DOI: 10.1002/cbic.200700633

Different Hydration Changes Accompanying Copper and Zinc Binding to Amyloid β-Peptide: Water Contribution to Metal Binding

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The pathological hallmark of Alzheimer's disease (AD) is the accumulation of extracellular amyloid plaques.[1] The primary component of the plaques,[2] amyloid β-peptide (Aβ) is a metalloprotein. Both copper and zinc can bind to Aβ and the levels of copper and zinc in the amyloid plaques are heavily increased and considered related to Aβ toxicity.[3,4] Recent NMR[5] and EPR[6] studies on the full-length Aβ have suggested that copper and zinc binding to Aβ cause deprotonation. Theoretical calculations also show that copper binding results in release of water.[7] Therefore, it is important for deciphering the role of metal ions in AD to uncover the hydration changes[8,9] upon metal binding to Aβ. However, to our knowledge, there is no report to show how hydration changes occur upon copper and zinc binding to Aβ. Herein we report that both copper and zinc binding to Aβ cause dehydration and their hydration changes were different as studied by the osmotic stress method. Zinc binding causes water molecules to be released more than twice as much as copper binding and leads to even more destabilised and aggregation prone Aβ than following copper binding.

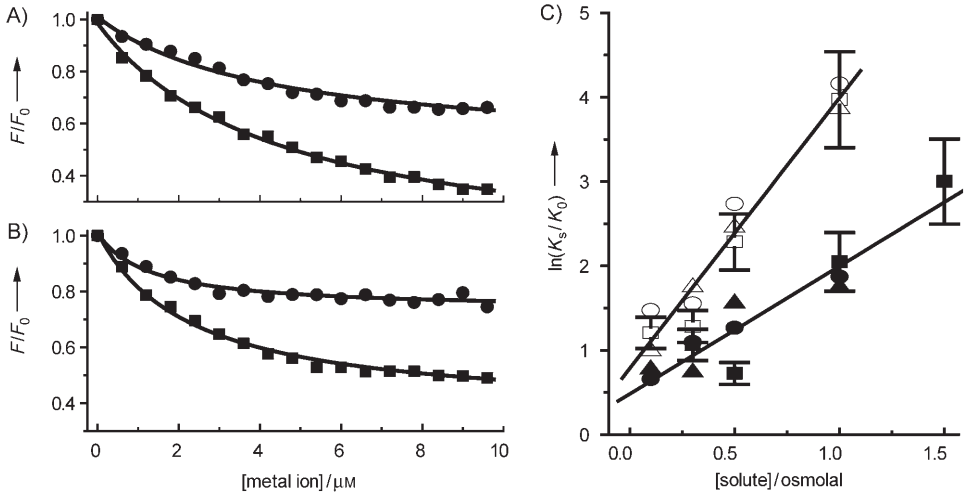

Figure 1. Binding isotherms for the interaction of Aβ with Cu^{2+} (■) and Zn^{2+} (●) in 20 mM Tris buffer in the absence A) or presence B) of osmolyte (0.5 M betaine). The Aβ peptide concentration was fixed at 3 μM in the titrations and 1:1 binding model was used to fit the fluorescence titration data. The sample was excited at 278 nm and the emission spectra were recorded. Details as described in experimental section. Experimental errors were 12% and 18% for Cu^{2+} and Zn^{2+}, respectively. C) Relationship between the binding constant and the osmolyte concentration. $\ln(K_s/K_0)$, the change in binding free energy was plotted against solution osmolality. Data for Cu^{2+} binding were shown as filled symbols. Data from Zn^{2+} binding were shown as open symbols. The different symbols indicate different osmolytes: triethylene glycol (■,□); betaine (●,○); sucrose (▲,△).

The osmotic stress method has been widely used as a direct in vitro probe to quantify hydration changes accompanying drug binding to DNA,[10–13] Ca^{2+} binding to protein,[14] and DNA–protein interactions.[15] We and others have shown that hydration changes are related to drug properties.[10,11] In the present study, we choose three commonly used osmolytes—sucrose, betaine, and triethylene glycol—whose size and physicochemical properties differ.[10,11] Fluorescence titrations were used to calculate the binding constants according to a 1:1 model.[5b,16,17] The sample was excited at 278 nm and the fluorescence emission spectrum was recorded. Typical data for Cu^{2+} and Zn^{2+} binding to Aβ in the absence or presence of an osmolyte (betaine) are shown in Figure 1A and B. From the preliminary data, the apparent binding constants can be estimated under our conditions. In the absence of osmolyte, the apparent binding constants were $4.6\times10^5\ M^{-1}$ and $5.9\times10^5\ M^{-1}$ for Cu^{2+} and Zn^{2+}, respectively, in agreement with previous reports that apparent dissociation constants in Tris buffer are in the micromolar range.[16,17] Intriguingly, the apparent Aβ binding constants for Cu^{2+} and Zn^{2+} were both significantly increased (Figure 1A and B) in the presence of betaine that perturbs water activity. The apparent binding constants for Cu^{2+} and Zn^{2+} binding to Aβ in the presence of three osmolytes (sucrose, betaine, and triethylene glycol) at several different osmolalities were determined (Figure 1C). Clearly, the apparent binding constants were increased with an increase in the osmolyte concentration and the three osmolytes exerted similar effects on the binding constants within experimental errors, showing that decreasing water activity enhanced Cu^{2+} and Zn^{2+} binding to Aβ. The solid lines through the data in Figure 1C were obtained by global fits to the data for all three different osmolytes used. From the slopes of the least-squares

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lines, it is possible to quantify the involvement of water in the metal–Aβ interaction. Assuming that the osmolytes are excluded from the vicinity of Aβ, which was supported by the similar effects of three different osmolytes,[10,11] the change in hydration is given by the equation:[10,11]

$$\partial \ln (K_s/K_0)/\partial[\mathrm{Osm}] = -\Delta n_w/55.5.$$

Where $\ln(K_s/K_0)$ is the change in binding free energy, "Osm" is the osmolality of the solution, and Δn_w is the difference in the number of bound water molecules between the complex and free reactants. In contrast with small drug binding to DNA,[10,11] the positive slopes of the best-fit lines in Figure 1 indicate that Δn_w is negative, showing that water is released upon complex formation. Within experimental errors, the Δn_w values are $-84(\pm 9)$ for Cu^{2+} and $-177(\pm 20)$ for Zn^{2+}, showing that a large number of water molecules were released upon metal binding.

Up till now, hydration contribution to metal binding to Aβ has not been considered. The entropy of releasing the ordered water molecules to bulk solvation is favourable for the complex formation. The value of the ΔS hydration term was calculated[18,19] by this equation: $\Delta S_{hydration} = 1.3 \pm 0.4\ \mathrm{cal\,K^{-1}\,mol^{-1}} \times \Delta n_w$, where $1.3 \pm 0.4\ \mathrm{cal\,K^{-1}\,mol^{-1}}$ is the average difference between the partial molar entropy of water in the bulk state and water in the hydration shells of amino acid residues at 298 K, and Δn_w is the estimates for the number of water molecules released to the bulk state upon the binding of Cu^{2+} and Zn^{2+} to Aβ. The estimated values are 109 ± 35 and $230 \pm 72\ \mathrm{cal\,K^{-1}\,mol^{-1}}$ for Cu^{2+} and Zn^{2+} binding, respectively. The hydration contributions to the binding free energy, $T\Delta S_{hydration}$ are 32.5 ± 10.4, and $68.5 \pm 22.4\ \mathrm{kcal\,mol^{-1}}$ for Cu^{2+} and Zn^{2+}, respectively (Table 1). These values are fourfold and eightfold larger than the net metal–Aβ binding free energy change, $\Delta G_b = -7.7$ (Cu^{2+}) and $-7.9\ \mathrm{kcal\,mol^{-1}}$ (Zn^{2+}), showing that hydration is playing an important role in controlling Cu^{2+} and Zn^{2+} binding to Aβ.

Many Aβ NMR signals ranged from E3–V18 which are not just limited to the coordination sites of His6, His13, and His14, become too weak to detect.[5] This is because of metal induced deprotonation,[5] and indicates that metal binding may disrupt the protein hydration shell. Theoretical calculation[7] shows that both Cu^{2+} and Aβ release water molecules when the complex is formed, indicating that a large number of water released can be the sum of the release from metal ions, Aβ, and metal-induced Aβ conformational transition. The large number of water release cannot originate from water dehydration of the metal only.[20] The major contribution may come from the Aβ and/or metal-induced Aβ conformational transition.[20]

Different dehydration changes may be due to the different properties of Cu^{2+} and Zn^{2+}. Previous studies have shown that the two metal ions have different binding modes to Aβ: Cu^{2+} coordinates to the Nπ atom of a histidine residue, whereas Zn^{2+} ligates to the Nτ atom; Cu^{2+} binding[20c] is more pH dependent than Zn^{2+}. We found that bis-ANS fluorescence was significantly enhanced and underwent a blue shift upon Zn^{2+} binding to Aβ. However, bis-ANS fluorescence hardly changed for Cu^{2+} binding to Aβ or Aβ alone (see Figure S1 in the Supporting Information), indicating that the Aβ–Zn^{2+} complex is more hydrophobic[21] and compact as shown in our CD studies (Figure 2A). This was in accordance with size-exclusion chromatography and pulse field gradient NMR diffusion results which show that the radius of the Aβ–Zn^{2+} complex is smaller and its structure is more compact than with copper binding.[22] These differences may cause more water molecules to be released upon Zn^{2+} binding.

Water is an integral part of protein structure. Water release would influence protein stability and conformation. As shown in Figure S2, Zn binding caused Aβ to be even more destabi-

Table 1. Summary of metal binding free energy change and hydration contribution to the binding at 25 °C.

Metal–Aβ Complex	ΔG_b[a] [kcal mol^{-1}]	Δn_w	$T\Delta S_{hydration}$[b] [kcal mol^{-1}]
Cu^{2+}–Aβ	−7.7	-84 ± 9	32.5 ± 10.4
Zn^{2+}–Aβ	−7.9	-177 ± 11	68.5 ± 22.4

[a] $\Delta G_b = -RT\ln K$; [b] $\Delta S_{hydration} = 1.3 \pm 0.4\ \mathrm{cal\,K^{-1}\,mol^{-1}}$ (the average difference between the partial molar entropy of water in the bulk state and water in the hydration shells of amino acid residues) $\times \Delta n_w$ at 298 K.

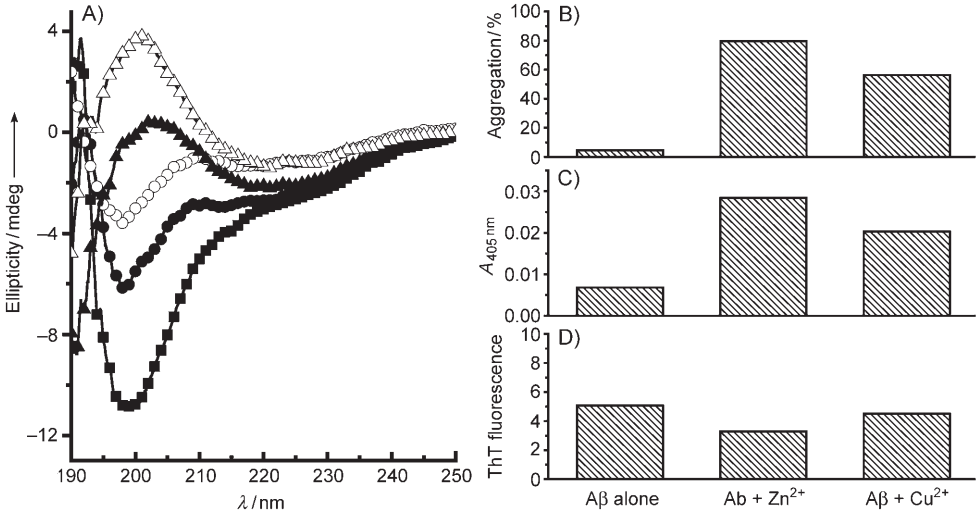
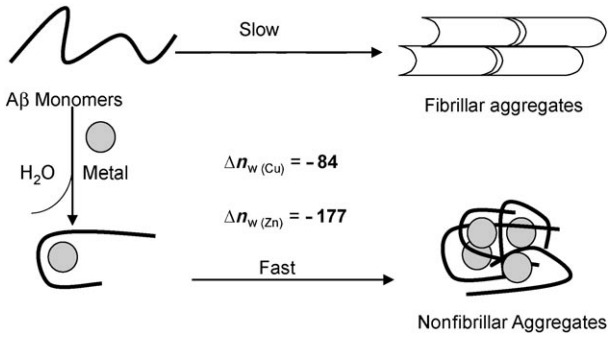


Figure 2. A) Far ultraviolet CD spectra of Aβ in the absence (black squares) or presence of metal ions. At 1:2 ratio of M^{2+}/Aβ: Cu^{2+} (●); Zn^{2+} (▲); At 1:1 ratio: Cu^{2+} (○); Zn^{2+} (△). Aβ1-40 concentration was 50 μM in Tris buffer. Effect of metal ions on the aggregation of Aβ: B) Total aggregation of Aβ measured by OD_{214} assay; C) Turbidity analysis performed by monitoring the absorption at 405 nm; D) The enhanced ThT fluorescence at 482 nm. M^{2+}/Aβ ratio at 2:1.

lised than Cu binding, and made Aβ more aggregation prone. This was consistent with the fact that more water molecules were released upon Zn^{2+} binding. Our CD data of Aβ before and after Cu^{2+} and Zn^{2+} binding also indicated that Zn^{2+} binding had a stronger effect on Aβ conformation although both Cu^{2+} and Zn^{2+} could regulate the secondary structure of Aβ (Figure 2A). Additional experiments with the OD214 assay and turbidity analysis (Figure 2B and C) showed that both Cu^{2+} and Zn^{2+} accelerated Aβ aggregation in several hours after incubation at 37 °C in comparison with Aβ alone, consistent with our previous results.[23] Cu^{2+}- or Zn^{2+}-induced aggregate was not a fibrous structure. This was supported by no fluorescence enhancement of ThT (a commonly used specific fluorescence dye for detection of fibrous structure formation) upon Cu^{2+} or Zn^{2+} binding compared to Aβ alone (Figure 2D), consistent with our AFM morphology studies in the absence or presence of Cu^{2+} or Zn^{2+} (Figure S3). We have carried out the aggregation and ThT assays with the same stoichiometry of metal to Aβ as the CD studies (Figure S4–S6). There was no ThT fluorescence enhancement observed for both metal ions. Aβ aggregations in the absence or presence of Cu^{2+} and Zn^{2+} can be described as follows (Scheme 1): Cu^{2+} and Zn^{2+} binding induced an Aβ conformational transition, released a large amount of water molecules by forming a Cu^{2+}– or Zn^{2+}–Aβ intermediate,[22] followed by rapid Aβ nonfibrillar amorphous aggregations. However, in the absence of Cu^{2+} and Zn^{2+}, the aggregation of Aβ is a nucleation-dependent process[24] and the ordered fibrils characterised by β-sheet conformation were formed in several days.[23] Cu^{2+} or Zn^{2+} induced Aβ destabilisation and rapid aggregation show that the less stable Cu^{2+}– or Zn^{2+}–Aβ intermediate[22] is more prone to aggregate than Aβ itself.



Scheme 1. Representative illustration of Aβ aggregation in the absence or presence of metal ions.

There is increasing evidence to show metal ions playing an important role in AD. Physiological levels of copper and zinc can accelerate Aβ aggregation,[3] and trace levels of copper and zinc may initiate seeding and oligomerisation of Aβ.[25] Metal chelation has been considered as a potential therapy for Alzheimer's disease and used in clinical trials.[25b] Dehydration occurs upon Zn^{2+} or Cu^{2+} binding to Aβ and Zn^{2+} binding causes even more water molecules to be released. These results indicate that water is a participant involved in metal–Aβ interactions and hydration plays a dominant role in metal binding. This would provide new insight to decipher the role of metal ions in Alzheimer's disease.

Experimental Section

Materials: Triethylene glycol, betaine, sucrose, and 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) were obtained from Acros Organics. Zinc chloride, copper chloride, 4,4′-dianilino-1,10-binaphthyl-5,5′-disulfonic acid (bis-ANS), and thioflavin T (ThT) were purchased from Sigma.

Sample preparation: Aβ40 was purchased from Sigma (lot no.091K49551) and prepared as previously described.[23] The Aβ40 peptide was first dissolved in 1,1,1,3,3,3-hexafluoropropan-2-ol (HFIP) at the concentration of 1 mg mL^{-1}. The solution was shaken at 4 °C for 2 h in a sealed vial for further dissolution and was then stored at −20 °C as a stock solution. Before use, the solvent HFIP was removed by evaporation under a gentle stream of nitrogen. Then, the peptide was dissolved in 20 mM Tris buffer, pH 7.4.

Absorption experiments: Thermal curves of protein were determined by using a Varian Cary300 Conc UV/Vis spectrophotometer.[26] Samples in the presence or absence of metal ions were heated at a rate of 1 °C min^{-1}, while continuously monitoring the absorption at 280 nm.

CD measurements: The influence of metal ions on protein secondary structure was recorded on a Jasco-J810 spectropolarimeter with a cell path of 0.1 cm.[23,26] The parameters were controlled as 0.1 nm intervals, 2 seconds response, and each sample was an average of three scans in a speed of 5 nm min^{-1} over the wavelength range from 195 nm to 250 nm.

Fluorescence detections and determination of binding constants: Fluorescence data were collected on a Jasco-FP6500 spectrofluorimeter.[26,27] The binding constants of metal ions with Aβ were determined by fluorescence titration at 25 °C.[16,17] The excitation wavelength was 278 nm and the emission intensity at 306 nm was monitored as a function of continuous increasing concentration of metal ions. Aβ40 peptide concentration was fixed at 3 μM whereas metal ion concentration was varied between 0.6 μM to 10 μM.

Bis-ANS was dissolved in Tris buffer (pH 7.4) to a concentration of 10 μM. Aβ in each sample was added to a final concentration of 1 μM in the experiments. The emission spectra were recorded from 420–620 nm with the excitation wavelength at 360 nm.[21]

Aggregation assay: Aβ (10 μM) in the presence or absence of metal ions (20 μM) was incubated at 37 °C for 3 h. Individual samples were then measured using different methods:

1) OD_{214} assay. The aggregated Aβ was sedimented by centrifugation. The optical density at 214 nm of the supernatant and the sample before centrifugation was measured. The fractions of aggregates were calculated.
2) Turbidity analysis. Turbidity analysis has been widely used to examine Aβ aggregations according to the absorption difference at 405 nm.[28,29] There is no absorption at 405 nm when the peptide is in soluble state, and the absorption at 405 nm gets increased when the peptide aggregates. All sample solutions were mixed by vortexing and the absorption at 405 nm was measured.

3) ThT fluorescence assay. Optimum fluorescence intensity measurements were obtained at the excitation and emission wavelengths of 444 nm and 482 nm, respectively, and the final concentration of ThT was 10 μM.
4) Atomic force microscope (AFM) imaging. Aliquots of 10 μL of each sample were placed on a freshly cleaved mica substrate.[23,27] After incubation for 5 min, the substrate was rinsed with water twice and dried before measurement. Tapping mode was used to acquire the images under ambient conditions.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (20331020, 20325101, and 20473084) and Funds from CAS and Jilin Province.

Keywords: beta-amyloid peptides · hydration change · metal ions · osmotic stress · water

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Received: October 23, 2007
Published online on February 22, 2008

