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Quantification of the Binding Properties of Cu²⁺ to the Amyloid-Beta Peptide: Coordination Spheres for Human and Rat Peptides and Implication on Cu²⁺-Induced Aggregation

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SUMMARY

There is no consensus on the coordinating ligands for Cu²⁺ by A β . Yet the differences in peptide sequence between human and rat have been hypothesized to alter metal ion binding in a manner that alters Cu²⁺-induced aggregation of A β . Herein, we employ isothermal titration calorimetry (ITC), circular dichroism (CD) and electron paramagnetic resonance (EPR) spectroscopy to examine the Cu²⁺ coordination spheres to human and rat A β and an extensive set of A β (16) mutants. EPR of the mutant peptides is consistent with a 3N1O binding geometry, like the native human peptide at pH 7.4. The thermodynamic data reveal an equilibrium between three coordination spheres, {NH₂, O⁻, N_{Im}^{His6}, amide N⁻}, {NH₂, O⁻, N_{Im}^{His6}, N_{Im}^{His13}} and {NH₂, O⁻, N_{Im}^{His6}, N_{Im}^{His14}} for human A β (16) but only one for rat A β (16), {NH₂, O⁻, N_{Im}^{His6}, N⁻}, at pH 7.4–6.5. ITC and CD data establish that the mutation R5G is sufficient for reproducing this difference in Cu²⁺ binding properties at pH 7.4. The substitution of bulky and positively charged Arg by Gly is proposed to stabilize the coordination {NH₂, O⁻, N_{Im}^{His6}, amide N⁻} that then results in one dominating coordination sphere for the case of the rat peptide. The differences in the coordination geometries for Cu²⁺ by the human and rat A β are proposed to contribute to the variation in the ability of Cu²⁺ to induce aggregation of A β peptides.

Keywords

amyloid beta; Cu²⁺ coordination; binding constant; binding enthalpy; peptide aggregation

INTRODUCTION

Amyloid beta (A β) peptides (39–43 amino acids in length) result from secretase-driven proteolytic processing of the amyloid precursor protein and are the major peptide components of senile plaques.^{1,2} The formation of these insoluble A β deposits in the human brain is the pathological hallmark of Alzheimer's disease (AD). Although the precise reason

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Supporting Information Available: Table S1 provides the binding enthalpies of A β mutants upon Cu²⁺ binding at 37 °C, pH 7.4 and ionic strength as 0.16 M in PIPES, HEPES, TEEN, and Chol buffers. The detailed analysis used to estimate the numbers of protons released from potential Cu²⁺-coordination spheres and the binding enthalpy differences between coordination **Hx** and **HxHy** are described. The thermodynamic model for obtaining the binding parameters of Cu²⁺ to human **Hx** and **HxHy** coordinations at pH 7.4 and 6.5 is detailed. Table S2 presents reported binding enthalpies for Cu²⁺ to specific amino acids at 25 °C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

for the formation of senile plaques in AD remains to be determined, the role of metal ions in promoting the aggregation of A β peptides *in vitro* has led to debate and extensive research addressing how metals bind to and induce aggregation of A β peptides.^{3–9} In particular, elevated concentrations of copper have been measured in the senile plaques,^{10,11} and the use of high-affinity metal ion chelators facilitate the dissolution of these plaques.^{12–14} This knowledge has provided momentum for the development of metal chelation-based therapies targeted toward treating AD.

Different from humans and numerous other mammals, rats and mice rarely form such plaques with age, despite the fact that rodents produce A β peptides in much the same manner as humans.^{15,16} *In vitro* the presence of Cu²⁺ significantly increases the aggregation of human A β (40), but this effect is much less profound for rat A β (40).¹² The defining difference, relative to human A β , is the presence of three specific substitutions in the primary amino acid sequence of the rat A β cleavage product: R5G, Y10F, and H13R.¹⁷ All three mutations lie within the hydrophilic N-terminal, which is also the domain where Cu²⁺ binds. The changes between the human and rat peptides are hypothesized to alter the metal binding in a manner that inhibits Cu²⁺-induced aggregation and, subsequently, neurotoxicity.^{12,18} It has been suggested that rat A β fragments (e.g. 1–6, 1–10 residues) bind to Cu²⁺ more strongly than the corresponding human A β fragments over a wide range of solution pH.¹⁹ In line with that work, the current paper finds that the binding constants of Cu²⁺ to rat A β are about 3 times higher than those of human A β peptides at pH 7.4, 37 °C and are similar at pH 6.5. This result suggests under similar conditions, rat A β can bind at least the same amount of Cu²⁺ as the human peptide. Because Cu²⁺-induced aggregation is more profound for human A β (40),¹² the differences between Cu²⁺ coordination spheres between the rat and human peptides must influence the aggregation process.

The binding sites for Cu²⁺ to human A β peptides have been extensively studied with various techniques, including: EPR, NMR, CD, RAMAN and potentiometry.^{4,5,9,19–27} The Cu²⁺ coordination to A β has been discussed in a recent review.²⁸ At physiological pH (~7.4), Cu²⁺ is coordinated by four ligands, 3N and 1O,^{4,5,9,19,21,22,24–27} with evidence that His6, His13, and His14 are involved, along with possible contributions from Tyr10, the carboxylate group of Asp1, the amide C=O of Ala2, and the N-terminus amine.^{4,5,9,19–25} However, the studies to date differ on the exact coordinating ligands. The different experimental conditions (e.g. low temperature compared to room temperature) and different sensitivity of the techniques used to certain binding motifs contribute to the conclusions that had been made. The different models proposed will be discussed in detail later within the context of the conclusion drawn from the current study. Herein we demonstrate that isothermal calorimetry, combined with CD and EPR, can provide a powerful approach for elucidating details of the coordinating ligands. The data further reveal a novel and interesting correlation between the binding geometry of Cu²⁺ and the propensity for Cu²⁺-induced aggregation of human and rat A β .

EXPERIMENTAL

Materials

Synthetic A β peptides – human A β (16), human A β (40), rat A β (40), mutants of human A β (16): H6A, H13A, H14A, H6AH13A, H6AH14A, H13AH14A, Y10F, and H13R, Ac-A β (16), and mutants of rat A β (16): rat H14A and rat H6A – were purchased from Bio-Synthesis, Inc. (Lewisville, TX) at >95% purity as custom peptides. Rat A β (16) and human R5G mutants were purchased from Department of Physiology, Tufts University, at purity > 98%. The sequences of the peptides are listed in Table 1. Glycine, 1,4-Piperazinediethanesulfonic acid (PIPES), and CuCl₂·(H₂O)₂ were purchased from Sigma-Aldrich (St. Louis, MO) at the highest purity grade available. N-(2-hydroxyethyl)piperazine-

N'(2-ethanesulfonic acid) (HEPES), (2-aminoethyl)trimethylammonium chloride hydrochloride (Chol), N,N,N',N'-tetraethylethylenediamine (TEEN), NaOH, and NaCl (for buffer preparation) were purchased from Fisher Scientific (Waltham, MA) at the highest purity grade available. All solutions were prepared in ultrapure water ($>18\text{ M}\Omega\text{-cm}$) purified using a Millipore Simplicity^{TM} system (Billerica, MA).

A $\beta(16)$ and its mutant stock solutions were prepared by dissolving the lyophilized synthetic peptide into ultrapure water ($\sim 5\text{ mg/mL}$) at room temperature upon receipt of the purified peptide. The peptide stock solutions were then sonicated for ~ 2 minutes in a water bath and centrifuged at $10\,000\text{ g}$ from 10 min to remove any large aggregates from the lyophilization process. The supernatants were carefully aliquoted into multiple tubes and frozen at $-20\text{ }^{\circ}\text{C}$ until experimental use. Our previous knowledge showed that with the presence of excess amount of glycine, one A $\beta(16)$ peptide molecule only binds to one Cu^{2+} at pH 7.4, $37\text{ }^{\circ}\text{C}$. Thus, peptide concentrations were determined with preliminary ITC run by setting the concentration of peptide to get a 1:1 binding stoichiometry when performing data analysis. A $\beta(40)$ solutions were prepared freshly from lyophilized synthetic peptide for each ITC measurement. Briefly, two tubes of $\sim 0.6\text{ mg}$ of peptide were first suspended in $100\text{ }\mu\text{L}$ of 1 mM NaOH and then 1 mL of buffer separately. After being vortex-mixed thoroughly, the solutions were sonicated at room temperature for 2 min and the centrifuged at $10\,000\text{ g}$ for 10 min to sediment possible aggregated A $\beta(40)$. The soluble peptide fractions of the two tubes were then combined and diluted to 2.3 mL , which was ready for degassing, pH checking and ITC measurements. The concentration was determined with Bradford assay (Pierce Biotechnology, Rockford, IL) and adjusted when fitting the ITC data. All buffer stock solutions were prepared at concentrations of 20 mM , with ionic strength adjusted to 160 mM by NaCl, titrated to desired pH at 37°C using 1 M NaOH or 1 M HCl, treated with Chelex100 overnight, and finally filtered through a $0.22\text{ }\mu\text{m}$ cellulose membrane to remove any biological contamination or large particulate matter. A Cu^{2+} stock solution was prepared in each buffer using $\text{CuCl}_2\cdot(\text{H}_2\text{O})_2$ with four equivalences of glycine in order to prevent the precipitation of $\text{Cu}(\text{OH})_2$ at physiological pH and with two equivalence NaOH in order to neutralize the protons released from glycine when it binds to Cu^{2+} .

Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were carried out on a Microcal VP-ITC (Northampton, MA) ultra-sensitive microcalorimeter. All solutions were degassed under vacuum at 37°C for 15 minutes prior to use. The reaction cell contained $\sim 60\text{ }\mu\text{M}$ peptide in buffer (20 mM , ionic strength as 160 mM , pH 7.4 at 37°C). The ligand solution, $\sim 700\text{ }\mu\text{M}$ CuCl_2 with 4 equivalences of glycine, contained in the injection syringe, was also diluted in a matching buffer solution identical to the reaction cell. Titrations were performed at 37°C , pH 7.4 or 6.5. The pH of each prepared solution was checked and adjusted, if necessary, after degassing at 37°C to minimize unwanted heats of dilution arising from buffer ionization. A control titration of glycine into peptide for each of the peptides showed that glycine was not interacting with any of the peptides to produce a detectable heat signal (data not shown). No visible precipitate or significant aggregation was observed at the end of each experiment.

ITC data analysis was performed using Igor Pro (Wavemetrics, Lake Oswego, OR) and Origin 7.0 (Northampton, MA) software. The binding constant K , enthalpy ΔH were determined by fitting ITC data using previously developed methods in which the competition of glycine to Cu^{2+} was explicitly taking into account.^{29,30}

Aggregation assay

The effects of Cu^{2+} on the aggregation of human A β (40), rat A β (40) and the R5G mutation on human A β (40), R5G(40) was examined following the procedures developed by Atwood et al.¹² In brief, ~ 2.5 μM peptide in 20 mM HEPES solution (pH6.5 or 7.4) was incubated with or without CuCl_2 at 37 °C for 30 minutes. The solution was subsequently subject to centrifugation at 10 000 g for 30 minutes to sediment the aggregates. The concentration of peptide in the supernatant was determined by the Micro Bradford Assay. Duplicate runs were performed for each condition and the deviations between runs are within 20%.

Circular dichroism spectroscopy (CD)

CD spectra were recorded on an Aviv model 202 CD spectrophotometer in the 760-400 nm range spectra with 2 nm sampling interval. A 1-cm cell path was used. The samples were prepared at ~ 100 μM of peptide in water. The pH values of the solutions were adjusted to pH7.4 or 6.5 using 0.1 M NaOH or HCl.

Electron Paramagnetic Resonance (EPR)

All samples were prepared with degassed buffer containing 25 mM 3-(N-morpholino) propanesulfonic acid (MOPS) and 30% glycerol (v/v) where the glycerol served as a cryoprotectant. Samples were adjusted to pH = 7.4 with HCl and/or KOH. X-band spectra (frequency of 9.43GHz, microwave power in the range of 0.6–5.0 mW, modulation amplitude of 5.0G) were acquired at approximately 125K using a Bruker EleXsys 500 spectrometer and an SHQ (Bruker) cavity equipped with a variable temperature controller. X-band spectra were analyzed in the manner described by Peisach and Blumberg.³¹

RESULTS and DISCUSSIONS

Cu^{2+} binding to A β (40) and A β (16)

The Cu^{2+} binding sites are within the hydrophilic N-terminal, the first 16 amino acids of A β (40),^{24,27} and so A β (16) is commonly used as model system for studying metal binding to the peptide. We first compare the Cu^{2+} binding thermodynamics for the 40- and 16- length peptides. Figure 1 shows the isotherms of the titration of a) human A β (40), b) human A β (16), c) rat A β (40) and d) rat A β (16) by Cu^{2+} (complexed with glycine) in PIPES buffer at pH 7.4, 37 °C and 0.16 M ionic strength. The binding constants and enthalpies determined from these data are listed in Table 2.

The numbers of protons exchanged (Table 3) are determined from the binding enthalpies of Cu^{2+} to the peptides in HEPES, TEEN, and Chol buffer solutions under identical experimental conditions. The enthalpy, ΔH , was determined by fitting ITC data using previously developed methods in which the competition of glycine binding to Cu^{2+} is explicitly taken into account.^{29,30} This tabulated binding enthalpies are therefore only sensitive to the nature of the binding site(s) and the proton exchanged between peptide and buffer upon the association of Cu^{2+} to the peptide. For the overall reaction

the enthalpy determined by ITC is given by

$$\Delta H = \Delta H_{\text{Cu-A}\beta} - n_{\text{H}^+} \Delta H_{\text{buff}}$$

where $\Delta H_{\text{Cu-A}\beta}$ is the enthalpy for Cu^{2+} binding to A β as

and ΔH_{buff} is the enthalpy of buffer ionization for the reaction

Thus n_{H^+} can be determined from the slope of a plot of ΔH vs. ΔH_{buff} (Figure 2).

As shown in Table 3, A $\beta(40)$ and A $\beta(16)$ of both human and rat species have the same binding enthalpies and numbers of protons exchanged, indicating the intermediate coordinating spheres are the same for the two peptides. This result is consistent with previous EPR, XANES studies.^{24,27} The binding constant to A $\beta(40)$ is a factor of ~2 smaller than that for A $\beta(16)$ for both human and rat peptides. Because the enthalpy of binding is the same, this must reflect entropic effects, and is consistent with the difference conformational changes of A $\beta(40)$ and A $\beta(16)$ upon Cu^{2+} binding reflected in the reported FTIR data.²⁴ In summary, the thermodynamic and spectroscopic data indicate a common coordination sphere for Cu^{2+} binding to A $\beta(40)$ and A $\beta(16)$.

Cu^{2+} binding to mutants of human A $\beta(16)$

Mutant peptides were prepared to examine the roles of His6, His13, His14, Tyr10 and the N-terminus amine in Cu^{2+} binding to the human A $\beta(16)$ peptide. Table 4 lists the binding constants, enthalpies and numbers of protons exchanged of Cu^{2+} binding to the peptides. (Table S1 provides the binding enthalpies in the four buffers used). Figure 3 shows two examples of the titration curves and associated fits for the H6A and Ac-N peptides in PIPES buffer. The mutation of His6, His13, His14 or the modification of N-terminus amine all have effects on Cu^{2+} binding of human A $\beta(16)$ (Table 4 and S1). Y10F, on the other hand, has few effects the binding enthalpies and results in a small decrease of binding constants.

Compared to the natural peptide, acylation of the N-terminus amine of human A $\beta(16)$ (Ac-N) results in a ~30-fold decrease in Cu^{2+} binding affinity (Table 4 and Figure 3), clearly indicating the involvement of N-terminus amine for the Cu^{2+} coordination. Single point mutations of each of the three histidine residues by Ala (HxA, x = 6, 13, 14) also result in reduced binding affinity of the peptide for Cu^{2+} , and changes in the associated binding enthalpy. H6A results in the largest decrease of binding constants of the three HxA mutants, exhibiting a ~3-fold decrease in Cu^{2+} binding affinity ($1.0 \times 10^9 \text{ M}^{-1}$, Table 4). This is consistent with a recent report on the binding of Cu^{2+} to the same three mutants of A $\beta(28)$.³² The thermodynamic data clearly suggest that N-terminus amine, His6, His13 and His14 are all involved in coordinating Cu^{2+} ions and thereby 4N contribute to the coordination. To achieve Cu^{2+} coordination sphere with the established 3N1O coordination sphere at physiological pH,^{4,5,9,19,21,22,24–27} there must have two or more 3N1O binding spheres present in equilibrium, consistent with the observed two types of Cu^{2+} EPR signals.^{4,5,9,19,32}

The significant change by the modification of N-terminus amine clearly indicates the N-terminus amine is bound to Cu^{2+} in the native peptide. Because of the significant difference in Gibbs energies between Cu^{2+} binding to the natural peptide and the Ac-N mutant, we conclude that all 3N1O binding geometries present share the feature that the N-terminus amine group is one of the N ligands. This conclusion is also supported by previous findings that the spectroscopic properties (UV-Vis, CD, and EPR) of the complex of Cu^{2+} and N-terminus acetylated peptides differ significantly from that of unmodified peptide.^{4,5} Because the similar binding enthalpies and numbers of proton released for Cu^{2+} coordination to Y10F and the human native peptide, Tyr10 can be ruled out as a potential ligand. This is in agreement with previous spectroscopic studies.^{4,5,23,27} However, the current work does not provide any insight into the identity of the O-ligand, and so cannot distinguish between the proposed COO^- of Asp1^{4,9,19,23,28} and the C=O of Ala2.^{4,25} Thus we focus on identify the remaining 2N of the 3N1O coordination.

Cu^{2+} coordinate to human A β (16) is equilibrated between two types of coordination, Hx and HxHy

The numbers of protons released upon Cu^{2+} binding to the peptide(s) provide insight into the coordinating ligands. The potential O-ligands don't have any ionizable protons at pH 7.4 and 37 °C so that part of the coordination shell does not contribute to n_{H^+} . The binding of Cu^{2+} to N-terminus amine and the imidazole ring of histidine releases ~0.6 and ~0.2 protons at pH 7.4 and 37 °C, respectively (the pK_a data used to determine these values is provided in supplemental materials). So a 3N coordination sphere comprised of the N-terminus and two imidazole nitrogens would result in $n_{\text{H}^+}=1.0$, significant less than that observed (1.5) for the binding of Cu^{2+} to human A β (16). To obtain $n_{\text{H}^+}=1.5$ protons and have an overall 3N1O binding geometry for human A β (16), Cu^{2+} coordination must therefore involve other residues that release proton(s).

Consider involvement of an amide N, which has been invoked for other protein and peptides.^{33–38} The binding of Cu^{2+} to an amide N will release of 1.0 proton at pH 7.4, 37 °C. If the 3N in the Cu^{2+} coordination sphere were assigned to the N-terminus amine, imidazole and amide N, then we would predict $n_{\text{H}^+}=1.8$, which is greater than that observed experimentally for human A β (16). These two cases, however, represent the only possible 3N coordinations, and so we conclude that there must be equilibrium between these two structures: $\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^x, \text{N}^-\}$ (denoted as **Hx**), where N_{Im} and N^- are the imidazole nitrogen of one of the three histidines, His x ($x=6, 13$ or 14), and an amide nitrogen from the peptide backbone, respectively; and $\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^x, \text{N}_{\text{Im}}^y\}$ (denoted as **HxHy**) where $\text{N}_{\text{Im}}^x, \text{N}_{\text{Im}}^y$ are from two of the three histidines. Because coordination of Cu^{2+} gives $n_{\text{H}^+}=1.8$ for **H6**, **H13** or **H14**, and $n_{\text{H}^+}=1.0$ for **H6H13**, **H6H14** and **H13H14**, the experimental range observed (1.5 to 1.9) for the peptides studied reflects differences in the equilibria between these possible coordination spheres. The reaction enthalpy in PIPES buffer at 37 °C of **HxHy** is ~ 8–9 kcal/mol more negative than that for **Hx** at pH 7.4 and is ~ 6 kcal/mol more negative than that for **Hx** at pH 6.5(Supplementary Materials). This difference in binding enthalpies together with determined values for n_{H^+} serve as constraints for determining the equilibrium populations of different coordination spheres.

The isotherms (Figures 1 and 2) reported herein have the characteristic shape of a single binding site, and so the multiple coordination spheres present are in fast equilibrium. The relative proportions of these different coordination spheres can then be determined as follows. The binding constant determined by ITC (K) is an apparent binding constant represented by the following sum of the possible complexes present:

$$K = (\sum_i [Cu^{2+} - A\beta]_i) / ([Cu^{2+}] \times [A\beta]) = \sum_i K_i \quad (1)$$

where “i” refers to the contributing coordination sphere (e.g., **H6**, **H6H14** etc.) and K_i is its binding constant. Correspondingly, the enthalpy ΔH and the number of protons n_{H^+} released are also averaged and can be expressed by

$$\Delta H = \sum_i \frac{K_i}{\sum K_i} \Delta H_i, \quad n_{H^+} = \sum_i \frac{K_i}{\sum K_i} (n_{H^+})_i. \quad (2)$$

where H_i and $(n_{H^+})_i$ are the enthalpy of and the number of protons released for the i^{th} coordination sphere, respectively. With these equations, we can thereby relate the properties of individual coordinations to the measure data by ITC.

We note that the HxA or HxAHyA mutants and human A β exhibit similar Cu^{2+} binding behavior. The EPR spectra of H13A and H14A contained two components and are similar to those of human A β (16).^{4,9,32} We find the EPR spectra of H6AH13A, H6AH14A and H13AH14A are characterized by $A_{||} \sim 160\text{--}163$ G and $g_{||} \sim 2.21\text{--}2.2$ (Table 5), which are also in the range reported for the component II (prevalent at high pH) in human A β .^{4,5,9,19} In addition, we found that (1) the two human mutants H13A and H13R have same binding properties, (2) rat H14A has same binding properties as rat A β (16) indicating His6 is the dominant coordinating histidine and (3) human mutant H6AH13A and rat H6A (R5G H6A Y10F H13R compared to human sequence) have same binding properties. These data suggest that if the mutated amino acid is not a coordinating ligand itself, or not immediately adjacent to a coordinating amide bonds, negligible effects on the binding thermodynamics are observed. We therefore conclude that HxA or HxAHyA and human A β (16) all possess a common 3N1O coordination sphere. With this knowledge, we are able to determine the equilibrium between different potential Cu^{2+} coordination through analysis of the thermodynamic parameters of the set of A β (16) mutants.

The detailed analysis using equations 1 and 2, the estimated number of protons released and binding enthalpy for **HxHy**, to determine the equilibrium concentrations of contributing coordination spheres is presented in supplemental materials. In brief, the thermodynamics of Cu^{2+} coordinated in the **H6**, **H13** or **H14** geometries are determined using the mutants H13AH14A, H6AH14A and H6AH13A, respectively. For these three double histidine mutants, only one histidine is present in the peptide. The thermodynamics of Cu^{2+} coordinated in the **H6H13**, **H6H14** or **H13H14** geometries are determined using the mutants H14A, H13A and H6A, respectively. In these single histidine mutants, however there are also potential contributions from **Hx** as well as **HxHy**.

Table 6 presents the resulting thermodynamic parameters (K , ΔH , n_{H^+}) associated with each of the possible contributing coordination sphere at pH7.4. There are several interesting observations to be made about the data in Table 6. First, although coordinations **H13** and **H14** are more enthalpy-favored (more negative binding enthalpies) than **H6**, the Cu^{2+} binding constant for **H6** is about three times higher than those for **H13** and **H14**. **H6** is more entropy-favored, which is reasonable because His6 is closer to the N-terminus and therefore requires less conformational re-arrangements of the peptide to form the coordination sphere of Cu^{2+} relative to His13 of His14. Second, the calculated binding enthalpies for **H6H13**, **H6H14** and **H13H14** are similar (-9.4 – -12.6 kcal/mol) and in good agreement with our estimated enthalpies, -10 – -12 kcal/mol for **HxHy** coordinations. Third, the numbers of

protons exchanged of **H6H13**, **H6H14** and **H13H14** are 1.1–1.2, which is also of good agreement of our estimations ~ 1.1 –1.2 proton released for the formation of **HxHy**.

CD spectra of H13AH14A, H6AH14A, H6AH13A H6A, H13A, H14A and human A β (16) with the presence of Cu^{2+} support the above conclusions (Figure 4). The CD band for H13AH14A is centered at 590 nm, which is consistent with previously reported 588–590 nm for the coordinations $\{\text{NH}_2, \text{CO}, \text{N}^-, \text{N}_{\text{Im}}^{\text{His6}}\}$ (**H6**) in human A β (1–6) or A β (1–10) where only one histidine (His6) is present.¹⁹ The CD bands for H6AH14A and H6AH13A are the same and centered at ~ 610 nm, which is consistent with the similar thermodynamic properties observed for the two peptides. The small red shift (20 nm) of the CD band and lack of the positive signals at 650–700 nm of H6AH13A or H6AH14A compared to that of H13AH14A is likely due to the different positions of the histidines in the sequences, resulting in different chelating loops around the coordinated Cu^{2+} . The spectra of H13A and H14A are similar and resemble that of H13AH14A (**H6** coordination), but differ from that of H6AH13A (**H14** coordination) or H6AH14A (**H14** coordination), confirming our earlier conclusion of the ubiquitous involvement of His6 in Cu^{2+} binding and that **H6** is the only **Hx** coordination in H13A or H14A. The CD band of human A β (16) also resembles that of H13AH14A, indicating the **H6** is the only **Hx** coordination in human A β (16).

Given that His6 is involved in all coordination spheres present in the equilibrium mixture, the possible contributing species are restricted to **H6**, **H6H13**, and **H6H14**. Using the thermodynamic parameters derived for each of these structures (Table 6), the apparent binding constant for the human peptide is calculated to be $(1.6+1.0+0.5) \times 10^9 \text{ M}^{-1} = 3.1 \times 10^9 \text{ M}^{-1}$. Calculation using equation (2) results an apparent binding enthalpy of -6.1 kcal/mol and the number of protons released as 1.5. These calculated parameters are in an excellent agreement with the experimental results (K as $3.0 \times 10^9 \text{ M}^{-1}$, ΔH as -5.6 kcal/mol and n_{H^+} as 1.5). We therefore conclude there is an equilibrium between three coordination spheres for human A β (16): **H6**, **H6H13**, **H6H14**, present in relative amounts 0.5:0.3:0.2 at pH 7.4 according to the binding constants.

Now consider Cu^{2+} binding to human A β (16) at pH 6.5 (Table 7). At pH 6.5, the binding constant of **H6** coordination is $1.3 \times 10^7 \text{ M}^{-1}$, ~ 2 -orders of magnitudes smaller than that at pH 7.4. The binding constant at pH 6.5 can be calculated from the value at pH 7.4. Specifically, the value at pH 6.5 is that at pH 7.4 multiplied by the ratio of $(1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{N-terminus}} \times (1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{His6}} \times (1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{N}^-}$ at pH 6.5 and pH 7.4. Taking the pK_a of N-terminus amine, imidazole of His6, amide N to be 7.6, 6.8, and 12 (Supplementary Material), we find the binding constant at pH 6.5 be lower than that at pH 7.5 by a factor of ~ 100 , in excellent agreements of the experimental results. The binding enthalpy of **H6** coordination is 1.0 kcal/mol, which is ~ 3 kcal/mol more than that at pH 7.4. This is expected because there is more proton dissociation from N-terminus, and Imidazole upon the formation of **H6** coordination at pH 6.5.

Carrying out the same calculations as we did for mutants at pH 7.4, the binding constant and enthalpy at pH 6.5 are $6.7 \times 10^7 \text{ M}^{-1}$ and -3.9 kcal/mol for coordination **H6H13** and $3.8 \times 10^7 \text{ M}^{-1}$ and -3.3 kcal/mol for coordination **H6H14**. The binding enthalpies are close to our estimations for the binding enthalpy of **HxHy** coordinations as -5 kcal/mol (Supplementary materials). Using the proportionality factor, the ratio of $((1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{N-terminus}} \times (1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{His6}} \times (1/(1+10^{(\text{pK}_a-\text{pH})}))_{\text{His13/His14}})$ of pH 6.5 and pH 7.4, the binding constants of **H6H13** and **H6H14** at pH 6.5 are $\sim 1/15$ of that at pH 7.4, which are also in excellent agreement with the experimental data. Moreover, **H6**, which releases more protons than **H6H14** and **H6H13**, should become less dominant with decreasing solution pH. Consistent with this prediction, the binding constants of peptides studied at pH 6.5 indicate that **H6** accounts for $\sim 10\%$ of the Cu^{2+} coordinations of human A β (16), smaller than that at

pH 7.4 (50%). In summary, the collective set of ITC data, CD data and EPR data of human A β (16) and its mutants establishes that the binding of Cu^{2+} to the human A β (16) peptide involves three distinctive binding coordination spheres present in equilibrium: **H6**, **H6H13** and **H6H14** over the pH range we studied (6.5–7.4) (Scheme 1a). The relative concentrations for **H6:H6H13:H6H14** are 0.5:0.3:0.2 and 0.1:0.6:0.3 at pH 7.4 and 6.5, respectively.

We now consider this result in the context of previous works. Previous ESR studies resulted in the conclusion that there are two types of binding structures, with one dominate at pH <7 and the other dominate at pH >10.⁵ The binding mode $\{\text{NH}_2, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$ was proposed be dominant at neutral pH and the possible involvement of amide NH was suggested for mode at higher pH. Recently the same group suggested coordination spheres $\{\text{NH}_2, \text{COO}^-, \text{COO}^-, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$, involving all three histidines (His6, His13, and His14) and two O-ligand and N-terminus, for both structures, with N-terminal amino group as axial ligand at pH 7.4 but in plane at pH 9.0.³² These two modes release the same numbers of protons (~1.4 protons), and therefore must have same pH-dependence. However, the EPR spectra reported vary with pH, suggestion that this assignment is unlikely.

Two recent related EPR studies identify two types of binding structures for Cu^{2+} association with A β (16): component I, $\{\text{NH}_2, \text{O}^- (\text{Asp1}), \text{N}_{\text{Im}}^6, \text{and } \text{N}_{\text{Im}}^{13} (\text{component Ia}) \text{ or } \text{N}_{\text{Im}}^{14} (\text{component Ib})\}$ and component II, $\{\text{C=O}, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$.^{9,25} Our proposed coordinations **H6H13** and **H6H14** are consistent with the assignment of component I. For the assignment of component II, however, as mentioned by the authors, ¹⁵N labeling His6 actually labels both the imidazole ring and the amide N, so that CW-EPR does not provide direct evidence concerning the involvement of an amide N in the coordination sphere of the bound Cu^{2+} . The later assignment is then based on the fact that the relative intensities of ¹⁵N_τ^{His6}, ¹⁵N_τ^{His13}, and ¹⁵N_τ^{His14} are the same at pH 8.0 in HYSCORE spectra. However, it is noticeable that there is considerable amount (~50%) of Component I at pH 8.0 according to the CW-EPR data at the same conditions. Thus, if component II is $\{\text{CO}, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$, the relative intensity of ¹⁵N_τ^{His6} should actually be larger than that of ¹⁵N_τ^{His13}, or ¹⁵N_τ^{His14} considering the increased contribution of His6 in component I. Their data show different intensity of the non-coordinating ¹⁴N_{am} at pH8 for the ¹⁵N-His6, ¹⁵N-His13 or ¹⁵N-His14 labeled A β (16). On the contrary, the intensity of the noncoordinating ¹⁴N_{am} of unlabelled A β (16) and ¹⁵N-His6,13,14 labeled A β (16) are the same. This inconsistency is likely due to different normalization. Thus, the assignment of component II needs further confirmation. We also note that for the proposed component II, the binding of Cu^{2+} to the peptide would release fewer protons compared to component I (at pH < 8). Thus, with increasing pH, component I would become dominant, but not component II as suggested by the work. Moreover, the EPR parameters of component II are close to the values for a CuL species of human A β (16) that releases ~ 2 protons upon its formation⁴ or that obtained for mutants H6AH13A, H6AH14A or H13AH14A of human A β (16) in this current work. These observations indicate that component II is indeed **Hx** coordination geometry. We suggest that although extremely low temperature could possibly shift the equilibrium, it is likely that **H6** is the component II detected in the EPR spectra at pH 8.⁹ With this information, the earlier study actually reveals the same three coordination spheres as concluded herein.

Potentiometric studies suggested that Cu^{2+} bound with human A β (16 or 28) are almost equally distributed between two types of binding geometries at physiological pH (7.4), CuHL assigned as $\{\text{NH}_2, \text{COO}^-, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$ and CuL assigned as $\{\text{NH}_2, \text{CO}, \text{N}^-, \text{N}_{\text{Im}}\}$ (for the latter geometry, His6 was proposed to dominate as the origin of N_{Im}).^{4,19} Our data indicated **H6** accounts for 50% of the bound Cu^{2+} , with **H6H13** and **H6H14** accounting for the other 50%. This observation is consistent with potentiometric data except that the

conclusion that two N_{Im} of CuHL complex are from His13 and His14.⁴ However, no specific evidence was provided to support this assignment in that work. This assignment was likely based on the fact that the coordination is only present in A β (16) but not present in A β (1–6) or A β (1–10) where His13 and His14 are absent. Our ITC data shows that the binding constant of **H13H14** is $\sim 0.4 \times 10^9 \text{ M}^{-1}$, only $\sim 10\%$ of that for human A β (16). Thus if it is the $\{\text{NH}_2, \text{O}, N_{Im}, N_{Im}\}$ component, this mode can only possess $\sim 10\%$ of the bound Cu^{2+} , which is much lower than the detected 50% and excludes the assignment.

Cu²⁺ binding to rat A β (16)

The rat A β wildtype sequence possesses an Arg at position 13 and thus cannot coordinate here through His as observed in the human sequence. Thus by taking an analog to human A β (16), there are only two possible coordination spheres, **H6** and **H6H14**. Table 8 list the binding constants, enthalpies and numbers of protons associated of Cu^{2+} binding to the mutant of rat A β (16): rat H6A and rat H14A at pH 7.4. Rat A β (16) and rat H14A mutant exhibit nearly identical Cu^{2+} binding parameters at pH 7.4. The Cu^{2+} binding properties of rat H6A, where only His14 is present, is similar to the human H6AH13A or H6AH14A mutant, suggested Y10F, R5G and H13R have negligible effects on the **H14** or **H13** coordination sphere. The CD spectra of rat A β (16) loaded with Cu^{2+} is also similar to that of rat H14A mutants, while the CD of rat H6A mutant is similar to that of human H6AH13A and H6AH14A (Figure 5). The similar Cu^{2+} binding thermodynamics and spectroscopic property of rat A β (16) and rat H14A clearly establish His6 as the dominant histidine bound to Cu^{2+} . Binding enthalpy and n_{H^+} for Cu^{2+} to rat A β (16) or rat H14A at pH 7.4, 37 °C are -4.0 kcal/mol and 1.8, respectively. The n_{H^+} is the same as we estimated for **Hx** coordination. The enthalpy is close to the observed binding enthalpies to **Hx** in human sequence as $-2 - -3 \text{ kcal/mole}$. Thus we conclude that **H6** is the dominant coordination for both rat A β (16) and rat H14A at pH 7.4.

If we assume that the three mutations of rat sequence (R5G, Y10F and H13R) have little effect on **H6H14**, which is reasonable because the amide N of His6 (next to Arg5) or His14 (next to His13) is not involved in binding for this coordination, the Cu^{2+} binding constant of rat **H6H14** is close to $0.6 \times 10^9 \text{ M}^{-1}$, the value for human sequence. This value however is on the order of the error in our measurements of the binding constant ($\sim 10 \times 10^9 \pm 10^9 \text{ M}^{-1}$), and so we cannot unambiguously determine whether **H6H14** contributes to Cu^{2+} binding in the rat peptide at pH 7.4. But if **H6H14** is present, it represents only $\sim 6\%$ of the total concentration. Thus in the rat peptide at pH 7.4 and 37 °C, **H6** is the dominant coordination sphere ($\sim 94\%$) with a potential minor contribution from **H6H14** ($\sim 6\%$) (Scheme 1b).

The binding constants, enthalpies and numbers of protons associated of Cu^{2+} binding to rat A β (16) and rat H14A at pH 6.5 in PIPES buffer are determined and listed in Table 9. The Cu^{2+} binding constants of rat H14A (rat **H6**) at pH 6.5 are ~ 2 -orders of magnitudes smaller than that at pH 7.4. As previously discussed, the binding constant of **Hx** at pH 6.5 is $\sim 1/100$ of that at pH 7.4. The experimental data is in excellent agreement of this calculation. Rat A β (16) has a slightly larger binding constant than that of rat H14A, but the difference ($2 \times 10^7 \text{ M}^{-1}$) is on the order of the error in determining binding constants over $1 \times 10^8 \text{ M}^{-1}$. But different from the data at pH 7.4, rat H14A has less negative binding enthalpies (-0.1 kcal/mol) than rat A β (16) (-0.8 kcal/mol), which clearly indicates a possible contribution of **H6H14** in rat A β (16). Because the difference between rat A β (16) and rat H14A ($2 \times 10^7 \text{ M}^{-1}$) is about the level of error for determining binding constants over $1 \times 10^8 \text{ M}^{-1}$, there may exist pretty large error in determine the binding constant of rat H6H14 this way. The binding constant of **H6H14** of human A β (16) at pH 6.5, is $\sim 3.8 \times 10^7 \text{ M}^{-1}$, which is close to the observed difference between rat A β (16) and rat H14A. Thus we suggest the binding constant of **H6H14** in rat A β (16) in the range of $(2-4) \times 10^7 \text{ M}^{-1}$. The binding enthalpy for this mode is estimated to be $-4 - -2.3 \text{ kcal/mol}$, which is close to the value for **H6H14** in

human A β (16) as -3.3 kcal/mol and also in good agreement of the estimated binding enthalpy for **HxHy** as ~ -5 kcal/mol. These analyses support that the presence of **H6H14** (20–30% according to binding constants) contributes to the more negative binding enthalpies in rat A β (16) as compared to that of rat H14A at pH 6.5.

The proposed coordination for rat A β (16) at pH 7.4 is consistent with earlier work, which showed that Cu^{2+} bound with rat A β (16) is dominantly in form CuL with only one histidine involved as $\{\text{NH}_2, \text{COO}^-, \text{N}^-, \text{N}_{\text{Im}}\}$ (**H6** coordination).^{4,19} But it differs from a recent study suggesting a coordination as $\{\text{NH}_2, 2\text{N}_{\text{Im}}\}$ with both His6 and His14 of rat A β (28) serving as anchoring sites for Cu^{2+} .³⁹ However, to mitigate the aggregation of the peptide, those experiments were conducted in micellar solutions with sodium dodecyl sulfate (SDS). SDS induces the peptide to form an α -helix, and thus in the presence of the surfactant, the peptides is in a different configuration.³⁹ The energetics of Cu^{2+} binding, therefore, depends on whether SDS is present in solution and it is not surprising to obtain different binding modes when SDS is present.

Comparison of Cu^{2+} binding to human and rat A β

The thermodynamic and spectroscopic analyses establish that there three coordination spheres, **H6**, **H6H13**, and **H6H14**, in human A β (16) and two, **H6** and **H6H14** in rat A β (16) (Scheme 1). The thermodynamic properties for Cu^{2+} to **H6H14** is similar for human and rat A β (16), while the properties for Cu^{2+} to **H6** is quite different for the two peptides. Rat **H6** exhibits approximately six times the affinity for Cu^{2+} as the human **H6** and releases less heat. This must be related to the amino acid difference between the two species. As mentioned in introduction, the rat and human peptides differ by three amino acids – R5G, Y10F and H13R. In order to determine whether a single mutation causes the different Cu^{2+} binding thermodynamics between rat and human A β peptides, we examined peptides containing each of the three individual mutations.

The binding constants, enthalpies and numbers of protons exchanged of Cu^{2+} binding to those mutants at pH 7.4 are listed in Table 10. The mutations Y10F and H13R result in a decreased Cu^{2+} binding constant. For Y10F the enthalpies of binding are similar to that of the native human peptide in the four buffers we studied. The reduction in Cu^{2+} binding constant by H13R is similar to that observed for H13A. On the other hand, R5G mutant exhibits the same binding constant, binding enthalpy, and numbers of protons released as the native rat peptide. The mutation R5G reproduces quantitatively the difference in Cu^{2+} binding thermodynamics between human and rat A β (16) at pH 7.4. This conclusion is further confirmed by CD studies. Figure 5 showed visible CD spectra of human A β (16), rat A β (16) and R5G in the presence of CuCl_2 at pH 7.4. The d-d transition band of human A β (16) is centered at 570 nm, ~ 100 nm shorter than that of rat A β (16) (670 nm). These data are consistent with previous reported CD spectra of the two peptides.⁴ CD spectrum of R5G mutant resembles with that of rat A β (16), supporting that R5G is responsible for the coordination difference between human and rat **H6**. It has been suggested that human and rat **H6** coordinations contain the amide of Ala2 and His6, respectively.^{4,19} The decreased bulkiness and positive charges that results from the R5G mutation enables formation of a stable six-member chelation ring (by His6, amide N and Cu^{2+}), which can account for the increased binding affinity and lower binding enthalpies observed for rat **H6** as compared to human **H6**.

Cu^{2+} - induced peptide aggregation

It is interesting to consider the ramifications of the Cu^{2+} coordination spheres within the context of amyloid aggregation. Figure 6 showed the extend of Cu^{2+} -induced aggregations of human A β (40), rat A β (40), and single mutation on human A β (40): R5G(40) at pH 7.4

and 6.5 after incubation at 37 °C for 0.5 hour. The presence of Cu²⁺ significantly increases the aggregation of human A β (40), but this effect is much less profound for rat A β (40). The Cu²⁺ induces aggregations of human A β (40) are pH-dependent, more profound at pH 6.5 than pH 7.4. These results on human and rat A β (40) at pH 7.4 and 6.5 are consistent with the reported data.¹² The R5G mutation reduced Cu²⁺-induced aggregations to the same levels as rat A β (40). For the experimental conditions of this assay (~ 20 μ M Cu²⁺ and 2.5 μ M A β (40)), more than 99.9% of all three peptides studied are loaded with Cu²⁺, since their Cu²⁺ binding constants are 10⁹–10⁸ M⁻¹ at pH 7.4–6.5. Combining the fact that R5G(40) shares the same coordinations as rat A β (40) and has the same level of Cu²⁺ induced aggregation as rat A β (40), we conclude it is the difference between binding sites rather than the amount of bound Cu²⁺ between the rat and human peptide influence the aggregation process.

By examining the pH-dependence of coordinations and pH-dependence of the Cu²⁺-induced aggregation, we observed an interesting positive correlation between the proportions of coordination **H6H13**, **H6H14** and the degree of aggregations. For example, in the rat peptide, **H6** is the dominant coordination sphere (~94%) with a possible minor contribution from **H6H14** at pH 7.4. In the human peptide, there are three Cu²⁺ coordinations in equilibrium: **H6** (50%), **H6H13** (30%), and **H6H14** (20%) at pH 7.4. Human A β (16) formed more aggregates than rat A β (16) at pH 7.4. When the solution pH is changed from 7.4 to 6.5, increased percentage of bound Cu²⁺ is in the form of **H6H13** and **H16H14** in human A β (16), and in the form of **H6H14** in rat A β (16). Accordingly, we observed increased Cu²⁺-induced aggregation at pH 6.5. The correlation indicates that the formation of **H6H13** or **H6H14** induces more aggregation than **H6**.

Compared to **H6**, the formation of **H6H13** and **H6H14** coordination spheres requires greater conformational rearrangements, which is reflected by their larger negative binding enthalpies but smaller binding constants. The conformational change resulted by the formation of **H6H13** or **H6H14** must play an important role in the Cu²⁺ induce aggregation of A β . Based on the aggregation assay on human A β (40), rat A β (40), and R5G(40), we conclude that the equilibrium between Cu²⁺ coordinations can greatly affect Cu²⁺-induced A β aggregation and that the conformational changes that accompany the formation of **H6H13** and **H6H14** coordination induce more or accelerate more of the Cu²⁺-initiated aggregation of the A β peptide than that accompany **H6**.

CONCLUSIONS

CD, EPR, and ITC studies on the coordination for Cu²⁺ to human and rat A β reveal that at pH 7.4, 37 °C and ionic strength as 0.16 M there are three Cu²⁺ coordination spheres for human A β (16) and one dominant coordination sphere for rat A β (16). The proportion of these coordination spheres varies with solution pH. Human and rat A β differ in three amino acids (R5G, Y10F and H13R) and R5G is responsible for the difference of Cu²⁺ coordinations between the two species. In addition, this single mutation of human A β (40) reduces Cu²⁺-induced aggregation of the peptide to the level of that for rat A β (40).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

A β	amyloid beta peptide
AD	Alzheimer's disease
ITC	isothermal titration calorimetry
CD	circular dichroism
EPR	electron paramagnetic resonance
PIPES	1,4-Piperazinediethanesulfonic acid
HEPES	N-(2-hydroxyethyl)piperazine-N'(2-ethanesulfonic acid)
TEEN	N,N,N',N'-tetraethylethylenediamine
Chol	(2-aminoethyl)trimethylammonium chloride hydrochloride
His	histidine
Gly	glycine
Arg	arginine
H6	coordination {NH ₂ , O, N _{Im} ⁶ , N ⁻ }
H13	coordination {NH ₂ , O, N _{Im} ¹³ , N ⁻ }
H14	coordination {NH ₂ , O, N _{Im} ¹⁴ , N ⁻ }
H6H13	coordination {NH ₂ , O, N _{Im} ⁶ , N _{Im} ¹³ }
H6H14	coordination {NH ₂ , O, N _{Im} ⁶ , N _{Im} ¹⁴ }
H13H14	coordination {NH ₂ , O, N _{Im} ¹³ , N _{Im} ¹⁴ }

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Figure 1.

ITC binding isotherms for Cu^{2+} binding to a) human $\text{A}\beta(40)$, b) $\text{A}\beta(16)$, c) rat $\text{A}\beta(40)$ and rat $\text{A}\beta(16)$ peptides. The top panels show the differential power signal measured for each injection throughout the experiment, and the bottom panels show the integrated peak areas corresponding to the measured heat released per injection. The theoretical fit is superimposed on the data, and the resulting parameters are listed in Table 2.

Figure 2.

The enthalpies resolved from ITC data, ΔH , are plotted as a function of the buffer ionization enthalpies, ΔH_{buff} , associated with the binding of Cu^{2+} to human A $\beta(16)$ in PIPES, HEPES, TEEN, and Chol. The slope of the best fit line through the experimental data is -1.5, meaning a net loss of 1.5 protons upon Cu^{2+} binding to the peptide.

Figure 3. ITC binding isotherms for Cu^{2+} binding to a) H6A and b) Ac-N mutated A β (16) peptides. The top panels show the differential power signal measured for each injection throughout the experiment, and the bottom panels show the integrated peak areas corresponding to the measured heat released per injection. The theoretical fit is superimposed on the data, and the resulting parameters are listed in Table 4.

Figure 4. CD spectra of human $A\beta(16)$ and its mutants (~ 100 μM) with the presence of excess amount of Cu^{2+} in water at pH7.4.

Figure 5. CD spectra of rat $A\beta(16)$ and its mutants (~ 100 μ M) with the presence of excess amount of Cu^{2+} in water at pH7.4.

Figure 6. Cu^{2+} -induced aggregations of human A β (40), rat A β (40), and R5G(40) in HEPES buffer at pH 7.4 and 6.5 after incubation at 37 °C for 0.5 hour.

Scheme 1.

Proposed Cu^{2+} coordinations for a) human and b) rat A β (16) at pH 7.4, 37 °C and ionic strength as 0.16 M. NH_2 represents the N-terminus amine. O represents the O-ligand, which was proposed as COO^- of Asp1 for human **H6H13** and **H6H14** and C=O of Ala2 for human **H6**. N represents amide N, which was proposed as from Ala2 for human **H6** and His6 from rat **H6**. In general **H6** only involves the first 6 residues of A β , while **H6H13** and **H6H14** involve the first 13 or 14 residues of A β .

Table 1

The primary structures of A β peptide and its mutants that were studied.

Peptide	Sequence
Human A β (40)	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Human A β (16)	DAEFRHDSGYEVHHQK
Ac-N	<u>Ac</u> -DAEFRHDSGYEVHHQK
H6A	DAEFR <u>A</u> DSGYEVHHQK
H13A	DAEFRHDSGYEV <u>A</u> HQK
H14A	DAEFRHDSGYEVH <u>A</u> QK
H6AH13A	DAEFR <u>A</u> DSGYEV <u>A</u> HQK
H6AH14A	DAEFR <u>A</u> DSGYEVH <u>A</u> QK
H13AH14A	DAEFRHDSGYEV <u>AA</u> QK
Rat A β (40) (R5G Y10F H13R)*	DAEFGHDSG <u>F</u> EV <u>R</u> HQKLVFFAEDVGSNKGAIIGLMVGGVV
R5G(40)	DAEFGHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV
Rat A β (16) (R5G Y10F H13R)*	DAEFGHDSG <u>F</u> EV <u>R</u> HQK
R5G	DAEFGHDSGYEVHHQK
Y10F	DAEFRHDSG <u>F</u> EVHHQK
H13R	DAEFRHDSGYEV <u>R</u> HQK
Rat H6A (R5G H6A Y10F H13R)*	DAEFG <u>A</u> DSG <u>F</u> EV <u>R</u> HQK
Rat H14A (R5G Y10F H13R H14A)*	DAEFGHDSG <u>F</u> EV <u>RA</u> QK

* Listed in the parenthesis are the mutations compared to human sequence.

Table 2

Binding constants (K) and enthalpies (ΔH) for the addition of Cu^{2+} to human A β and rat A β in PIPES buffer at 37 °C, pH 7.4 with ionic strength adjusted as 0.16 M.

Peptide	K/ 10^9 M^{-1}	$\Delta H/\text{kcal/mol}$
Human A β (40)	1.9	-5.7
Human A β (16)	3.0	-5.6
Rat A β (40)	5.6	-4.0
Rat A β (16)	9.7	-3.9

Table 3

Binding enthalpies of human A β (40), A β (16), rat A β (40), A β (16) upon Cu²⁺ binding at 37 °C, pH 7.4 and ionic strength as 0.16 M in multiple buffers. Buffer ionization heats, ΔH_{buff} , are also listed (NIST). All values are reported as kcal/mol. The number of protons transferred, n_{H^+} (error $< \pm 0.1$) for the binding of Cu²⁺ to the peptides are determined from the slope of a plot of ΔH as a function of ΔH_{buff} (Figure 2).

Peptide	PIPES	HEPES	TEEN	Chol	n_{H^+}	R %
Human A β (16)	-5.6	-9.9	-11.7	-18.6	1.5	99.6
Human A β (40)	-5.7	-9.8	-11.5	-18.3	1.5	99.5
Rat A β (16)	-3.9	-8.5	-11.4	-19.6	1.8	99.9
Rat A β (40)	-4.0	-8.7	-11.4	-19.4	1.8	99.8
Buffer Ionization	3.0	5.4	7.4	11.5		

* The binding enthalpies for all other peptides studied in the four buffers can be found in Table S-1.

Table 4

Binding constants and enthalpies for the addition of Cu^{2+} to human A $\beta(16)$ -related mutants in PIPES buffer at 37 °C, pH 7.4 with ionic strength adjusted as 0.16 M.

Peptide	$K/10^9 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$	n_{H^+}
Human A $\beta(16)$	3.0 ± 0.2	-5.6 ± 0.2	1.5
H13A	2.1 ± 0.1	-4.2 ± 0.1	1.7
H14A	2.6 ± 0.1	-4.9 ± 0.1	1.6
H6A	1.0 ± 0.1	-5.6 ± 0.2	1.6
Y10F	2.7 ± 0.1	-6.0 ± 0.2	1.5
H13AH14A	1.6 ± 0.1	-1.7 ± 0.2	1.8
H6AH13A	0.6 ± 0.1	-3.1 ± 0.2	1.9
H6AH14A	0.6 ± 0.1	-3.2 ± 0.1	1.9
Ac-N	0.1	-2.6	-

Table 5EPR parameters determined for Cu²⁺-peptide complex at pH7.4.

Sample	A (G)	g
H6AH13A	162	2.22
H6AH14A	160	2.22
H13AH14A	162	2.21

Table 6

The binding parameters for the human coordination spheres at pH 7.4, 37 °C, and ionic strength as 0.16M. The thermodynamic parameters were determined as described in the supplementary materials.

Coordination	Coordination sphere	$K/10^9 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$	n_{H^+}
H6	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^6, \text{N}^-\}$	1.6	-1.7	1.8
H13	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^{13}, \text{N}^-\}$	0.6	-3.2	1.9
H14	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^{14}, \text{N}^-\}$	0.6	-3.1	1.9
H6H13	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{13}\}$	1.0	-10.0	1.1
H6H14	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^6, \text{N}_{\text{Im}}^{14}\}$	0.5	-12.2	1.1
H13H14	$\{\text{NH}_2, \text{O}, \text{N}_{\text{Im}}^{13}, \text{N}_{\text{Im}}^{14}\}$	0.4	-9.4	1.2

Table 7

Binding constants and enthalpies for the addition of Cu^{2+} to A $\beta(16)$ -related mutants in PIPES buffer at pH 6.5 and 37 °C with ionic strength as 0.16 M. The calculated parameters of the **H6H13** and **H6H14** complexes are also listed.

Peptide	K/ 10^7 M^{-1}	$\Delta\text{H}/\text{kcal/mol}$
Human A $\beta(16)$	13	−3.3
H13AH14A	1.6	0.4
H13A	5.4	−2.2
H14A	8.3	−3.1
Coordination	K/ 10^7 M^{-1}	$\Delta\text{H}/\text{kcal/mol}$
H6	1.3 [§]	1.0 [§]
H6H13	6.7	−3.9
H6H14	3.8	−3.3

[§]Due to the contribution of $\{\text{NH}_2, \text{COO}^-, \text{N}_{\text{Im}}\}$, the binding constant of **H6** is about 80% of that for H13AH14A and the enthalpy is about 0.6 kcal/mol more than H13AH14A. See supplementary materials for details.

Table 8

Binding constants and enthalpies for the addition of Cu^{2+} to rat H6A and rat H14A mutants in PIPES buffer at 37 °C, pH 7.4 with ionic strength adjusted as 0.16 M. To facilitate the comparison, data of rat A β (16) are also provided.

Peptide	$K/10^9 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$	n_{H^+}
Rat A β (16)	9.7 ± 0.3	-3.9 ± 0.2	1.8
Rat H6A	0.6 ± 0.1	-3.0 ± 0.1	1.8
Rat H14A	10.2 ± 0.5	-3.8 ± 0.1	1.8
Coordination	$K/10^9 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$	n_{H^+}
Rat H6	10.2	-3.8	1.8
Rat H14	0.6	-3.0	1.8

Table 9

Binding constants and enthalpies for the addition of Cu^{2+} to rat A β (16), rat H14A and rat H6A mutants in PIPES buffer at 37 °C, pH 6.5 with ionic strength adjusted as 0.16 M.

Peptide	$K/10^8 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$
Rat A β (16)	1.2 ± 0.2	-0.8 ± 0.1 *
Rat H6A	0.09	-0.9
Rat H14A	1.0 ± 0.1	-0.1 ± 0.1 *
Peptide	$K/10^8 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$
Rat H6	1.0	-0.1
Rat H14	0.09	-0.9
Rat H6H14	0.2–0.4	$-4.0 \sim -2.3$

* The deviations of the measured enthalpies between runs are less than 5% (0.1 kcal/mol vs. $-2.3 \sim -3$ kcal/mol). Since the dissociation enthalpy of $\text{Cu}(\text{Gly})_2$ and buffer ionization were taking account, the resolved Cu^{2+} binding enthalpy of rat H14A or rat A β (16) is ~ -0.1 or ~ -0.8 kcal/mol. The $\pm$ values in the table are thereby more representing the range of the enthalpy, but not the error of measurements.

Table 10

Binding constants and enthalpies for the addition of Cu^{2+} to mutants designed to study the effects of the amino acid different between human and rat A β (16) on Cu^{2+} binding. The experiments were conducted in PIPES buffer at 37 °C, pH 7.4 with ionic strength adjusted as 0.16 M. To facilitate the comparison, data of human and rat A β (16) are also provided.

Peptide	$K/10^9 \text{ M}^{-1}$	$\Delta H/\text{kcal/mol}$	n_{H^+}
Rat A β (16)	9.7 ± 0.3	-3.9 ± 0.2	1.8
Human A β (16)	3.0 ± 0.1	-5.6 ± 0.1	1.5
R5G	9.1 ± 0.7	-4.2 ± 0.1	1.8
Y10F	2.7 ± 0.1	-6.0 ± 0.2	1.5
H13R	2.1 ± 0.1	-4.1 ± 0.4	1.6