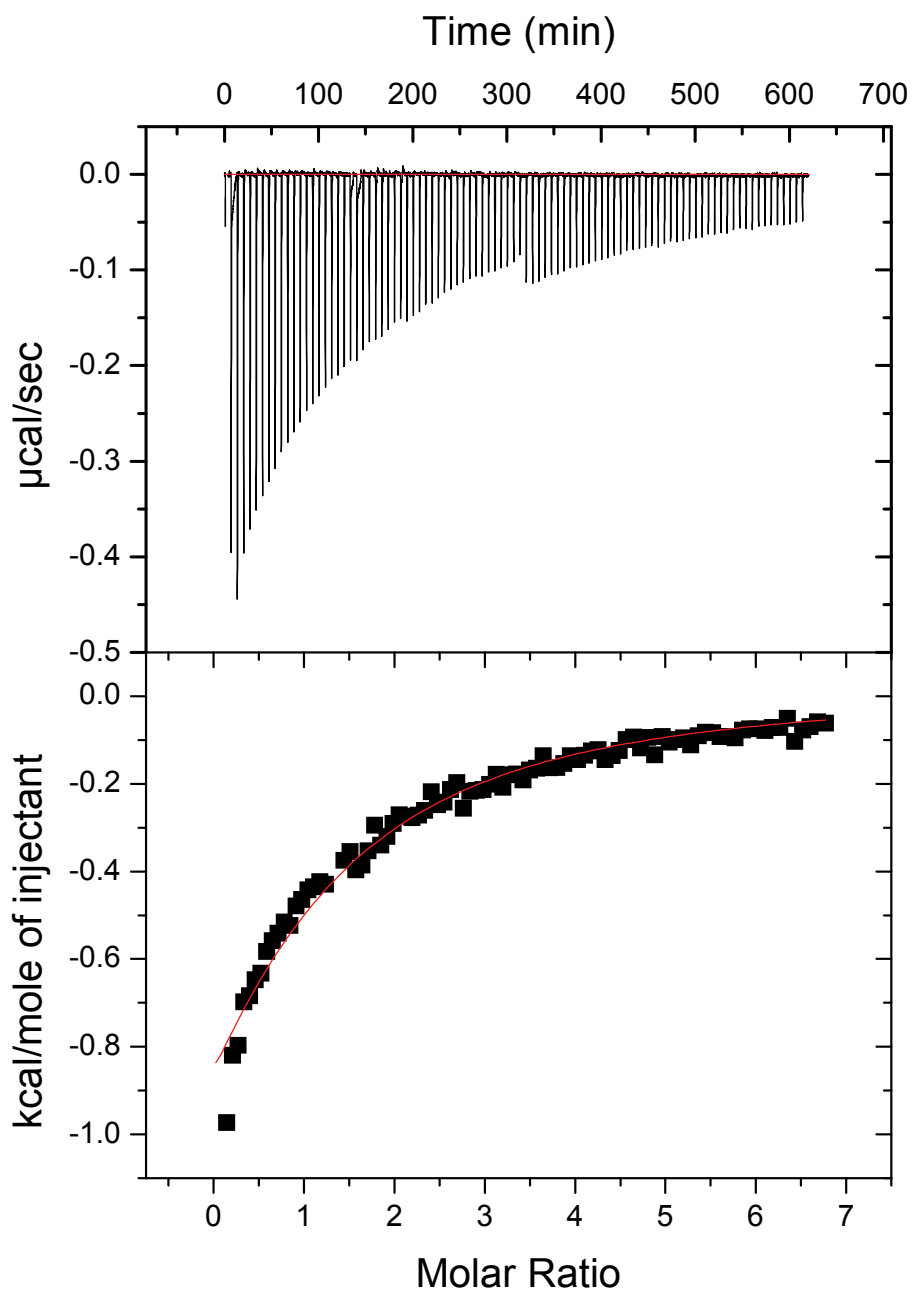


Figure 3.3. ITC titration of 2.1 mM copper into 136 μM Ac-A β 16 (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 1.00$ (fixed), $K = 4.18 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.07 \text{ kcal/mole}$, $\Delta G = -5.13 \text{ kcal/mole}$, $T\Delta S = 3.06 \text{ kcal/mole}$.

iii. Discussion

ITC titrations have been performed on copper binding to Ac-A β 16. As mentioned previously, there are discrepancies whether the N-terminal amine or the carboxylate of the aspartic acid (Asp-1) is the ligand that binds to copper. It is suspected that blocking or hindering a possible binding site may alter the thermodynamics. The thermodynamics of Ac-A β 16 and A β 16 are compared in **Table 3.2**. Comparing the binding isotherms in **Figure 3.2** and **Figure 3.3**, a difference in the binding curve of the isotherm can be observed between A β 16 and Ac-A β 16, respectively. It can be seen that copper does not bind or interact with acetylated A β 16 as it does with non-acetylated A β 16.

The stoichiometry, n , is 0.88 which signifies roughly one copper ion is binding per Ac-A β 16 molecule. The apparent binding constant has a value of $1.37 (\pm 0.95) \times 10^4 \text{ M}^{-1}$ which is lower than free A β 16 by roughly a factor of 6. This demonstrates there is a weaker binding between copper and Ac-A β 16 than free peptide. The ΔG of the reaction, $-5.66 (\pm 0.53) \text{ kcal/mol}$, is less than that of A β 16 indicating that copper binding to the acetylated peptide is energetically less favored. Calculating the $\Delta\Delta G$'s for the A β 16 and Ac-A β 16 reveals that A β 16 is more favorable by -1.23 kcal/mol . The ΔH , $-1.40 (\pm 0.67) \text{ kcal/mol}$, is decreased in the acetylated peptide which indicates metal binding to the Ac-A β 16 is enthalpically less favored. The $T\Delta S$ for Ac-A β 16, $4.26 (\pm 1.20) \text{ kcal/mol}$, is positive indicating it is entropically favored. It cannot be interpreted at this time if there is a significant difference in $T\Delta S$ for A β 16 and Ac-A β 16. A decreased $T\Delta S$ could indicate a weaker binding.

As mentioned previously, two different modes of copper binding may exist in equilibrium for A β 16. One mode suggests three histidines and the carboxylate of Asp-1 while the

second mode suggests two histidines, the N-terminus and the carboxylate of Asp-1. Both modes exist with the free peptide while the acetylated peptide no longer has a free N-terminus. With the thermodynamic information from using a one set of sites model, our data shows the N-terminal amine is essential for binding as indicated by a more favorable ΔG .

	<u>Aβ16</u>	<u>Ac-Aβ16</u>
K/M ⁻¹	7.77 (\pm 1.8) x 10 ⁴	1.37 (\pm 0.95) x 10 ⁴
ΔH /kcal · mole ⁻¹	-2.37 (\pm 0.19)	-1.40 (\pm 0.67)
ΔG /kcal · mole ⁻¹	-6.88 (\pm 0.17)	-5.66 (\pm 0.53)
T ΔS /kcal · mole ⁻¹	4.51 (\pm 0.06)	4.26 (\pm 1.2)

Table 3.2. Comparison between the thermodynamics of copper binding to A β 16 and Ac-A β 16 established by ITC using a one set of sites model.

3.5 Characterization of Copper Binding to A β 28

i. Experimental

In a 10 mL volumetric flask, 16.35 mg of pure, lyophilized peptide was dissolved in 18 M Ω H₂O. The stock concentration was determined to be 0.567 mM using UV-Vis spectroscopy. A volume of 0.5 mL A β 16 was added to microcentrifuge tubes, flash frozen with liquid N₂ and kept in a freezer until ready for use. To make 5 mL of a 140 μ M solution, 1.23 mL of stock peptide was needed, 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 113 μ M A β 28.

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 350s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 350s spacing, and a 2s filter period. The representative results were an average of three titration experiments. The ITC figure presented in **Figure 3.4** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other two ITC plots can be found in **Appendix A** along with the fitting parameters for those titrations.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 113 μ M A β 28 is shown in **Figure 3.4**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately 1. The results were an average of three titration experiments. Fitting the data to a “single set of sites

model” provides the following data: $n = 0.96 (\pm 0.04)$, $K = 4.72 \times 10^4 (\pm 0.57) \text{ M}^{-1}$, and $\Delta H = -3.02 (\pm 0.13) \text{ kcal/mol}$. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-6.61 (\pm 0.08) \text{ kcal/mol}$ and $3.59 (\pm 0.19) \text{ kcal/mol}$, respectively.

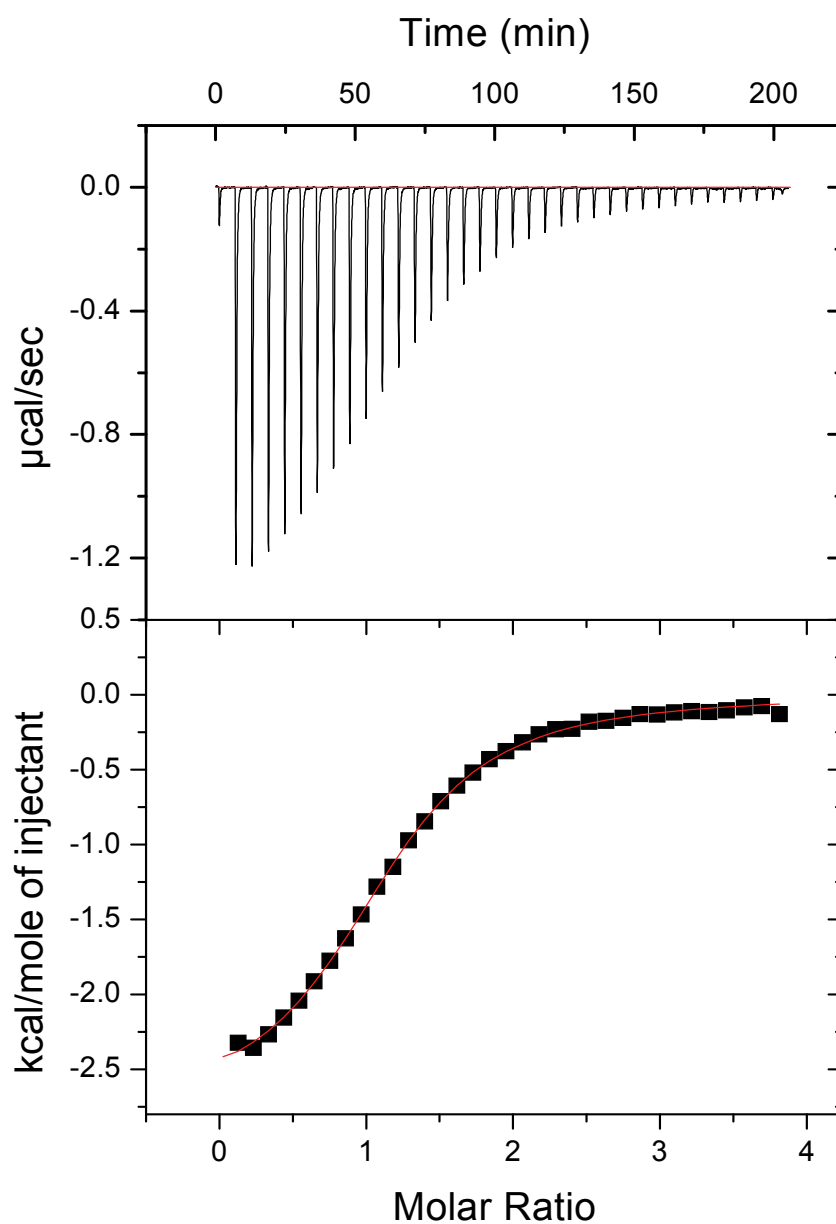


Figure 3.4. ITC titration of 2.1 mM copper into 113 μ M A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 0.93$, $K = 5.34 \times 10^4$ M^{-1} , $\Delta H = -2.77$ kcal/mole, $\Delta G = -6.70$ kcal/mole, $T\Delta S = 3.93$ kcal/mole.

iii. Discussion

ITC titrations have also been performed on the longer A β 28 (**Figure 3.4**). Thermodynamics of the free peptide and acetylated peptide are compared in **Table 3.3**. The stoichiometry, n , is 0.96 which signifies one copper ion is binding per A β 28 molecule. The apparent binding constant of free A β 28 is higher than that of nonacetylated A β 16, but the order of magnitude is the same which indicates the metal binding affinity is similar to A β 16. This confirms that the copper binding is within the first 16 residues as proposed. The ΔG of the reaction is very similar to A β 16 which suggests the reaction is also an energetically favorable process. The ΔH of the reaction was found to be $-3.02 (\pm 0.13)$ kcal/mol. This value is increased compared to the ΔH of A β 16 ($-2.37 (\pm 0.19)$ kcal/mol). This indicates metal binding to A β 28 is enthalpically more favorable. Recall enthalpy reflects the energy changes associated with bonds breaking and forming. Since A β 28 is a longer peptide it will have different ΔH values associated with the peptides' equilibria (**Table 2.1**) compared to shorter A β 16. The $T\Delta S$ value is decreased compared A β 16 therefore the reaction is entropically less favorable than the shorter peptide. The $T\Delta\Delta S$ of A β 16 and A β 28 is 0.92. Recall that hydrophobic interactions can give rise to an increase in entropy upon solvent release. Interestingly, a decreased $T\Delta S$ is observed for the longer peptide that contains a hydrophobic region. Conversely, entropy can decrease upon addition of a ligand (copper) to a peptide.

	<u>Aβ16</u>	<u>Aβ28</u>
K/M ⁻¹	7.77 (± 1.8) x 10 ⁴	4.72 (±0.57) x 10 ⁴
ΔH/kcal · mole ⁻¹	-2.37 (± 0.19)	-3.02 (± 0.13)
ΔG/kcal · mole ⁻¹	-6.88 (± 0.17)	-6.61 (±0.08)
TΔS/kcal · mole ⁻¹	4.51 (±0.06)	3.59 (± 0.19)

Table 3.3 Comparison between the thermodynamics of copper binding to Aβ28 and Ac-Aβ28 established by ITC using a one set of sites model.

3.6 Characterization of Copper Binding to Ac-A β 28

i. Experimental

In a 10 mL volumetric flask, 16.45 mg of pure, lyophilized peptide received was dissolved in 18 M Ω H₂O. The stock concentration was determined to be 0.716 mM using UV-Vis spectroscopy. A volume of 0.5 mL A β 16 was added to microcentrifuge tubes, flash frozen with liquid N₂ and kept in a freezer until ready for use. To make 5 mL of a 140 μ M solution, 0.978 mL of stock peptide was needed, 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 143 μ M Ac- A β 28.

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 350s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 400s spacing, and a 2s filter period. Ac-A β 28 titrations had to be concatenated to ensure peptide was saturated with copper. The representative results were an average of three titration experiments. The ITC figure presented in **Figure 3.5** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other two ITC plots can be found in **Appendix A** along with the fitting parameters for those titrations.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 143 μ M Ac-A β 28 is shown in **Figure 3.5**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately

1. The results were an average of three titration experiments. Fitting the data to a “single set of sites model” provides the following data: $n = 1.01 (\pm 0.013)$, $K = 4.56 (\pm 0.65) \times 10^3 \text{ M}^{-1}$, and $\Delta H = -2.47 (\pm 0.21) \text{ kcal/mol}$. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-5.17 (\pm 0.085) \text{ kcal/mol}$ and $2.71 (\pm 0.28) \text{ kcal/mol}$, respectively

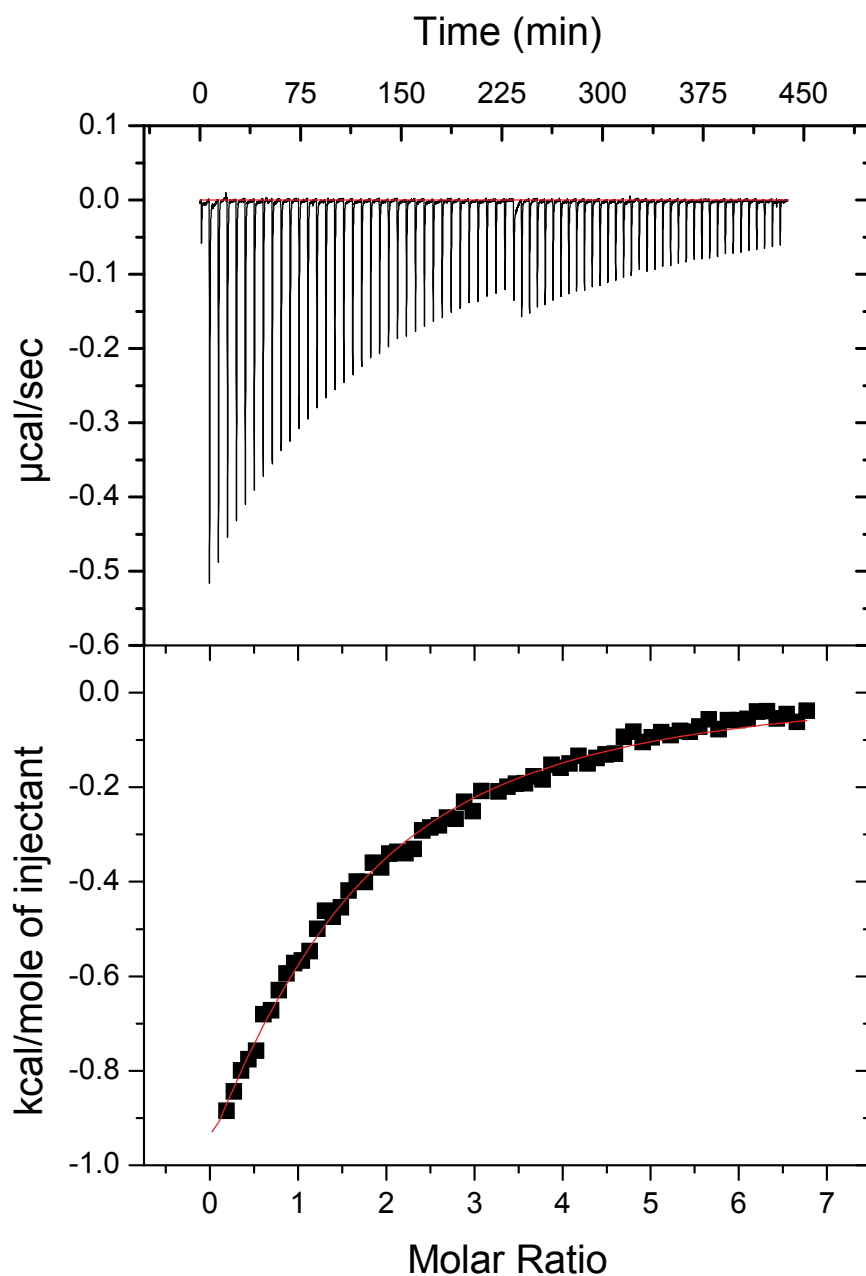


Figure 3.5. ITC titration of 2.1 mM copper into 143 μM Ac-A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 1.04$, $K = 5.78 \times 10^3 \text{ M}^{-1}$, $\Delta H = -2.06 \text{ kcal/mole}$, $\Delta G = -5.33 \text{ kcal/mole}$, $T\Delta S = 3.27 \text{ kcal/mole}$.

iii. Discussion

ITC titrations have been performed on the acetylated form of A β 28. The thermodynamics of Ac-A β 28 and A β 28 are compared in **Table 3.4**. ITC titration of Ac-A β 28 shows a similar binding curve to Ac-A β 16. It can be seen that copper does not bind or interact with the acetylated A β 28 as it does with free A β 28. The stoichiometry, n , is 1.01 which signifies one copper ion is binding per Ac-A β 28 molecule. The apparent binding constant for acetylated A β 28, $4.56 (\pm 0.65) \times 10^4 \text{ M}^{-1}$, is also an order of magnitude smaller than A β 28, $4.72 \times 10^4 (\pm 0.57) \text{ M}^{-1}$. This would imply copper binding to the acetylated forms of A β is weaker than free peptide. The ΔG of the reaction, $-5.17 (\pm 0.085) \text{ kcal/mol}$, is lower than that of A β 28, $-6.61 (\pm 0.08) \text{ kcal/mol}$, which indicates an energetically less favorable binding event thus a weaker copper binding. The $\Delta\Delta G$ of the A β 28 and Ac-A β 28 was calculated to be 1.44 kcal/mol . Also, according to the ΔH and $T\Delta S$, the values show that the reaction is enthalpically and entropically less favorable than copper binding to the free peptide which also indicates a weaker metal binding.

Copper binding to Ac-A β 28 has been studied by circular dichroism (CD) in the visible region by Syme, et al. at a pH 7.5.¹⁴ CD spectra in the visible region can give information regarding relative positions of copper ligands with changes in sign and intensity. The copper $d-d$ transitions can be seen in the visible region when copper is bound in a chiral environment. Their CD spectrum for Ac-A β 28 shows a very different signal compared to A β 28 indicating the N-terminus is involved in copper binding. Also reported are CD studies in the UV region on copper binding to Ac-A β 28. CD studies in the UV region can be used to monitor structural changes of peptides upon addition of a ligand. A distinct CD spectrum for Ac-A β 28 is observed compared

to A β 28. This suggests copper coordination to Ac-A β 28 induces a conformational change distinct from A β 28 which implies the N-terminus is involved as a ligand.¹⁴

	<u>Aβ28</u>	<u>Ac-Aβ28</u>
K/M ⁻¹	4.72 (\pm 0.57) x 10 ⁴	4.56 (\pm 0.65) x 10 ³
Δ H/kcal · mole ⁻¹	-3.02 (\pm 0.13)	-2.47 (\pm 0.21)
Δ G/kcal · mole ⁻¹	-6.61 (\pm 0.08)	-5.17 (\pm 0.09)
T Δ S/kcal · mole ⁻¹	3.59 (\pm 0.19)	2.71 (\pm 0.28)

Table 3.4. Comparison between the thermodynamics of copper binding to A β 28 and Ac-A β 28 established by ITC using a one set of sites model.

3.7 Characterization of Copper Binding to A β 28(H6A)

i. Experimental

Small amounts (14-18 mg) of crude A β 28(H6A) were dissolved in 2 mL 18 M Ω H₂O and purified using RP-HPLC. Lyophilized peptide was dissolved in small amounts of water until an appropriate concentration was reached using UV-Vis spectroscopy. This peptide solution was used as the stock solution and diluted to make 5 mL of a 140 μ M solution with 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 140 μ M A β 28(H6A).

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 350s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 350s spacing, and a 2s filter period. The representative results were an average of three titration experiments. The ITC figure presented in **Figure 3.6** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other two ITC plots can be found in **Appendix A** along with the fitting parameters for those titrations.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 140 μ M A β 28(H6A) is shown in **Figure 3.6**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately 1. The results were an average of three titration experiments.

Fitting the data to a “single set of sites model” provides the following data: $n = 0.82 (\pm 0.053)$, $K = 3.33 (\pm 0.077) \times 10^4 \text{ M}^{-1}$, and $\Delta H = -2.15 (\pm 0.053) \text{ kcal/mol}$. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-6.41 (\pm 0.014) \text{ kcal/mol}$ and $4.36 (\pm 0.052) \text{ kcal/mol}$, respectively.

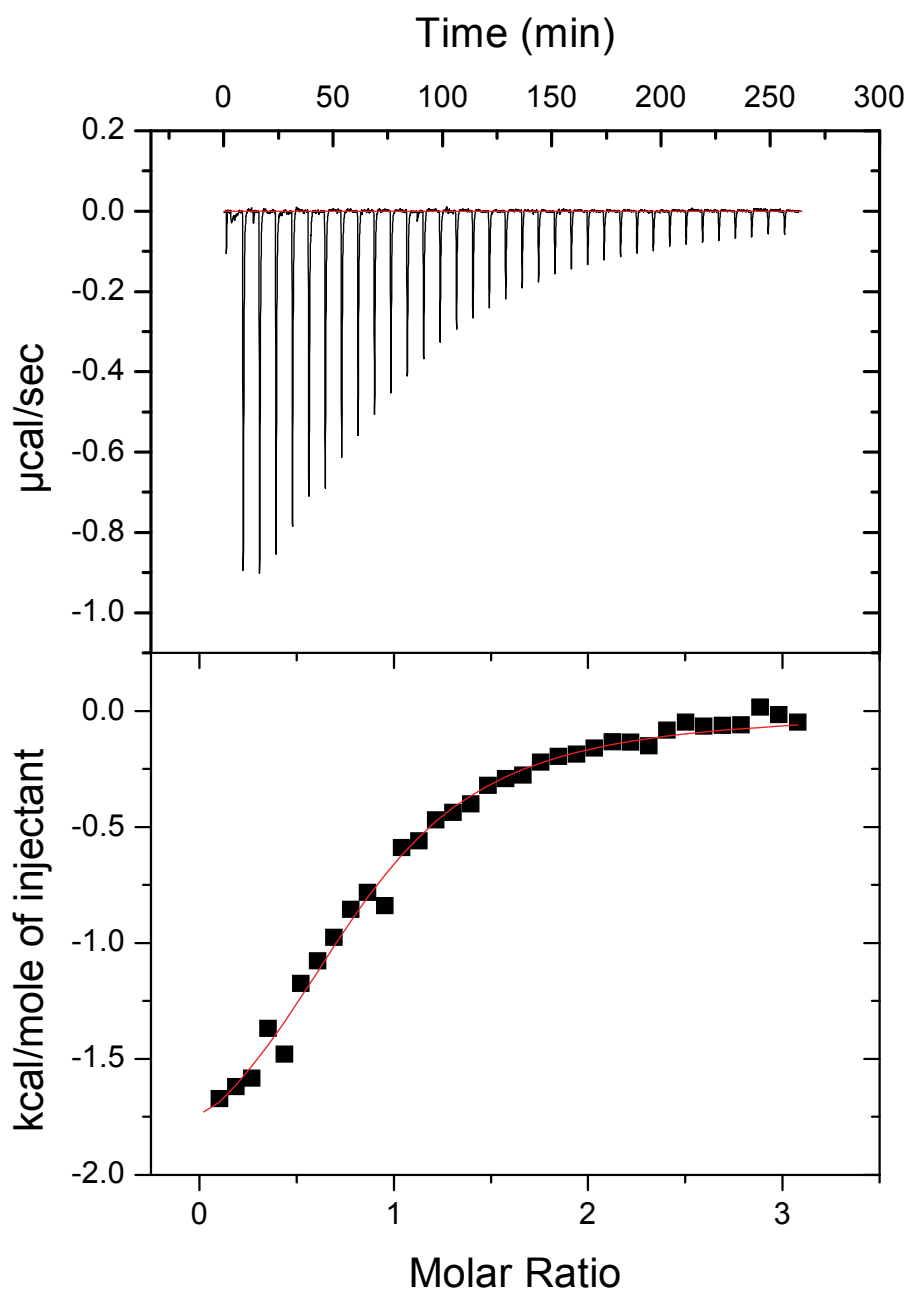


Figure 3.6. ITC titration of 2.1 mM copper into 140 μM A β 28(H6A) (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 0.83$, $K = 3.22 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.20 \text{ kcal/mole}$, $\Delta G = -6.39 \text{ kcal/mole}$, $T\Delta S = 4.18 \text{ kcal/mole}$.

iii. Discussion

ITC titrations have also been performed on A β 28(H6A). In this peptide, histidine-6 was mutated to the non-binding amino acid, alanine. His-6 is an important amino acid to probe because it has been implicated in binding copper.¹⁶ Electron paramagnetic resonance (EPR) studies have shown copper coordination to three nitrogens and one oxygen or four nitrogens. Ligands that provide readily available nitrogens in the pH range of 7.4 are the N-terminal amine and the three histidines residues as position 6, 13 and 14. Therefore the thermodynamics of copper binding to A β 28 without His-6 would be of interest. As mentioned previously, two different binding modes for copper are thought to exist in equilibrium. It is suggested that His-6 is present in both sites.¹² **Table 3.5** compares the thermodynamics of copper binding to A β 28 and A β 28(H6A).

The stoichiometry, n , is 0.82 which signifies roughly one copper ion is binding per A β 28(H6A) molecule. The apparent binding constant, $3.33 (\pm 0.077) \times 10^4 \text{ M}^{-1}$ is within error of A β 28 ($4.72 \times 10^4 (\pm 0.57) \text{ M}^{-1}$). The ΔH of the reaction, $-2.15 (\pm 0.053) \text{ kcal/mol}$, is lower than free peptide, $-3.02 (\pm 0.13) \text{ kcal/mol}$, indicating this reaction is enthalpically less favored than A β 28. The ΔG , $-6.41 (\pm 0.014) \text{ kcal/mol}$, is slightly smaller than A β 28, $-6.61 (\pm 0.08) \text{ kcal/mol}$, which indicates the energetics of the binding event are slightly decreased than wild-type peptide. The $T\Delta S$ is increased for A β 28(H6A) indicating this reaction is entropically more favorable than copper binding to A β 28.

Copper binding to A β 28(H6A) has also been studied by CD in the visible region by Syme, et al. at a pH 7.5.¹⁴ Their work describes little changes in visible CD spectra indicating a small difference in copper binding relative to A β 28. However, in the same work, CD studies in

the UV region on copper binding to A β 28(H6A) show a distinct spectrum compared to A β 28. This implies copper coordination to A β 28(H6A) induces a conformational change distinct from A β 28. Therefore His-6 is implicated as a ligand involved in copper binding.¹⁴ It is interesting to note that in our A β 28(H6A) studies the entropy increases while enthalpy decreases relative to the native peptide. Changes in peptide backbone can be observed in the CD upon copper coordination and the increase in entropy indicates a conformational change in the peptide.¹⁴ It is possible A β 28(H6A) is twisting in such a way that causes a loss in enthalpy.

	<u>Aβ28</u>	<u>Aβ28(H6A)</u>
K/M ⁻¹	4.72 (\pm 0.57) x 10 ⁴	3.33 (\pm 0.77) x 10 ⁴
Δ H/kcal · mol ⁻¹	-3.02 (\pm 0.13)	-2.15 (\pm 0.05)
Δ G/kcal · mol ⁻¹	-6.61 (\pm 0.08)	-6.41 (\pm 0.01)
T Δ S/kcal · mol ⁻¹	3.59 (\pm 0.19)	4.25 (\pm 0.05)

Table 3.5 Comparison between the thermodynamics of copper binding to A β 28 and A β 28(H6A) established by ITC using a one set of sites model.

3.8 Characterization of Copper Binding to A β 28(H13A)

i. Experimental

Small amounts (14-18mg) of crude A β 28(H13A) were dissolved in 18 M Ω H₂O and purified using RP-HPLC. Lyophilized peptide was dissolved in small amounts of water until an appropriate concentration was reached using UV-Vis. This peptide solution was used as the stock solution and diluted to make 5 mL of a 140 μ M solution with 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 140 μ M A β 28(H13A).

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 450s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 450s spacing, and a 2s filter period. The representative results were an average of three titration experiments. The ITC figure presented in **Figure 3.7** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other ITC plot can be found in **Appendix A** along with the fitting parameters for that titration.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 140 μ M A β 28(H13A) is shown in **Figure 3.7**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately 1. The results were an average of three titration experiments. Fitting the data to a “single set of sites model” provides the following data: $n = 0.81 (\pm 0.04)$, $K = 5.72 (\pm 1.6) \times 10^4$

M^{-1} , and $\Delta H = -1.99 (\pm 0.07)$ kcal/mol. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-6.72 (\pm 0.17)$ kcal and $4.72 (\pm 0.24)$ kcal/mol, respectively.

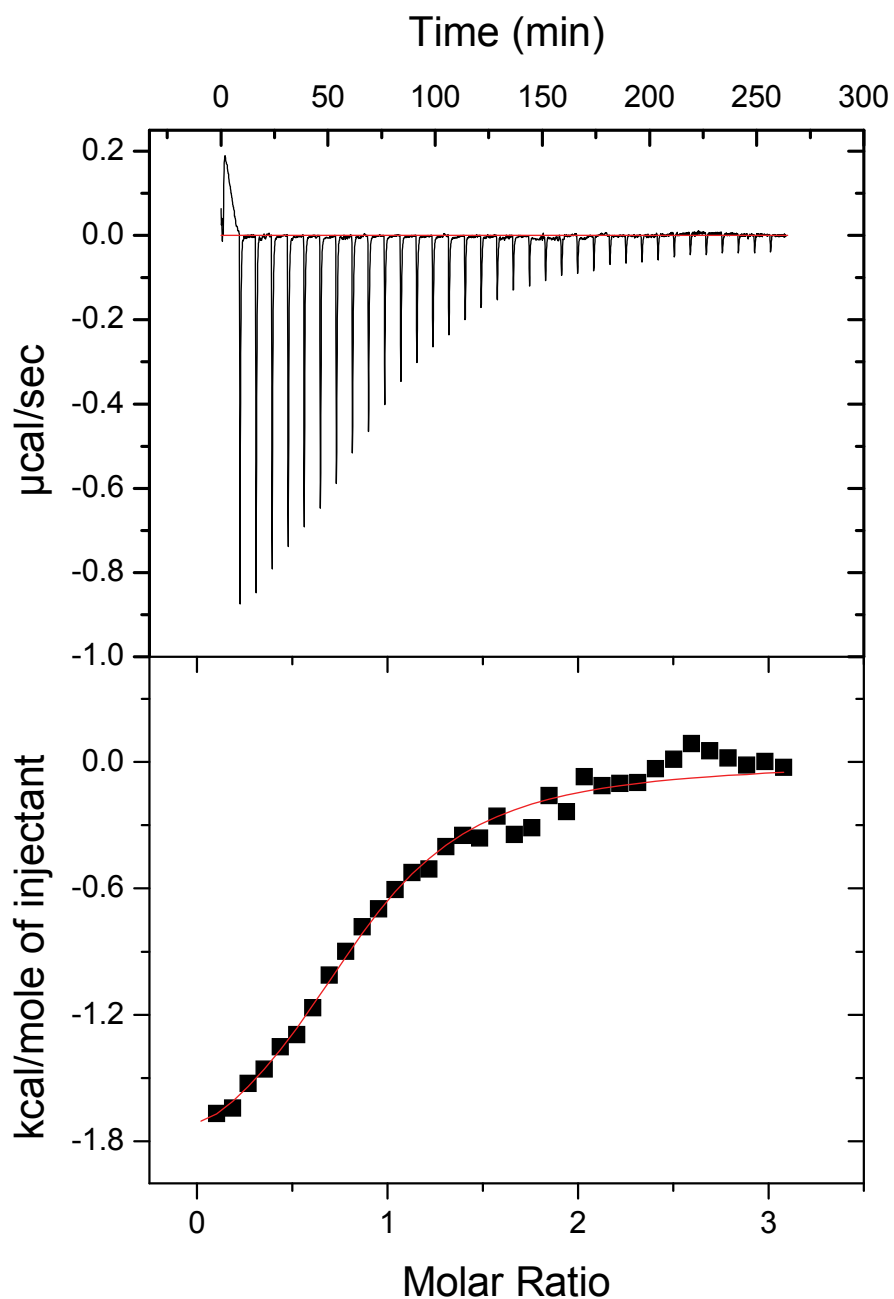


Figure 3.7. ITC titration of 2.1 mM copper into 140 μM A β 28(H13A) (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 0.85$, $K = 4.14 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.06 \text{ kcal/mole}$, $\Delta G = -6.54 \text{ kcal/mole}$, $T\Delta S = 4.49 \text{ kcal/mole}$.

iii. Discussion

ITC titrations have also been performed on A β 28(H13A). In this peptide, histidine-13 was mutated to the non-binding amino acid, alanine. His-13 has been implicated as a possible ligand for copper coordination.¹⁶ His-13 is lacking in A β produced by rats and has been found that rats without His-13 do not develop AD.¹⁷ In the proposed binding sites that may exist for copper in equilibrium, His 13 is found in one site while it can be either His-13 or His-14 in another site.²⁹ **Table 3.6** compares the thermodynamics of copper binding to A β 28 and A β 28(H13A).¹⁸

The stoichiometry, n , is 0.81 which signifies roughly one copper ion is binding per A β 28(H13A) molecule. The apparent binding constant, $4.72 (\pm 1.6) \times 10^4 \text{ M}^{-1}$, is within error of A β 28 ($5.72 (\pm 0.57) \times 10^4 \text{ M}^{-1}$) implying the binding affinity for copper to A β 28(H13A) is similar to the wild-type peptide. The ΔG is similar to A β 28 which indicates the energetic of the binding event may be similar. The ΔH of the reaction is lower than free peptide indicating this reaction is enthalpically less favored than A β 28. The $T\Delta S$ is increased for A β 28(H13A) indicating this reaction is entropically more favorable than copper binding to A β 28. The $T\Delta\Delta S$ of A β 28 and A β 28(H13A) is 1.13 kcal/mole. This change in entropy shows that histidine 13 is involved in copper coordination to A β 28.

Copper binding to A β 28(H13A) has also been studied by circular dichroism (CD) in the visible region by Syme, et al. at a pH 7.5.¹⁴ Their CD spectrum shows variations in signal intensity implying copper coordinates to His-13. In the same work, CD studies have been performed in the UV region. A distinct spectrum is seen for copper binding to A β 28(H13A) compared to A β 28. This indicates copper coordination to A β 28(H13A) induces a conformational

change distinct from A β 28 thus confirming His-13 is a ligand. Similar to A β 28(H6A), the entropy increases while enthalpy decreases relative to the native peptide. Again, it is possible A β 28(H13A) is twisting in such a way that causes a loss in enthalpy.

	<u>Aβ28</u>	<u>Aβ28(H13A)</u>
K/M ⁻¹	4.72 (\pm 0.57) x 10 ⁴	5.72 (\pm 1.6) x 10 ⁴
Δ H/kcal · mol ⁻¹	-3.02 (\pm 0.13)	-1.99 (\pm 0.07)
Δ G/kcal · mol ⁻¹	-6.61 (\pm 0.08)	-6.72 (\pm 0.17)
T Δ S/kcal · mol ⁻¹	3.59 (\pm 0.19)	4.72 (\pm 0.24)

Table 3.6. Comparison between the thermodynamics of copper binding to A β 28 and A β 28(H13A) established by ITC using a one set of sites model.

3.9 Characterization of Copper Binding to A β 28(H14A)

i. Experimental

Small amounts (14-18mg) of crude A β 28(H6A) were dissolved in 18 M Ω H₂O and purified using RP-HPLC. Lyophilized peptide was dissolved in small amounts of water until an appropriate concentration was reached using UV-Vis. This peptide solution was used as the stock solution and diluted to make 5 mL of a 140 μ M solution with 0.50 mL of 0.2 M ACES, and 0.50 mL of 1M NaCl. The actual concentration was 140 μ M A β 28(H14A).

The ITC injection parameters consisted of 35 injections. The first injection was 2 μ L of titrant with a 4s duration, 450s spacing, and a 2s filter period. The last 34 injections were 8 μ L with a 16s duration, 450s spacing, and a 2s filter period. The representative results were an average of three titration experiments. The ITC figure presented in **Figure 3.8** represents one titration. The thermodynamics of that specific ITC reaction can be found in the figure caption. The other two ITC plots can be found in **Appendix A** along with the fitting parameters for those titrations.

ii. Results

A representative ITC isotherm for 2.1 mM Cu²⁺ titrated into 140 μ M A β 28(H14A) is shown in **Figure 3.8**. The top section of the graph represents the raw data in μ cal/sec vs time (min) while the bottom represents integrated heats of each injection in kcal/mol of injectant vs molar ratio. The ITC binding isotherm shows a single phase with an inflection point at approximately 1. The results were an average of three titration experiments. Fitting the data to a “single set of sites model” provides the following data: $n = 0.74 (\pm 0.028)$, $K = 4.84 (\pm 1.1) \times$

10^4 , and $\Delta H = -2.35 (\pm 0.12)$ kcal/mol. ΔG and $T\Delta S$, obtained from **Eq.s 2.2 and 2.3**, are $-6.61 (\pm 0.14)$ kcal and $T\Delta S = 4.26 (\pm 0.25)$ kcal/mol, respectively.

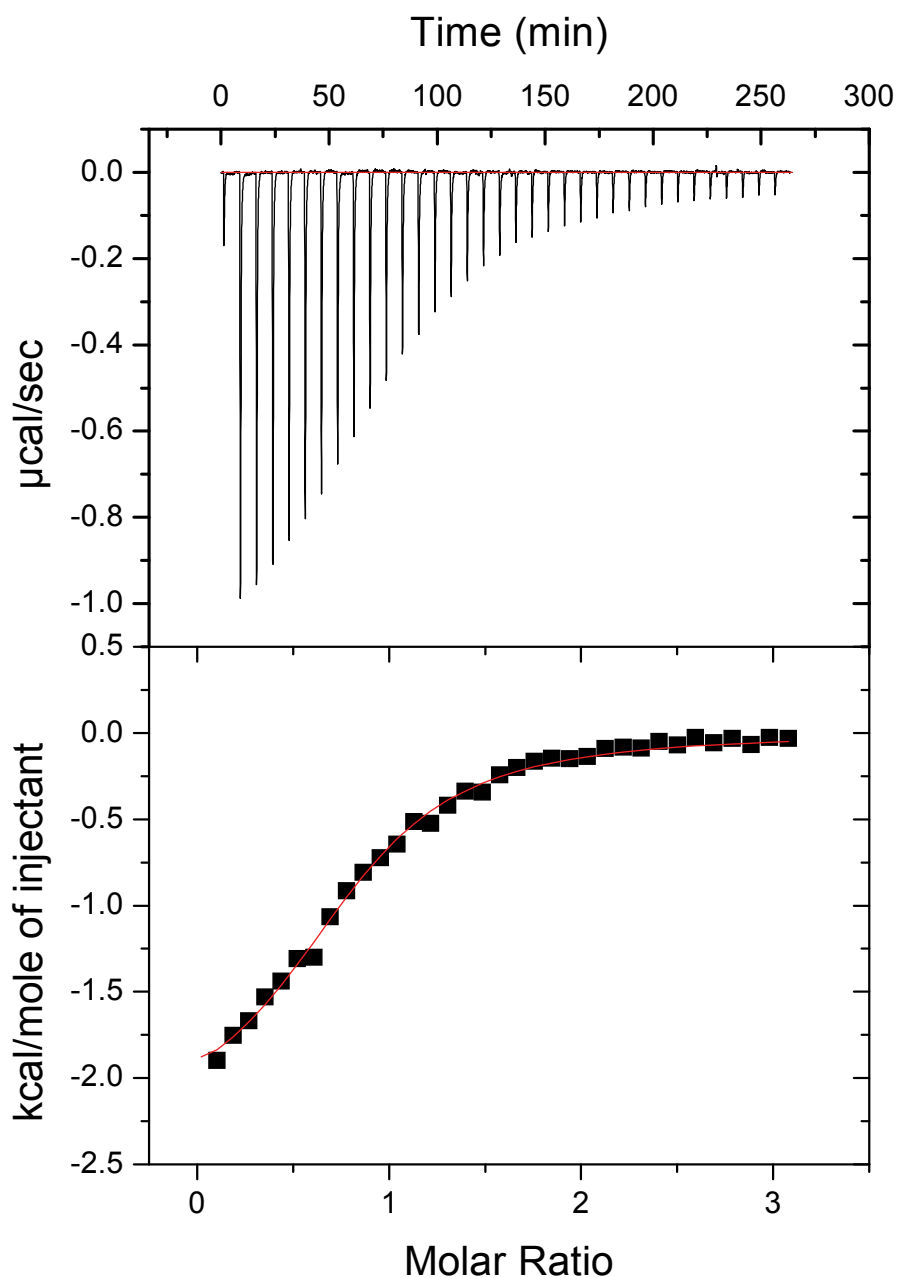


Figure 3.8. ITC titration of 2.1 mM copper into 140 μM A β 28(H14A) (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters generated from ITC data displayed in the figure gave $n = 0.80$, $K = 4.16 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.29 \text{ kcal/mole}$, $\Delta G = -6.54 \text{ kcal/mole}$, $T\Delta S = 4.25 \text{ kcal/mole}$.

iii. Discussion

ITC titrations have also been performed on A β 28(H14A). In this peptide, histidine-14 was mutated to the non-binding amino acid, alanine. His-14 has been implicated as a possible ligand for copper coordination.¹⁴ In the proposed binding sites that may exist for copper in equilibrium, His 14 is found in one site while it can be either His-13 or His-14 in another site.²⁹

Table 3.7 compares the thermodynamics of copper binding to A β 28 and A β 28(H14A).¹⁸

The stoichiometry, n , is 0.74 which signifies roughly one copper ion is binding per A β 28(H14A) molecule. The apparent binding constant, $4.84 (\pm 1.1) \times 10^4 \text{ M}^{-1}$, is within error of A β 28 ($4.72 (\pm 0.57) \times 10^4 \text{ M}^{-1}$) implying the binding affinity for copper to A β 28(H14A) is similar to the wild-type peptide. The ΔH of the reaction is lower than free peptide indicating this reaction is enthalpically less favored than A β 28. The ΔG is the same as A β 28 indicating the energetics of the binding event is similar. The $T\Delta S$ is increased for A β 28(H14A) indicating this reaction is entropically more favorable than copper binding to A β 28.

Copper binding to A β 28(H14A) has also been studied by circular dichroism (CD) in the visible region by Syme, et al. at a pH 7.5.¹⁴ Their CD spectrum shows small variations in signal intensity compared to A β 28. In the same work, CD studies have been performed in the UV region. A distinct spectrum is seen for copper binding to A β 28(H14A) compared to A β 28. This indicates copper coordination to A β 28(H14A) induces a conformational change distinct from A β 28 thus implicating His-14 in copper coordination to A β . Similar to A β 28(H6A) and A β 28(H13A), the entropy increases while enthalpy decreases relative to the native peptide. Again, it is possible that the entropy increase observed for A β 28(H14A) is due to the peptide twisting in such a way that causes a loss in enthalpy.

	<u>Aβ28</u>	<u>Aβ28(H14A)</u>
K/M ⁻¹	4.72 (±0.57) x 10 ⁴	4.84 (± 1.1) x 10 ⁴
ΔH/kcal · mol ⁻¹	-3.02 (± 0.13)	-2.35 (±0.12)
ΔG/kcal · mol ⁻¹	-6.61 (±0.08)	-6.61 (± 0.14)
TΔS/kcal · mol ⁻¹	3.59 (± 0.19)	4.26 (±0.25)

Table 3.7. Comparison between the thermodynamics of copper binding to Aβ28 and Aβ28(H14A) established by ITC using a one set of sites model.

<i>n</i>	<u>Aβ28</u> 0.96 (± 0.04)	<u>Aβ28(H6A)</u> 0.82 (± 0.05)	<u>Aβ28(H13A)</u> 0.81 (± 0.04)	<u>Aβ28(H14A)</u> 0.74 (± 0.03)
K/M ⁻¹	4.72 (±0.57) x 10 ⁴	3.33 (± 0.77) x 10 ⁴	5.72 (± 1.6) x 10 ⁴	4.84 (± 1.1) x 10 ⁴
ΔH/kcal · mol ⁻¹	-3.02 (± 0.13)	-2.15 (±0.05)	-1.99 (±0.07)	-2.35 (±0.12)
ΔG/kcal · mol ⁻¹	-6.61 (±0.08)	-6.41 (± 0.01)	-6.72 (± 0.17)	-6.61 (± 0.14)
TΔS/kcal · mol ⁻¹	3.59 (± 0.19)	4.25 (±0.05)	4.72 (±0.24)	4.26 (±0.25)

Table 3.8. Comparison between the thermodynamics of copper binding to Aβ28 and the Aβ28 mutants established by ITC using a one set of sites model.

Chapter 3.10 Conclusions

The comparison of the thermodynamic parameters between A β 16 and Ac-A β 16 have shown that acetylation of the N-terminus alters the thermodynamics. A decrease in the binding constant, ΔH , ΔG , and $T\Delta S$ was observed for copper binding to Ac-A β 16. A decrease in these parameters indicates a weaker copper binding. The ΔG of Ac-A β 16 is less energetically favorable which suggests the N-terminal amine is essential for copper binding.

The thermodynamics of copper binding to the mutated peptides, A β 28(H13A), A β 28(H6A) and A β 28(H14A), shows relatively different thermodynamics compared to the other mutated peptides. Similar binding constants and binding energetics have been observed for the mutant peptides compared to the native peptide indicating that the loss of one ligand might allow for the substitution of another ligand. Also an increase in entropy and decrease in enthalpy have been observed for the mutant peptides relative to the native peptide. This implies a change in the conformational degrees of freedom possibly causing the peptide to structurally change due to the loss of a ligand. It is possible the peptide is distorting in a way that it can still bind energetically but the loss of enthalpy may indicate longer bond lengths caused by distortion of the peptide. In order to assess significant changes in the peptide upon copper addition, another technique must be used to describe these interactions.

Additionally, the second binding site of copper was not observed. This is most likely due to the competition between ACES and A β . The second binding site has a lower affinity and therefore could not be detected using ITC under our conditions. Cumulatively, our results show copper binding to the A β peptide is a complex process.

In order to effectively develop a copper chelator for Alzheimer's disease, copper's affinity for the A β peptide must be established. Once it is established, a chelator affinity must be higher than that of the Cu-A β binding constant. Copper chelators could decrease plaque formation and also aid in the reduction of ROS generated from redox active metals.

3.11 Future Directions

ITC cannot be the only technique used to describe copper coordination to A β . In order to understand this dynamic process, circular dichroism can be used to describe copper and peptide interactions. Syme and coworkers have produced UV and visible CD studies that show signal changes in A β 28(H6A), A β 28(H13A) and A β 28(H14A) upon copper coordination.¹⁴ Experiments that may provide insight are CD spectra of ITC reactions. For example, a UV or visible CD signature of copper addition to A β after copper saturation via ITC may prove useful. Due to the difference in entropy between A β 28 and the mutated peptides, one can probe the structural differences of these peptides in the UV region. Entropy gives insight into hydrophobic interactions and conformational degrees of freedom. This change in entropy may be observed structurally. Visible CD studies can give information regarding the ligands bound to copper. Therefore, altering a ligand proposed in copper binding may be observed in the CD signature.

Another future direction includes computational studies of copper binding to the A β peptide. Dr. Lee Bartolotti at East Carolina University is planning to computationally study these interactions. Using molecular dynamics (MD), the motion of the peptides can be studied by generating the lowest energy configuration for each molecule. A comparison between the wild-type and mutant peptides can give information regarding the lowest energy state and which ones are thermodynamically preferred.

Lastly, another technique that may be useful is infrared spectroscopy. This technique is based on the fact that molecules vibrate at certain frequencies. These vibrational frequencies may be altered upon addition of copper. For instance, one could assess the frequency of a histidine

residue that has copper bound or unbound. This would give insight to what ligands are bound to copper.

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APPENDIX A

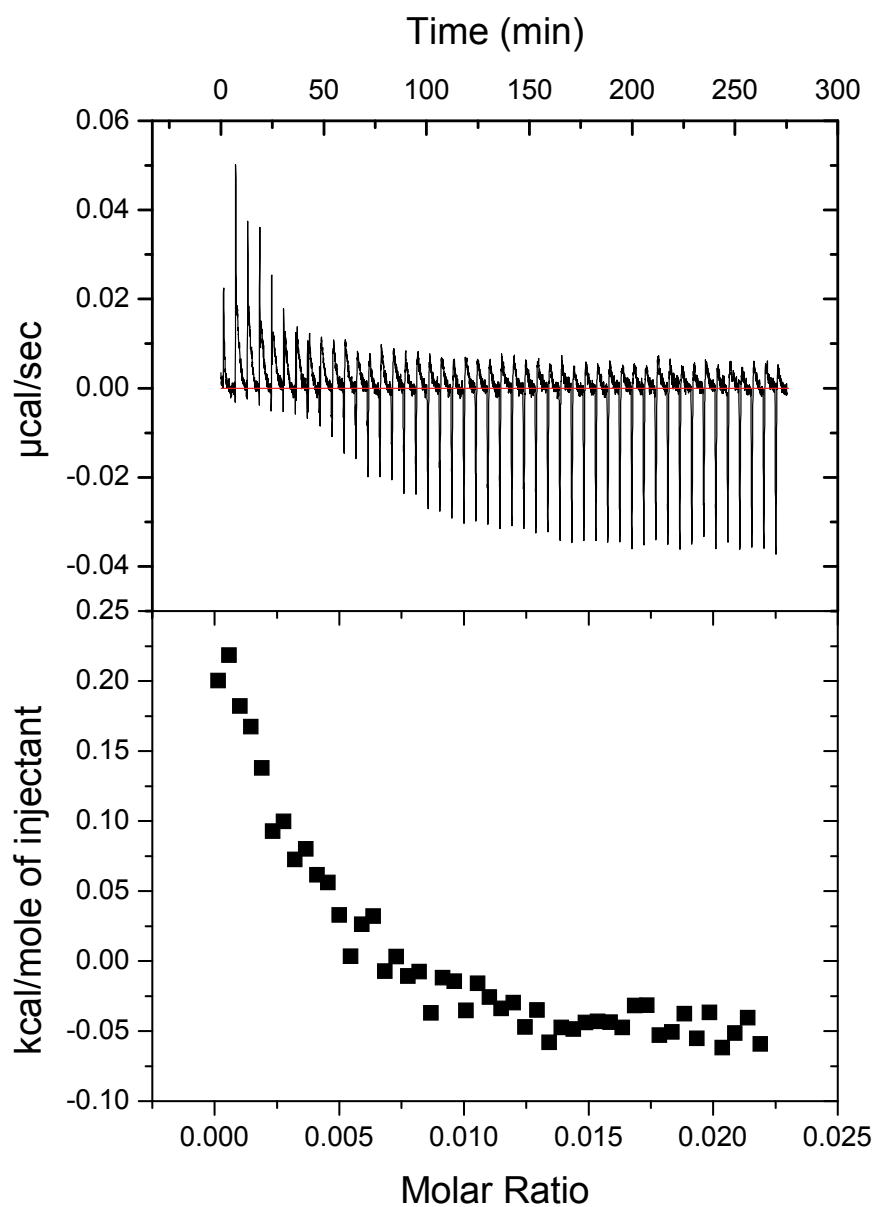


Figure A.1. Supplement ITC of **Figure 3.1**. ITC titration of 2.1 mM copper into 20 mM ACES (100 mM NaCl, pH 7.4 at 37°C). The heat of dilution was found to be -45.84 cal/mole of injectant.

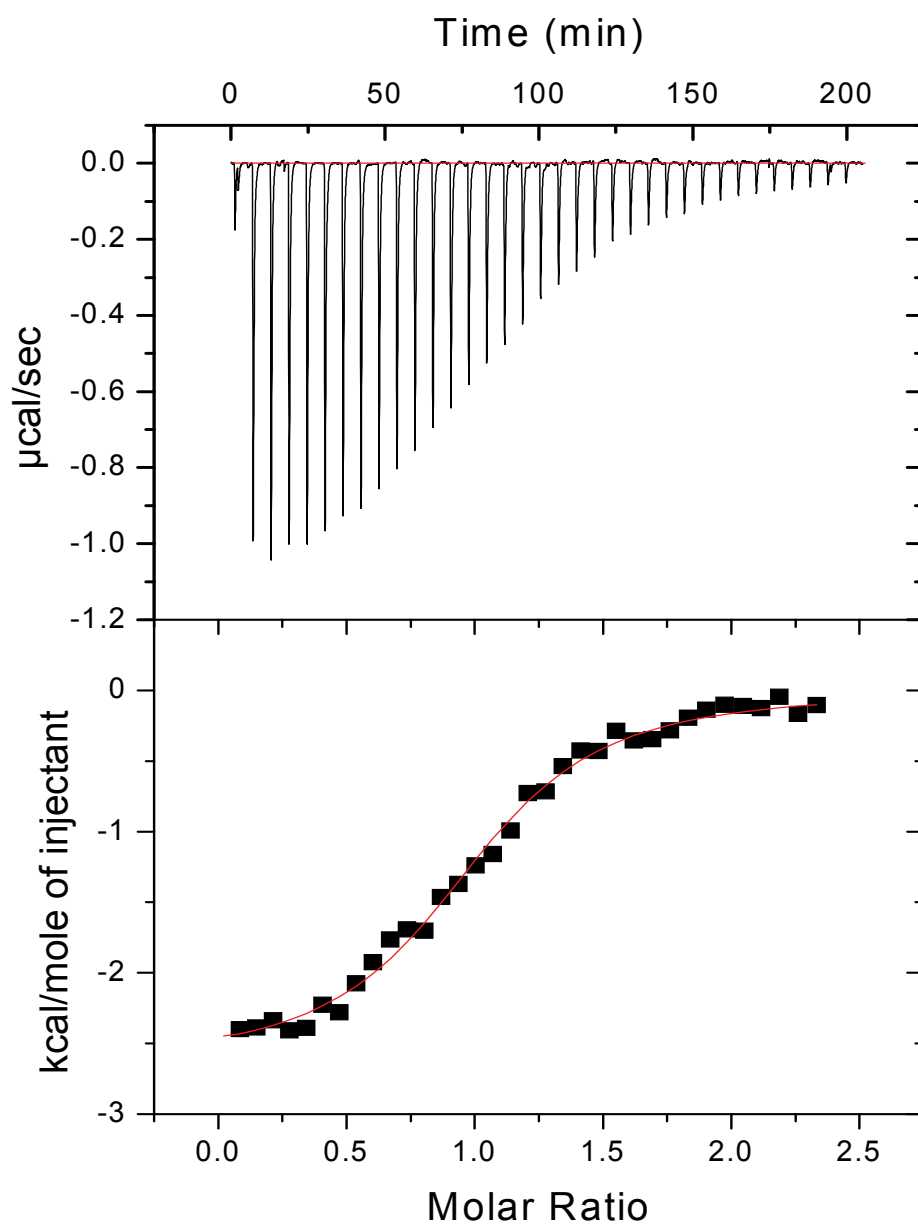


Figure A.2. Supplement ITC of **Figure 3.2**. ITC titration of 2.1 mM copper into 136 μM A β 16 (20 mM ACES, 100 mM NaCl, pH 7.4 at 37°C). The solid red line represents the fit generated from the one set of sites model. Fitting parameters gave $n = 1.01$, $K = 9.27 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.64$ kcal/mole, $\Delta G = -7.04$ kcal/mole, $\Delta S = 4.39$ kcal/mole.

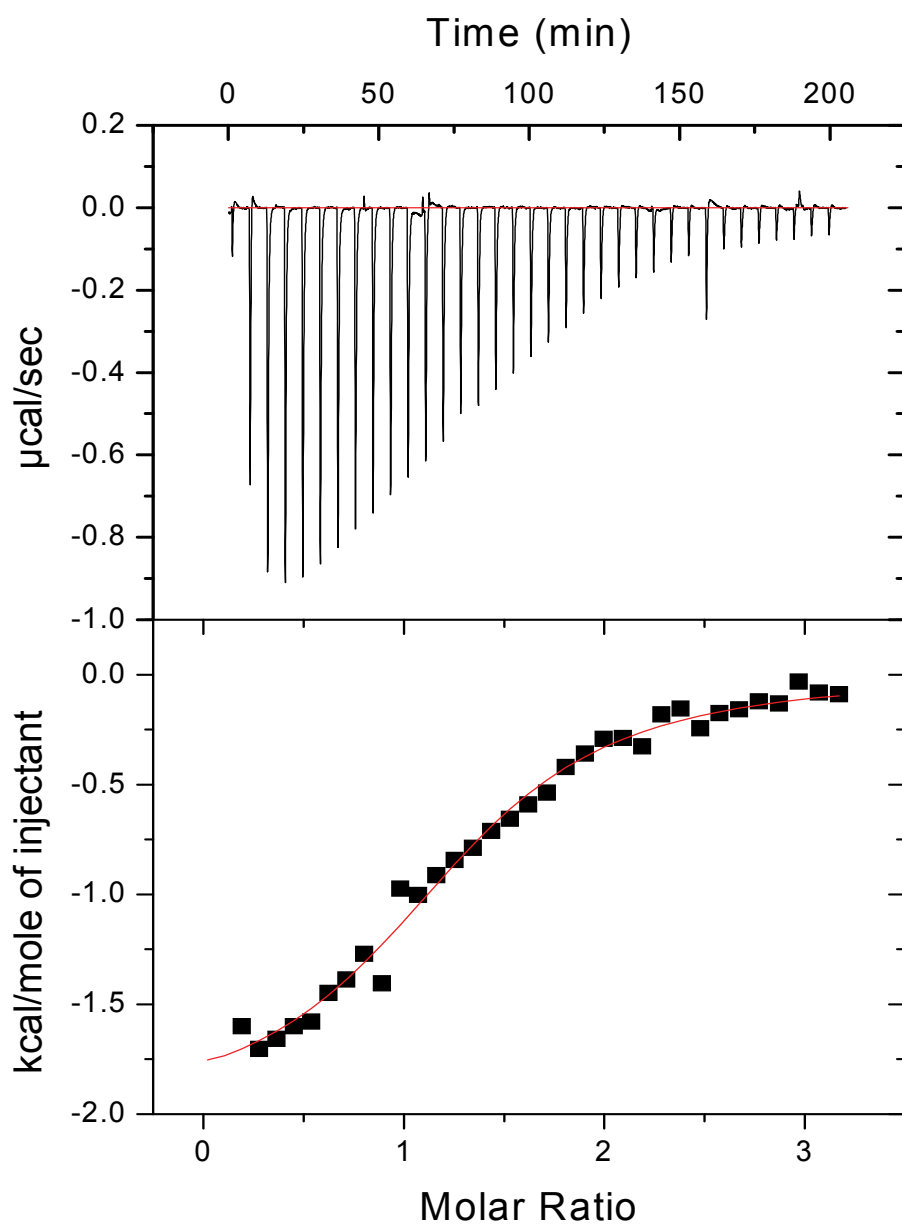


Figure A.3. Supplement ITC of **Figure 3.2**. ITC titration represents 2.1 mM copper into 136 μM A β 16 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 1.28$, $K = 4.10 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.00 \text{ kcal/mole}$, $\Delta G = -6.54 \text{ kcal/mole}$, $\Delta S = 4.53 \text{ kcal/mole}$.

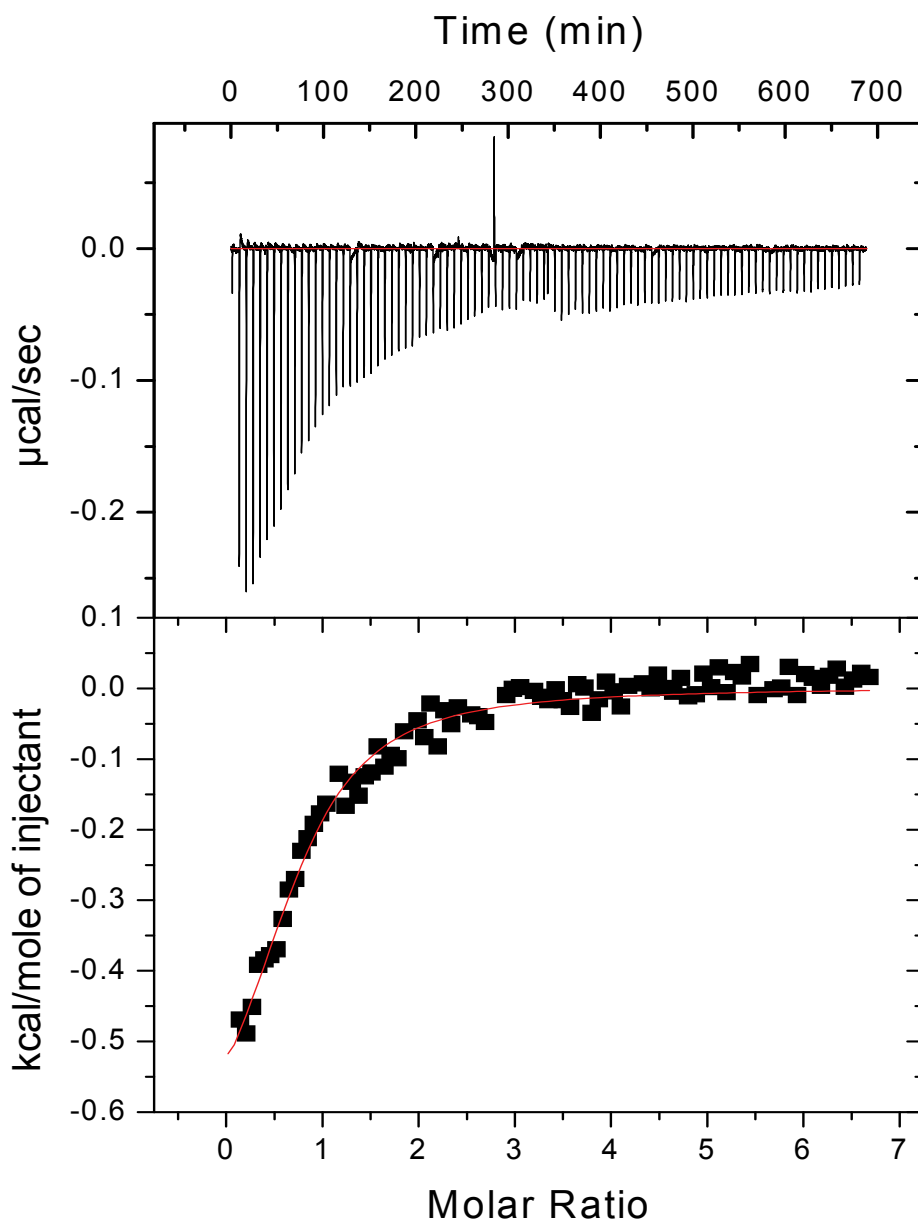


Figure A.4. Supplement ITC of **Figure 3.3**. ITC titration represents 2.1 mM copper into 136 μM Ac-A β 16 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 1.03$, $K = 9.93 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.47 \text{ kcal/mole}$, $\Delta G = -6.19 \text{ kcal/mole}$, $\Delta S = 5.45 \text{ kcal/mole}$.

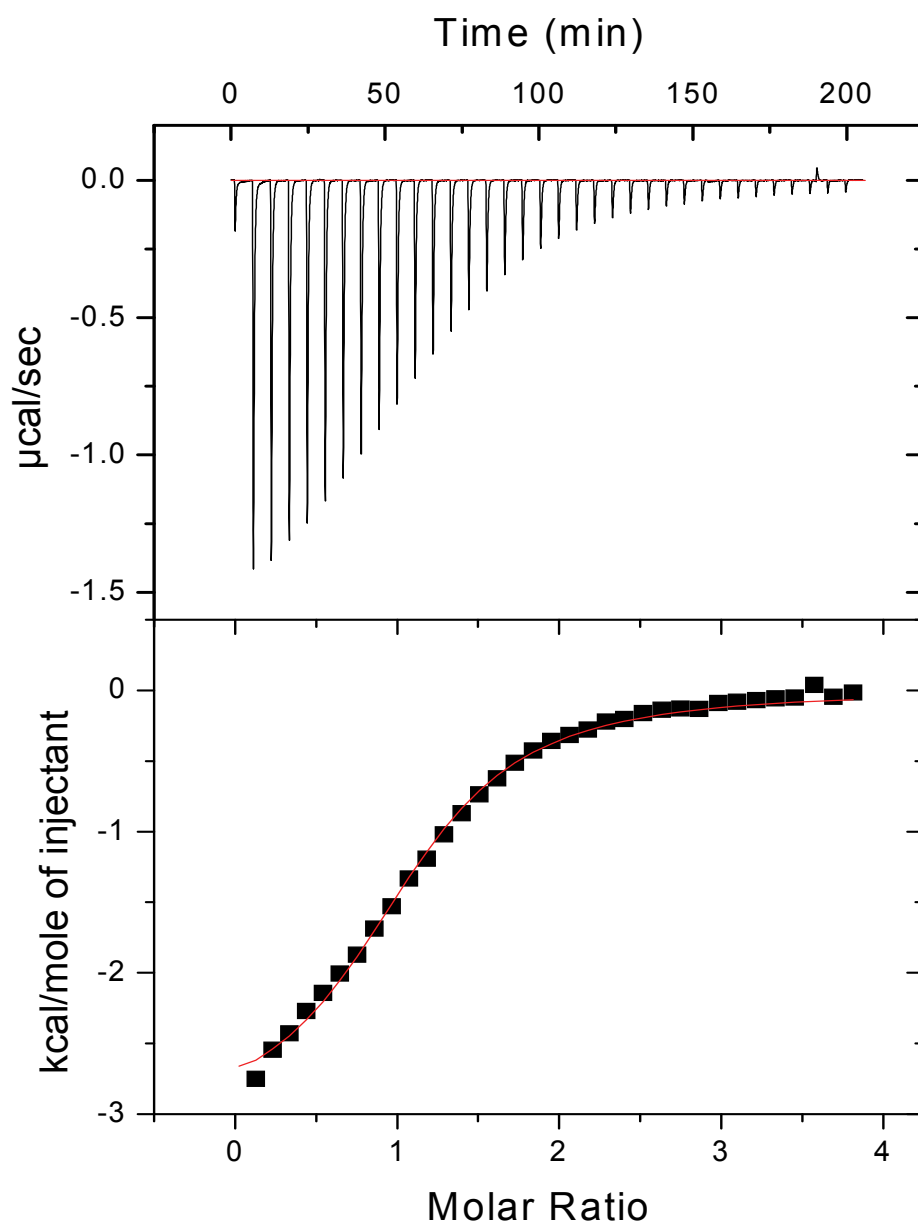


Figure A.5. Supplement ITC of **Figure 3.4**. ITC titration represents 2.1 mM copper into 113 μM A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.902$, $K = 5.23 \times 10^4 \text{ M}^{-1}$, $\Delta H = -3.09 \text{ kcal/mole}$, $\Delta G = -6.69 \text{ kcal/mole}$, $\Delta S = 3.60 \text{ kcal/mole}$.

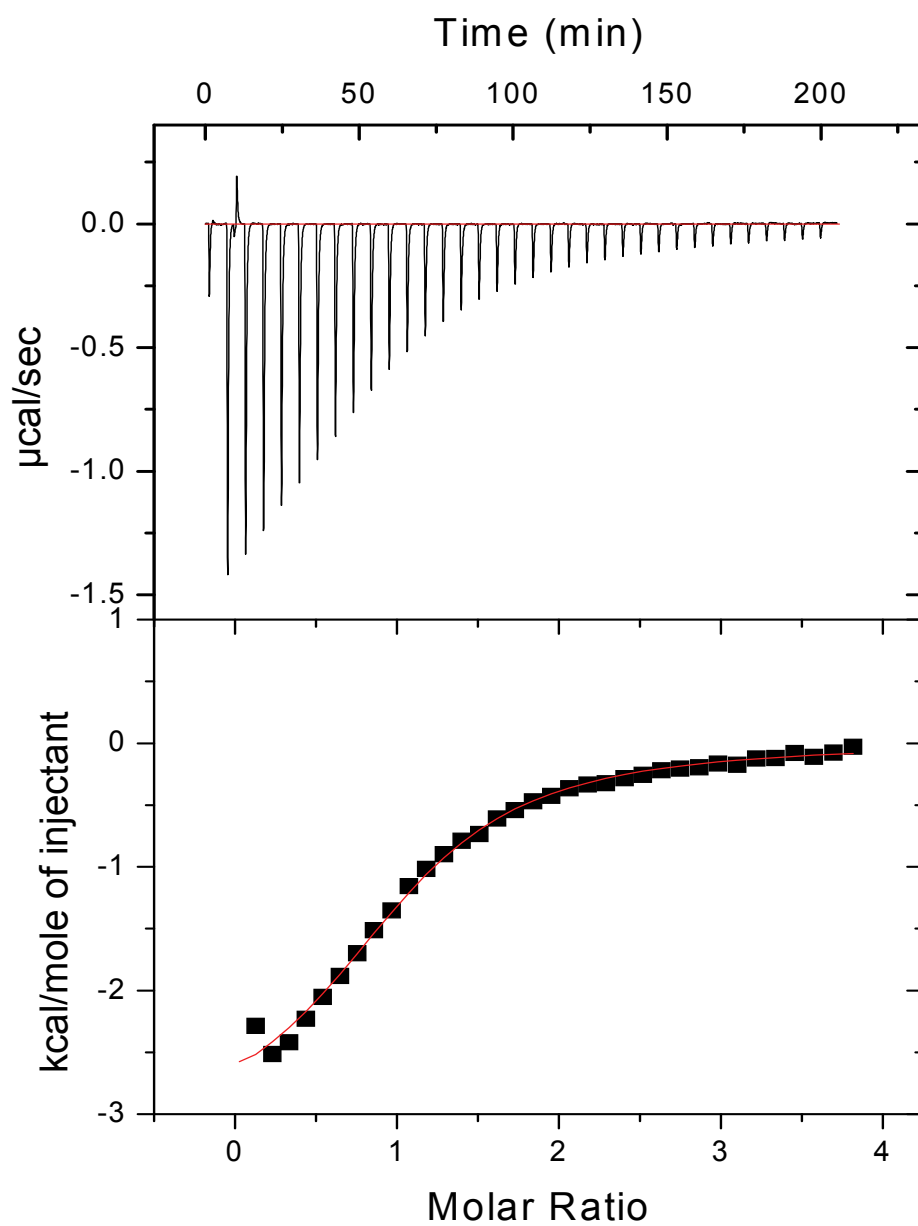


Figure A.6. Supplement ITC of **Figure 3.4**. ITC titration represents 2.1 mM copper into 113 μM A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 1.04$, $K = 3.59 \times 10^4 \text{ M}^{-1}$, $\Delta H = -3.20 \text{ kcal/mole}$, $\Delta G = -6.45 \text{ kcal/mole}$, $\Delta S = 3.25 \text{ kcal/mole}$.

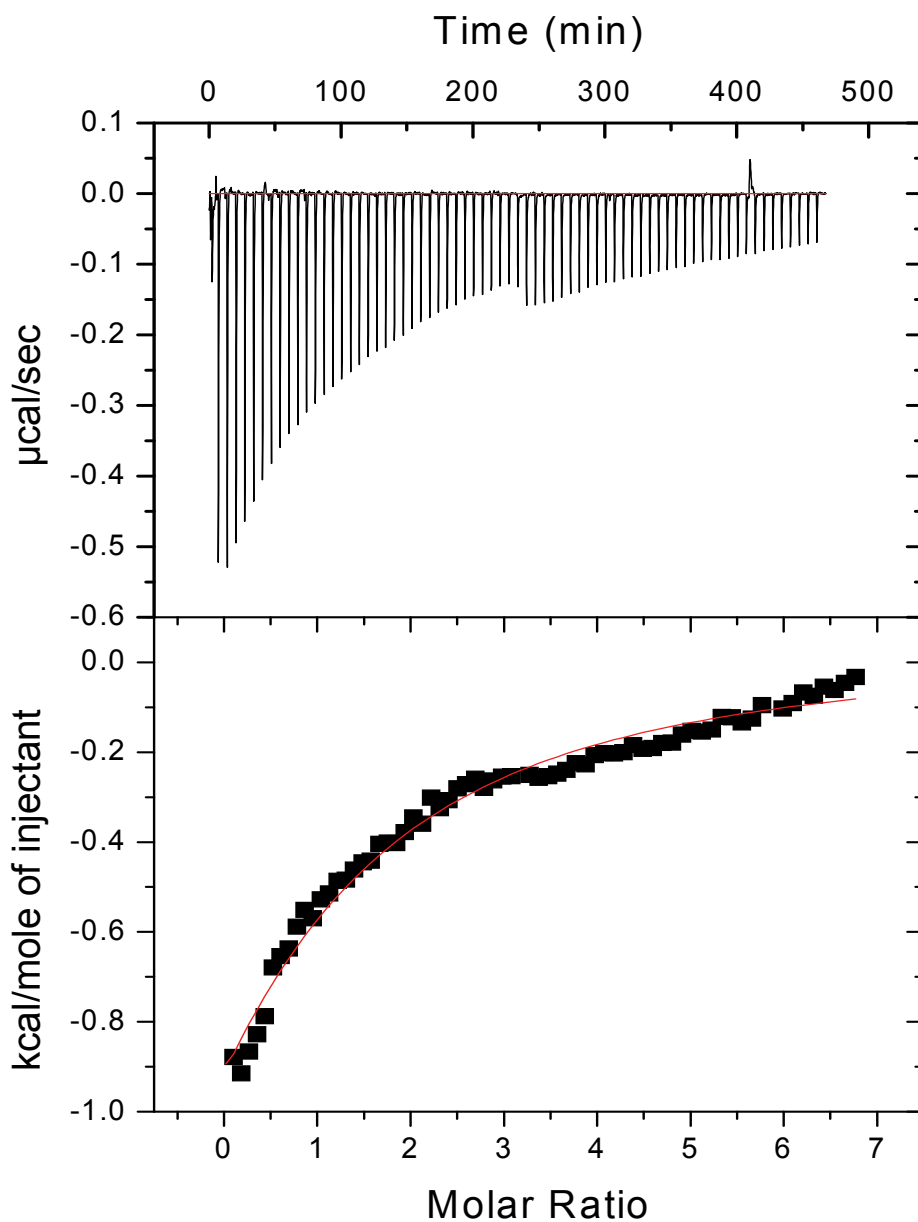


Figure A.7. Supplement ITC of **Figure 3.5**. ITC titration represents 2.1 mM copper into 143 μM Ac-A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 1.00$ (fixed), $K = 3.58 \times 10^3 \text{ M}^{-1}$, $\Delta H = -2.61 \text{ kcal/mole}$, $\Delta G = -5.04 \text{ kcal/mole}$, $\Delta S = 2.42 \text{ kcal/mole}$.

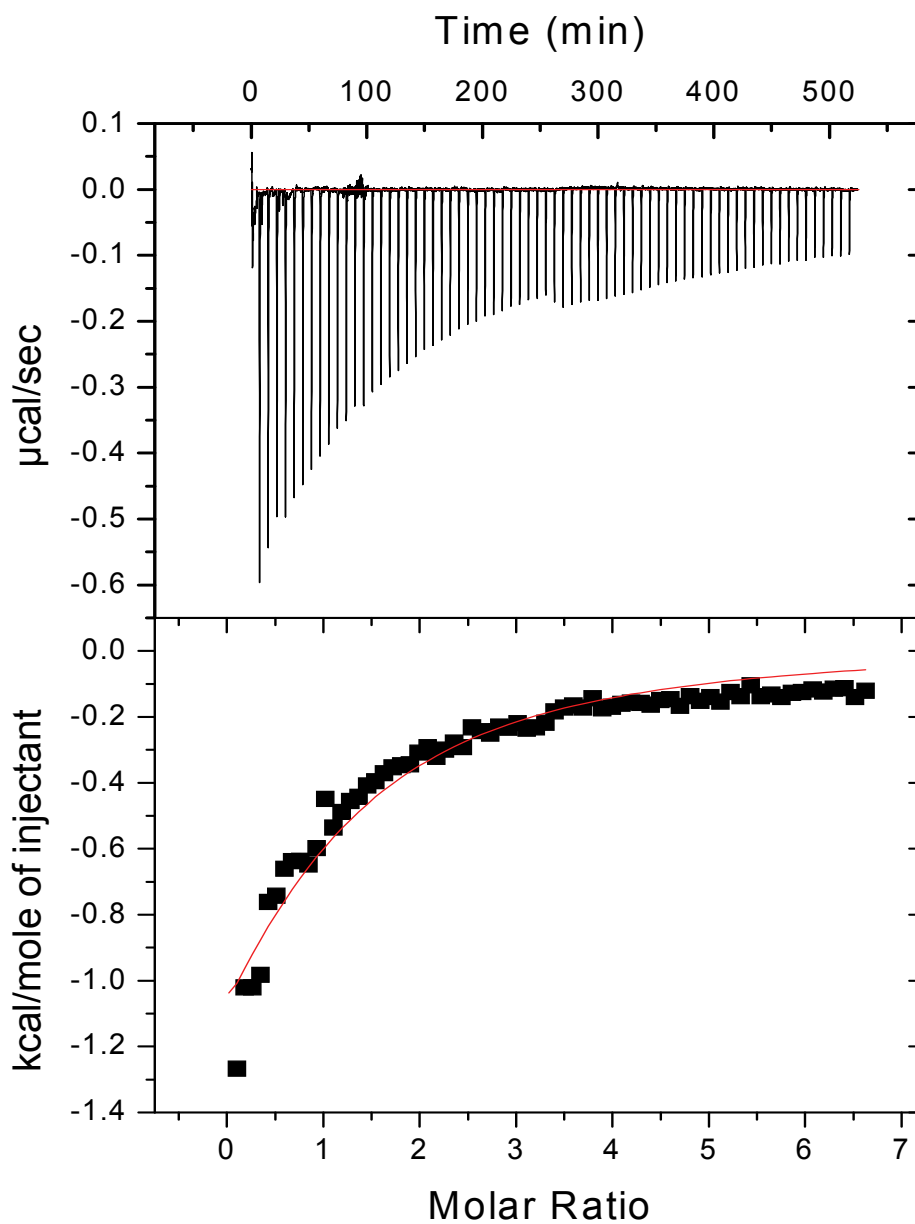


Figure A.8. Supplement ITC of **Figure 3.5**. ITC titration represents 2.1 mM copper into 113 μM Ac-A β 28 (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 1.00$ (fixed), $K = 4.33 \times 10^3 \text{ M}^{-1}$, $\Delta H = -2.73 \text{ kcal/mole}$, $\Delta G = -5.15 \text{ kcal/mole}$, $\Delta S = 2.43 \text{ kcal/mole}$.

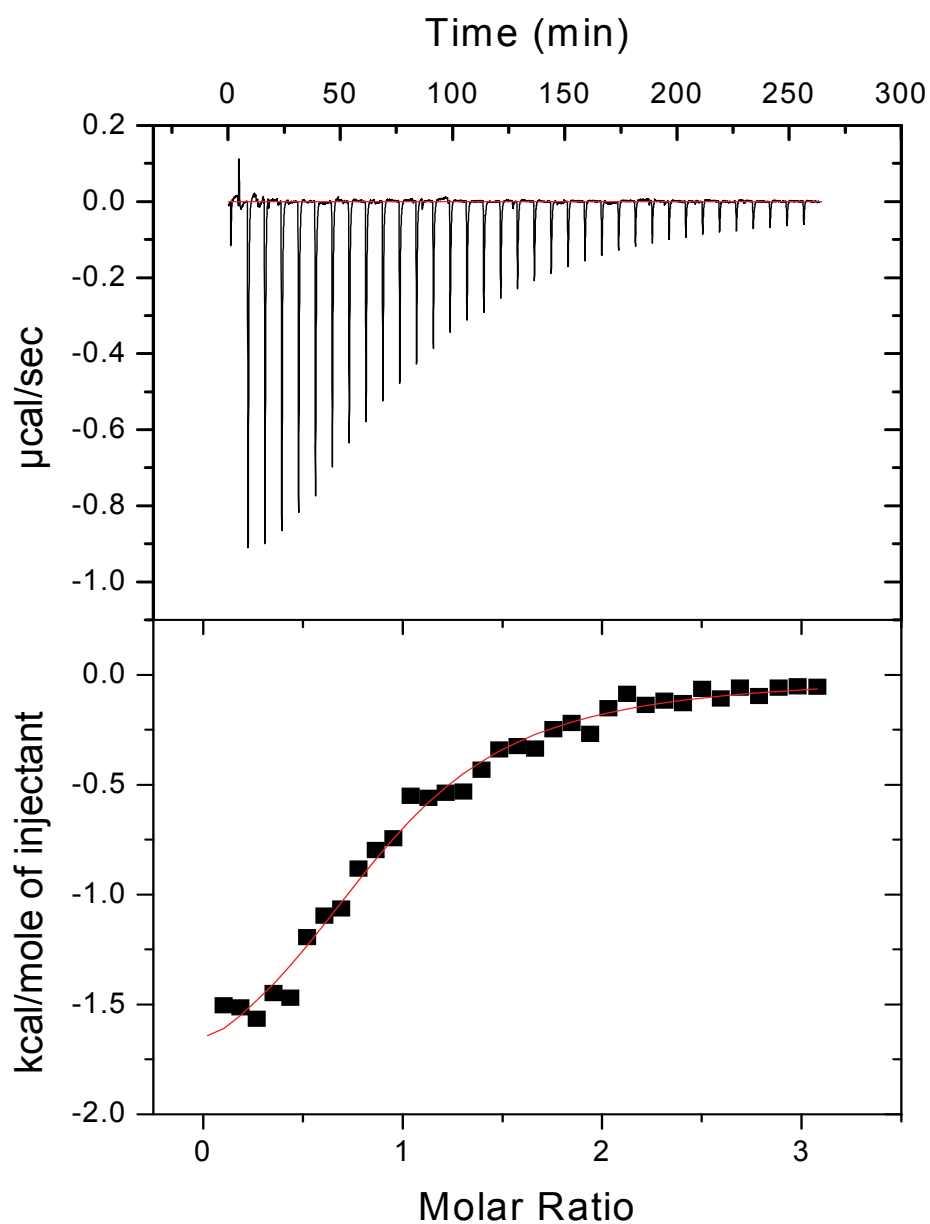


Figure A.9. Supplement ITC of **Figure 3.6**. ITC titration represents 2.1 mM copper into 140 μM A β 28(H6A) (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.902$, $K = 3.30 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.05 \text{ kcal/mole}$, $\Delta G = -6.40 \text{ kcal/mole}$, $\Delta S = 4.36 \text{ kcal/mole}$.

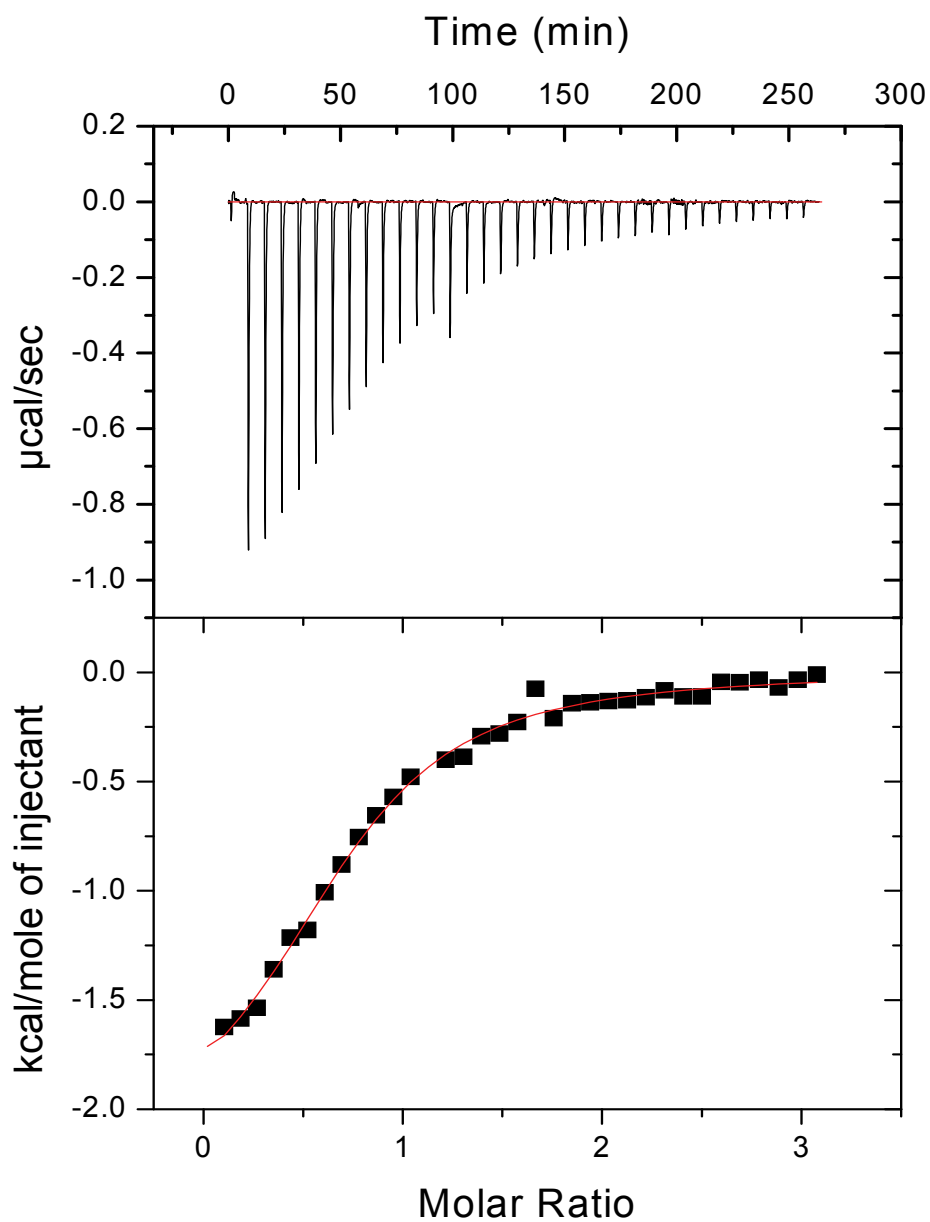


Figure A.10. Supplement ITC of **Figure 3.6**. ITC titration represents 2.1 mM copper into 140 μM A β 28(H6A) (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.719$, $K = 3.48 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.21 \text{ kcal/mole}$, $\Delta G = -6.43 \text{ kcal/mole}$, $\Delta S = 4.22 \text{ kcal/mole}$.

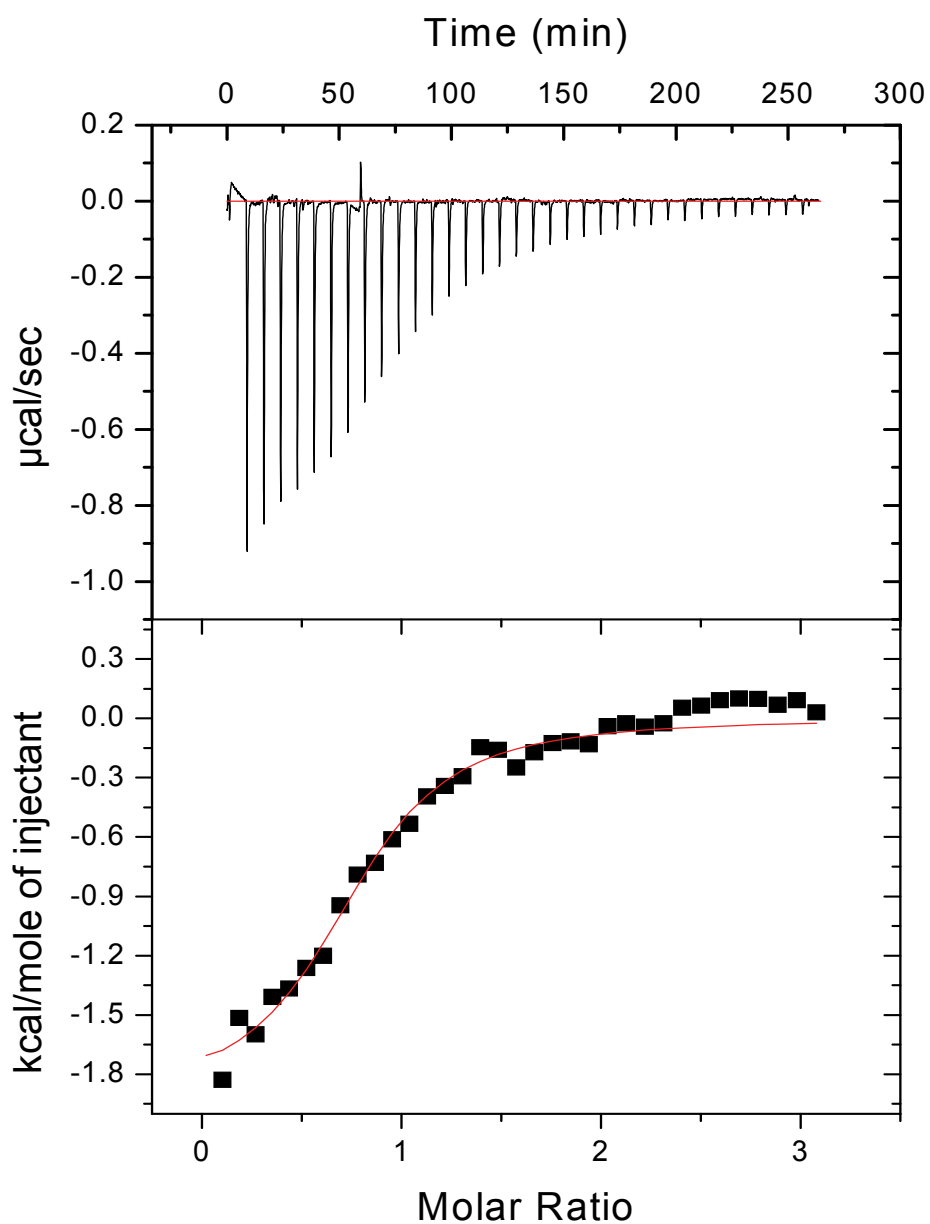


Figure A.11. Supplement ITC of **Figure 3.7**. ITC titration represents 2.1 mM copper into 140 μM A β 28(H13A) (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.78$, $K = 7.30 \times 10^4 \text{ M}^{-1}$, $\Delta H = -1.93 \text{ kcal/mole}$, $\Delta G = -6.89 \text{ kcal/mole}$, $\Delta S = 4.97 \text{ kcal/mole}$.

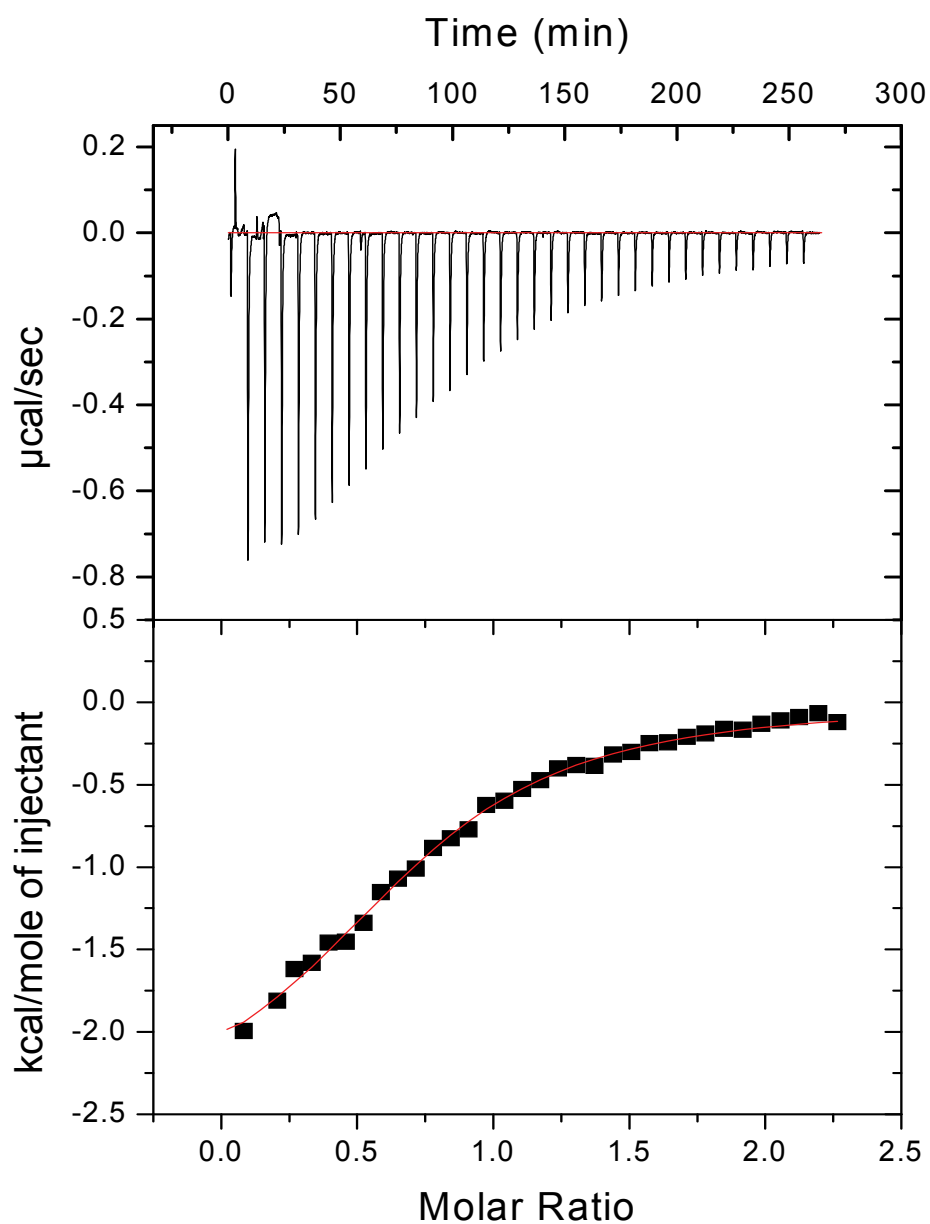


Figure A.12. Supplement ITC of **Figure 3.8**. ITC titration represents 2.1 mM copper into 140 μM A β 28(H14A) (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.724$, $K = 3.34 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.59 \text{ kcal/mole}$, $\Delta G = -6.41 \text{ kcal/mole}$, $\Delta S = 3.82 \text{ kcal/mole}$.

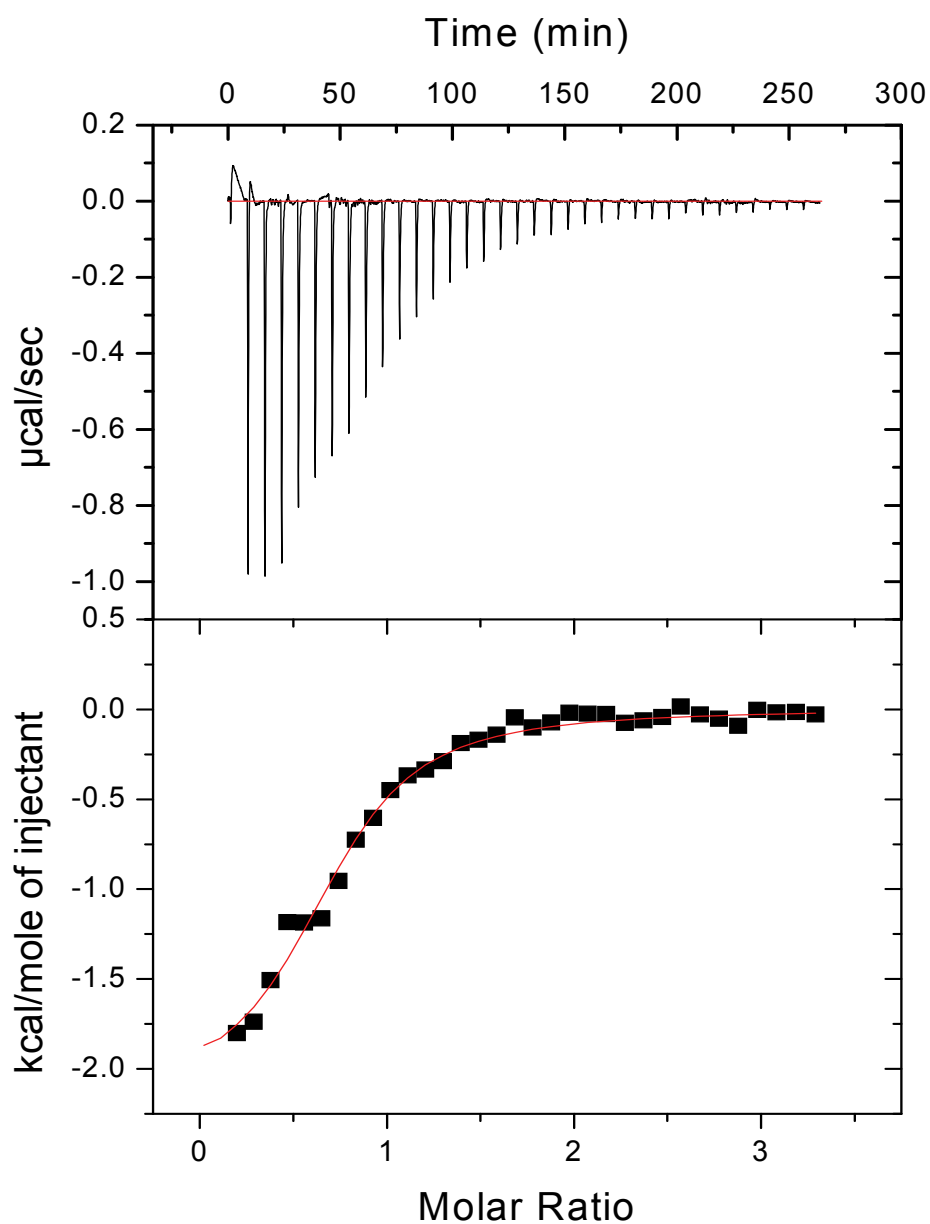


Figure A.13. Supplement ITC of **Figure 3.8**. ITC titration represents 2.1 mM copper into 140 μM A β 28(H14A) (20 mM ACES, 100 mM NaCl, pH 7.4) at 37°C. The solid red line represents the one set of sites model with $n = 0.707$, $K = 7.03 \times 10^4 \text{ M}^{-1}$, $\Delta H = -2.16 \text{ kcal/mole}$, $\Delta G = -6.87 \text{ kcal/mole}$, $\Delta S = 4.70 \text{ kcal/mole}$.

APPENDIX B

Determination of Buffer Independent Binding Affinity for Cu-A β 16 Complex³¹

The apparent binding constant, K_{ITC} , for A β 16 can be expressed as:

$$K_{ITC} = \frac{[ML]}{[M]_{ITC}[L]_{ITC}}$$

Eq. B.1

where [ML] is the concentration of the Cu-A β 16 complex, $[M]_{ITC}$ is the free concentration of copper and $[L]_{ITC}$ is the free concentration of A β 16 in solution. The K_{ITC} was found to be $7.77 \times 10^4 \text{ M}^{-1}$. $[M]_{ITC}$ can be defined as

$$[M]_{ITC} = [M] + [MB_2]$$

Eq. B.2

where [M] is the free metal and $[MB_2]$ is metal bound to buffer. **Eq. B.2** assumes that before titration occurs, all copper binds to ACES in a 1:2 stoichiometry. This ignores speciation because it is unsure how many protons are being displaced from the protein upon metal binding. The equilibrium expression that describes metal (M) binding to buffer (B) is represented as

$$K_{MB} = \frac{[MB]}{[M][B]} \quad K_{MB_2} = \frac{[MB_2]}{[M][B]^2}$$

Eq. B.3

where K_{MB} is $2.09 \times 10^4 \text{ M}^{-1}$ and K_{MB_2} is $5.89 \times 10^8 \text{ M}^{-1}$.³⁰ [B] is 0.02 M and $[B]^2$ is 0.0004 M.

In addition, the equilibrium expression for metal binding to peptide can be described as:

$$K_{ML} = \frac{[ML]}{[M][L]_{ITC}}$$

Eq. B.4

Solving for [MB] and [MB₂] in Eq. B.3 and substituting it into Eq. B.2 gives the following expression:

$$[M]_{ITC} = [M](1 + K_{MB}[B] + K_{MB_2}[B]^2)$$

Eq. B.5

Eq. B.5 can be substituted into Eq. B.1 to give:

$$K_{ITC} = \frac{[ML]}{[M](1 + K_{MB}[B] + K_{MB_2}[B]^2)[L]_{ITC}}$$

Eq. B.6

The above equation can be rearranged to give the following:

$$K_{ITC} = \frac{[ML]}{[M][L]_{ITC}} \times \frac{1}{(1 + K_{MB}[B] + K_{MB_2}[B]^2)}$$

Eq. B.7

Substituting Eq. B.4 into Eq. B.7 provides:

$$K_{ITC} = K_{ML} \times \frac{1}{(1 + K_{MB}[B] + K_{MB_2}[B]^2)}$$

Eq. B.8

Solving for K_{ML} gives the expression:

$$K_{ML} = K_{ITC}(1 + K_{MB}[B] + K_{MB_2}[B]^2)$$

Eq. B.9

Solving for K_{ML} gives the value of $1.85 \times 10^9 \text{ M}^{-1}$.