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Cu(II)-Zn(II) Cross-Modulation in Amyloid-Beta Peptide Binding: an X-ray Absorption Spectroscopy Study

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Abstract

In this work we analyze at a structural level the mechanism by which Cu(II) and Zn(II) ions compete for binding to the A β peptides that is involved in the etiology of Alzheimer's disease. We collected X-ray Absorption Spectroscopy data on samples containing A β with Cu and Zn at different concentration ratios. We show that the order in which metals are added to the peptide solution matters and that, when Zn is added first, it prevents Cu from binding. On the contrary, when Cu is added first, it does not (completely) prevent Zn binding to A β peptides. Our analysis suggests that Cu and Zn ions are coordinated to different numbers of histidine residues depending on the [ion]:[peptide] concentration ratio.

INTRODUCTION

The hypothesis that competition between metal ions for coordination to protein is critical for homeostasis is becoming more and more well established^{1–2}. Metal homeostasis is of particular relevance in the central nervous system, where ion imbalance has been implicated in several severe neurological diseases. In the context of the Alzheimer's disease (AD)^{3–5}, the possible role of Cu(II) and Zn(II) in aggregation has been extensively studied^{6–7}. Recent Electron Spin Resonance (ESR) data⁸ and X-ray Absorption Spectroscopy (XAS)^{9–10} measurements, carried out in the related case of the prion protein (PrP), confirmed that there

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Notes

The authors declare no competing financial interest.

Supporting information

Comparison among the Cu K-edge EXAFS spectra prepared with the same concentration ratios but different procedure (Figure S1). Comparison among the Cu K-edge XANES spectra prepared with the same procedure but different concentration ratios (Figure S2). Comparison among the Zn K-edge EXAFS spectra prepared with the same concentration ratios but different procedure (Figure S3). Comparison between the EXAFS spectra of the S3_Cu and S5_Cu samples (Figure S4). Comparison among the Zn K-edge and Cu K-edge XANES spectra (Figure S5). Comparison among the Zn K-edge and Cu K-edge EXAFS spectra (Figure S6). EXAFS spectra comparison between 3His and 2His models (Figure S7). Comparison of the Fourier Transform of the fitted EXAFS spectra of the 3His and 2His models (Figure S8). This information is available free of charge via the Internet at <http://pubs.acs.org>

is a competition for PrP binding between the two ions, thus suggesting the existence of a general mechanism of fine regulation of metal binding possibly selected to prevent cell damage from accumulated free ions. In this general framework, it appears to be of the utmost importance to understand and clarify whether and how Cu(II) and Zn(II) cross-interact with amyloidogenic peptides. In this work we analyze at a structural level the mechanism by which different metal ions compete in the binding to the A β peptides, which is involved in AD.

Several Nuclear Magnetic Resonance (NMR)^{11–13}, ESR^{14–19} and XAS^{20–21} studies have been carried out in the last years to investigate the Cu(II)- and/or Zn(II)-A β coordination modes. In particular, the ESR work of Silva et. al.¹⁴ and the multi-technique (ESR, XAS, NMR, potentiometry) investigations by Alies²² and Damante²³ analyzed the structures of A β -Cu(II) and A β -Zn(II) complexes when both metal ions are simultaneously present and showed that the presence of Zn affects the Cu(II) coordination mode.

The work presented here is aimed at providing a structural characterization of the local environment around Cu(II) and Zn(II) when they are simultaneously present in solution with the A β peptide. This was done by performing a systematic XAS study of a set of samples in which Cu(II) and Zn(II) ions are added to the A β peptide in different orders and at different concentrations. Our results show that the metal-peptide coordination mode depends not only, as already pointed out by Silva¹⁴, on the relative metal ions concentrations, but also on the order in which the two metal ions are added to the A β solution.

MATERIALS AND METHODS

As a natural extension of the recent ESR experimental results¹⁴ on A β -[Cu/Zn] complexes and those^{9–10} obtained using XAS on the similar PrP-[Cu/Zn] complexes, we performed a thorough XAS study of A β -[Cu/Zn] complexes with the aim of elucidating at the atomic level the cross-interaction dynamics when both ions are simultaneously present. Continuous-wave ESR (CW-ESR) measurements are also carried out to support the XAS results. In this work, the 1-16 fragment of the A β peptide is subjected to investigation. Although it has been proposed that the remaining portion of the peptide may have a direct or indirect role in metal coordination^{24–26}, this is indeed the region where the highest affinity binding sites of Cu and Zn are known to be located^{23,27–32}.

Sample preparation

A β peptide (1-16) were purchased from Sigma-Aldrich Co. (The Woodlands, Texas). N-Ethylmorpholine(NEM) was purchased from Sigma-Aldrich Co. (St. Louis, MO).

A β peptide samples were prepared following the protocol described in Silva et al.¹⁴. All samples were prepared dissolving the peptide in a solvent containing 100 mM NEM buffer (pKa = 7.8) in 50% (v/v) glycerol. The latter is added to help stabilise the sample³³. The pH of the solution was kept constant at 7.4 by adding appropriate amounts of sulfuric acid (H₂SO₄). The peptide concentration used for ESR and XAS measurement was 1.25 mM. For the samples subjected to XAS measurements, Cu(II) and Zn(II) were added as CuSO₄ and ZnSO₄ salts (purchased from Sigma-Aldrich Co.), respectively. Cu(II) concentration was

kept constant at 1 equivalent (eq), namely equal to the 1.25 mM peptide concentration. Zn(II) was added at two different concentrations, i.e. 1 or 4 eq (see Table 1). Isotopically enriched (98.6%) $^{63}\text{CuCl}_2$, purchased from Cambridge isotope laboratory, and anhydrous ZnCl_2 powder (99.995% metal basis), purchased from Sigma-Aldrich Co. (St. Louis, MO), were used for CW-ESR measurements. The enriched ^{63}Cu isotope was employed to minimize inhomogeneous broadening of the ESR signal. One eq Cu(II) and one eq Zn(II) were added to the peptide solution.

In order to investigate the nature of the competition between Cu(II) and Zn(II) ions for peptide binding, for each metal/peptide concentration ratio, three different sets of samples were subjected to XAS measurements. The first set was prepared by adding to the solution containing the peptide, Cu(II) and Zn(II) ions simultaneously 15 hours before we started taking XAS data. This set of samples is exactly the same as the one studied in Silva et al.¹⁴. The others two sets of samples were prepared by dissolving either Cu(II) or Zn(II) first, and then, after a 15 hours incubation, the second ion a few minutes before quenching at 77K in the XAS sample-holder. Furthermore, we collected control spectra (c_Cu and c_Zn) where only one of the two metals is present in solution with the peptide (see Supporting information).

Note that all samples (with the obvious exception of the Cu and Zn buffers, b_Cu and b_Zn, and control spectra, c_Cu and c_Zn) are measured at both, Cu and Zn, K-edges. In the following, we use the notation Sn_Cu when the spectrum of n-th sample is measured at the Cu K-edge and Sn_Zn when the spectrum is measured at the Zn K-edge.

X-ray data collection

XAS experiments have been carried out at the BM30B beamline of the European Synchrotron Radiation Facility (Grenoble, France)³⁴. The beam energy was selected using a Si(220) double-crystal monochromator with a resolution of ~ 0.5 eV³⁵. The beam spot on the sample was approximately $300 \times 200 \mu\text{m}^2$ ($H \times V$, FWHM). Spectra were recorded in fluorescence mode by the use of a 30-element solid-state Ge detector. To avoid photo-degradation and spectra evolution during XAS measurements, all the samples were first rapidly brought from room temperature to 77 K and then cooled and kept at 13 K with the help of a liquid helium cryostat throughout XAS measurement. For the same reason, in the process of data acquisition, samples were systematically moved to a different position for each scan, thus hitting a different portion of the sample. Each scan never exceeded 40 minutes.

XAS data analysis

The absorption coefficient, $\mu(E)$, and the fine structure signal, $\chi(k)$, are extracted from the raw data by means of the ATHENA software³⁶. The definition of $\chi(k)$ and its relationship with the absorption coefficient, $\mu(E)$, are discussed in the appendix of ref.⁶. XAS data are presented by separating the low-energy (the so-called XANES, X-ray Absorption Near Edge Structure) and the high-energy (the so-called EXAFS, Extended X-ray Absorption Fine Structure) regions of the spectrum. The XANES region is analyzed comparing the spectra of the various samples collected at the same K-edge. Structural information from the EXAFS

region is obtained using the EXCURV98 software³⁷. We exploited the so-called constrained refinement strategy that consists in treating the lateral chains of amino acid residues as rigid bodies therefore allowing for a substantial reduction of the number of free parameters in the fitting procedure³⁸.

ESR data collection

A 200 μ L aliquot of the sample was transferred into a quartz tube (inner diameter 3 mm). CW-ESR experiments were performed using a Bruker ElexSys E680 X-band FT/CW spectrometer equipped with a Bruker EN4118X-MD4 resonator. The temperature was controlled using an Oxford ITC503 temperature controller and an Oxford LLT 650 low-loss transfer tube. All samples were frozen quickly using liquid nitrogen. Then the samples were inserted into the sample cavity which was precooled to 80 K. Experiments were carried out at 80 K with a microwave frequency of approximately 9.69 GHz. As much as 1024 data points were acquired in the magnetic field interval ranging from 2600 to 3600 G. A time constant of 40.96 ms, a conversion time of 81.92 ms, a modulation amplitude of 4 G, a modulation frequency of 100 kHz, and a microwave power of 0.1993 mW were the other instrumental parameters used.

RESULTS AND DISCUSSION

Qualitative XAS data analysis

In order to get an orientation in data analysis we decided to start by carrying out a qualitative comparison of the XANES spectra of all the samples that we measured. In this way, besides being able to select the samples that deserved further quantitative analysis of the EXAFS region, we are able to get interesting indications on how the concentration of metal ions and their addition order affect the Cu(II) and Zn(II) coordination mode.

The qualitative comparison of the XANES spectra strongly suggests that the order in which metal ions are added to the peptide solution has a significant influence on the metal-peptide binding mode. Figure 1 shows XANES spectra collected at the Cu K-edge subdivided according to metal ion concentration. In the left panel we show the spectra of the samples having concentrations in the ratio $\text{A}\beta\text{:Cu:Zn} = 1\text{:}1\text{:}1$ and in the right panel those with $\text{A}\beta\text{:Cu:Zn} = 1\text{:}1\text{:}4$ concentrations ratios. One can immediately notice that, when Zn(II) is added to the peptide solution before Cu(II), the spectra (dark grey and burgundy, respectively) are identical to that of the Cu(II) in buffer (blue). This fact clearly indicates that, in the presence of bound Zn, Cu(II) is unable to bind to the peptide thus remaining free in solution. This conclusion is confirmed by the CW-ESR measurements described in the *ESR data analysis* paragraph below.

A similar behavior is observed when an excess of Zn is added simultaneously to stoichiometric Cu (sample S2_Cu - light gray in the right panel). In this case the XANES spectrum of S2_Cu is not identical to that of b_Cu, but its general features still suggest that there are contributions from Cu(II) in solution. Comparisons among Cu K-edge spectra in the EXAFS region lead to identical conclusions and are shown in Figure S1.

A standard way to determine the possible simultaneous presence of absorbing metals in different local arrangements is to assume that the total spectrum results from the weighted linear sum of experimentally measured spectra of samples where the absorber is known to exist in the different local arrangements in consideration^{36,39}. We employed this method to quantify the fraction of Cu and Zn bound to the peptide and free in solution.

We apply this strategy to the S2_Cu XANES data assuming that in this sample Cu(II) finds itself partly in the S5_Cu coordination mode and partly in the b_Cu coordination mode. The best fit to the relative weight of the two configurations is obtained by taking the combination (0.43 ± 0.05) S5_Cu + (0.57 ± 0.05) b_Cu. As one can see by looking at the magnitude of the difference spectrum shown in gold in Figure 2, the fit is extremely good (quality-factor = 0.07% as computed by the ATHENA software). We conclude that about half of Cu remains in solution when the two metals are added simultaneously. In contrast, in the case where Zn is added first, the totality of Cu remains free in solution. The same procedure is not applied to S1_Cu sample (A β :Cu:Zn = 1:1:1, Simultaneously) because the spectrum of the latter is almost identical to that of S3_Cu (A β :Cu:Zn = 1:1:1, Cu first, see Figure 1, left panel) and the fit would lead to an obvious 100% contribution of the S3_Cu signal. In other words, S1_Cu and S3_Cu contain about the same amount of Cu(II) in buffer. In order to obtain information about the Cu coordination mode in the S3_Cu and S5_Cu samples, we performed a quantitative analysis of the EXAFS region of the spectrum as described below.

A comparison between S3_Cu and S5_Cu data is shown in Figure S2 (left panel). These samples were prepared by adding Cu(II) first and then either 1 or 4 equivalents of Zn(II), respectively. Interestingly S3_Cu and S5_Cu have identical XANES spectra, thus demonstrating that an excess of Zn, when added after Cu, does not further affect the Cu(II) coordination mode. However, when both the metal ions are added simultaneously, the Cu(II) coordination mode is significantly affected as shown in Figure S2 (right panel). This observation is consistent with ESR experiments¹⁴, where the two metals are added simultaneously. In this case the Cu(II) coordination mode is significantly influenced by the amount of Zn(II) added (right panel of Figure S2).

An attractive feature of the present spectroscopic study is that we are in a position to also get some insight into Zn coordination. Direct insights on Zn coordination are impossible by ESR because Zn is ESR-silent. The XANES data at the Zn K-edge is shown in Figure 3. As it is visible in Figure 3, when Cu(II) is added to the peptide solution prior to Zn(II), the XANES spectra appear to be all significantly different from that of Zn(II) in buffer. This observation means that Cu(II) is not able to completely prevent the binding of Zn(II) to the peptide. Comparisons among Zn K-edge spectra in the EXAFS region lead to identical conclusions and are shown in Figure S3.

We note that for both concentration ratios, the Zn K-edge spectrum of the sample in which Zn(II) is added to the peptide solution prior to Cu(II) is very similar (though not identical) to the that of the sample where Zn(II) and Cu(II) are added simultaneously (namely, S1_Zn similar to S4_Zn and S2_Zn similar to S6_Zn).

Analogously to what we saw at the Cu K-edge, one can notice that at the Zn K-edge and in the presence of 4 eq of Zn(II), the spectrum of the sample prepared adding Cu(II) and Zn(II) simultaneously (S2_Zn) is more similar to that of Zn(II) in buffer than to that of the S6_Zn sample prepared with Zn added first. Our interpretation of this fact is that, when Zn is added first, it binds more easily to the peptide (lacking any competition with Cu) than when the two metals are added simultaneously. So we expect to find more Zn in solution in sample S2_Zn than in sample S6_Zn. This qualitative observation can be tested by the same kind of analysis we performed before in the case of the S2_Cu sample. We thus try a fit of the S2_Zn sample XANES data as a linear combination of S6_Zn and b_Zn data. The best fit displayed in Figure 4 is very good (quality-factor = 0.14%) and gives for the S2_Zn sample a combination with (75±5)% of S6_Zn and (25±5)% of Zn buffer. The same observation we made above for the case of S1_Cu is in order here for S1_Zn, as no useful information could be obtained by trying to represent S1_Zn data as a linear combination of S4_Zn and b_Zn data.

Quantitative EXAFS data analysis

Reliable structural information on the actual Cu(II) and Zn(II) coordination geometry can be obtained from a quantitative analysis of the EXAFS region spectra. EXAFS spectra were analyzed using the EXCURV98 package³⁷. The theoretical absorption coefficient is calculated by taking into account single and multiple scattering contributions. Starting coordination models were built exploiting available ESR data and structures deposited in the MESPEUS structural database⁴⁰. The so-called constrained refinement strategy proposed in Binsted et al.³⁸ was followed, in which the amino acidic side chains are treated as rigid bodies. In the case at hand, we considered the imidazole ring of His residues and the side chains of Asp1 and Ala2 as un-deformable. Thus, in the fitting step we refined only the distance of the bound nitrogen and the orientation of the imidazole ring plane. This enabled significant reduction of the number of free variables in the fitting procedure³⁸. In order to deal with signals with minimal contamination from non-bound metal we need to consider the samples that have the lowest possible fraction of the absorber free in solution. This constraint selects the 1:1:1 samples, S3_Cu at the Cu K-edge, which was prepared by adding Cu before Zn, and S4_Zn at the Zn K-edge, which was prepared, instead, by adding Zn before Cu.

S3_Cu sample

The quantitative analysis of the S3_Cu sample is performed starting from a structural model that embodies the information coming from the available ESR^B results obtained on the same set of samples we are considering here^{14,15}.

According to ESR there is a coexistence of two different local Cu environments, termed Component I and Component II by the Authors. In Component I Cu(II) is bound to two His residues, one N atom belonging to the N-terminal and one carboxylic oxygen from the N-terminal amino acid Asp^{16–17,19}. In Component II Cu(II) is bound to one His residue, one N

^BIt is worth pointing out that, since samples S3_Cu and S5_Cu have identical EXAFS spectra (see Figure S4), the considerations presented in the following on sample S3_Cu also apply to sample S5_Cu.

atom belonging to the N-terminal, one carboxylic oxygen from the N-terminal amino acid Asp and one N atom belonging to an Ala^{15,16}. Since axial oxygens are not visible in ESEEM and HYSCORE experiments, as the ones here considered^{14,15}, following Kim et al.⁴¹, we completed the preferred penta-coordinated Cu first shell by including an oxygen atom from a water molecule. A two-component model of this kind requires, besides the optimization of the structural parameter of the two metal sites, a fit of the relative weights by which each structural component contribute to the total spectrum.

The best-fit curve is shown in Figure 5 and is in good agreement with the experimental data. The best-fit values of the geometrical parameters characterizing the two Cu sites are reported in Table 2, together with the relative weight of each Component for which we find $w_I = (66 \pm 5)\%$ and $w_{II} = (34 \pm 5)\%$, in line with the ESR results of Silva et al.¹⁴, $w_I = 53\%$ and $w_{II} = 47\%$.

ESR data refer to a sample with the same 1:1:1 concentration ratios as our S3_Cu sample. The small difference between the two sets of percentages should be attributed to the fact that, in ESR samples, where possible Cu in solution is invisible, the two metals were simultaneously added to the A β solution, while Cu was added first in our case thus strongly limiting the amount of Cu free in solution.

S4_Zn sample

In the analysis of the EXAFS spectra acquired at the Zn K-edge we cannot rely on input from ESR experiments as Zn(II) is ESR silent. We therefore tried various structures in which Zn(II) is coordinated to different numbers of His residues and oxygen atoms, the latter coming from water. A thorough analysis of many cases (whose details we do not give here) shows that the best fit (see Figure 6) is obtained by taking Zn(II) in a tetra-coordinated geometry with three His residues and one oxygen. The best fit values of the parameters characterizing the Zn(II) site are given in Table 3. Interestingly all the three His residues belonging to the A β peptide sequence are involved in Zn(II) binding. This fact has the consequence that, when Cu(II) is added after Zn incubation to the sample, the latter is not able to bind the A β peptide as there are no His's available for binding. In the ESR experiments¹⁴ carried out by keeping the A β and Cu(II) ratio constant and increasing the Zn(II) ratio from 0 to 10 equivalents it is found that up to a concentration of 4 equivalents, Zn(II) is able to substitute Cu(II) and modify its coordination mode. Above this concentration no effects of adding more Zn(II) is visible in pulsed ESR¹⁴.

ESR data analysis

CW-ESR experiments on equimolar complexes of Cu(II)/Zn(II) and A β (1-16) in 100 mM NEM (pH 7.4) buffer were specifically performed to cross-check the XAS results described above. In CW-ESR experiments only the Cu(II) signal can be observed as Zn(II) is a diamagnetic ESR silent species. At the physiological pH in NEM buffer, free Cu(II) precipitates and does not contribute to the ESR signal. Hence, only Cu(II) bound to the peptide can possibly give a signal. Figure 7 shows two experimental CW-ESR spectra, one obtained from a sample where Cu(II) is added first, subsequently followed by adding Zn(II) 60 seconds later (blue curve), and a second one from a sample where Zn(II) is added first,

followed by Cu(II) after 60 seconds (red curve). When Cu(II) is added first, the subsequent addition of Zn(II) does not completely displace the Cu(II) bound to the peptide and one gets the CW-ESR spectrum represented by the blue curve in Figure 7. On the contrary, when Zn(II) is added first, Cu(II) is no longer capable of binding to the peptide and, as seen in Figure 7, the CW-ESR signal is absent (red curve). These results are consistent with what we found from XAS experiments and fully confirm the conclusion that the order of metal addition is critical in A β peptide metal binding (see the **Qualitative XAS data analysis** paragraph).

CONCLUSIONS

By exploiting the unique possibility offered by XAS for the structural study of the local environment around metal ions even in non-crystalline samples, we have been able to investigate the way Cu(II) and Zn(II) bind the A β peptide, and clarify their cross-interaction when they are simultaneously present in solution.

We want to stress that the analysis we have presented has once more demonstrated the power of XAS in yielding accurate details of the geometry of the metal coordination site in non-crystallized samples of biological interest. We have shown that, in these situations, ESR and XAS techniques are complementary and, where there is an overlap, XAS results are in full agreement with the conclusions of ESR experiments.

Besides confirming ESR results, XAS has made possible to clarify the mechanism of Cu(II) vs Zn(II) competition for A β -peptide binding. In particular, our results indicate that in our experimental conditions and after a long incubation time, at variance with what was previously reported by Alies²² and by Danielsson et al.²⁶ the order in which metal ions are added to the peptide solution does matter. Damante et.al.²³ suggested that the addition of Zn(II) does not release Cu(II) ions, but modifies the Cu(II) distribution in Components I and II, contrary to what we observed in this study.

In Figure 8 the effect of adding Cu and Zn in different orders is schematically depicted. One can notice that when Zn is added first (top panel), Cu binding practically does not occur and Cu remains free in solution. The XAS results are confirmed by CW-ESR measurements, which actually suggest that even after a shorter incubation time Zn(II) is able to bind the A β and prevent the formation of A β -Cu(II) complexes. On the contrary, when Cu is added first, it does not (completely) prevent Zn binding (bottom panel), so one finds Zn bound to the peptide as well as free in solution. In the case of the simultaneous addition of metals, there is an intermediate situation with both Cu and Zn partially bound to the peptide and free in solution (middle panel).

A detailed quantitative analysis of the measured EXAFS spectra shows that the binding of Cu(II), as well as that of Zn(II), to the A β peptide occurs always through His residues. This is a further indirect, but strong indication that the two metals do compete for A β binding.

It is worth pointing out that, despite the reported higher affinity of Cu(II) with respect to Zn(II) for A β peptide binding^{26,42-44}, in our experiments we observe that, once Zn(II) is bound, Cu(II) is not able to bind anymore to the A β peptide. A possible interpretation of this

fact is that the addition of Zn starts an irreversible reaction process leading to the formation of Zn(II)-A β complexes that are likely not accessible for Cu(II) ions.

In this study we have been able not only to improve our knowledge of the structure of the Cu(II)-A β binding site, but also to extend the analysis to the characterization of the structure of the Zn(II) binding site in the presence of Cu(II). Specifically we observe that, when Zn(II) is added first, all the three A β His residues are involved in the binding and are not available for Cu(II) binding.

As pointed out in the Introduction, the presence of metal ions is known to be relevant in the development of several neurodegenerative diseases, including AD, Parkinson's disease and transmissible spongiform encephalopathies (TSEs).

In this connection - although not specifically related to the question of metal ion coordination which is the main focus of this paper - it is worth mentioning interesting relations between AD and TSEs emerging from the works of refs.⁴⁵⁻⁴⁷. They have to do with the observation⁴⁸ that there seems to exist a direct interaction between the prion protein and the A β peptide, with the former serving as a receptor for the latter and mediating the pathogenic effects of A β oligomers.

On the interesting question of possible TSEs-AD functional relations, the results presented in this work can be also compared to the analogous results obtained by our group⁹⁻¹⁰ on the prion protein. In that case, it was found that once Cu(II) is bound to the prion protein, Zn(II) is not able to bind it. In the case of the A β peptide, in a way the roles of Zn and Cu are exchanged. We observe, in fact, that, when Zn(II) is bound to the A β peptide and Cu(II) is added after some time (hours), Cu(II) is not able to bind to the A β . In view of the established different affinity of A β and prion protein for specific metal ion binding, one can speculate that the two peptides can possibly play complementary roles in the homeostasis of Cu(II) and Zn(II) ions in cells. In this sense we would like to look at our work as a modest step toward elucidating the role of metal dis-homeostasis in devastating neurodegenerative diseases like AD and TSEs.

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ABBREVIATIONS

AD	Alzheimer's Disease
Aβ	amyloid beta peptide
CW-ESR	Continuous Wave Electron Spin Resonance
ESR	Electron Spin Resonance
EXAFS	Extended X-ray Absorption Fine Structure

NMR	Nuclear Magnetic Resonance
PrP	Prion Protein
TSEs	transmissible spongiform encephalopathies
XANES	X-ray Absorption Near Edge Structure
XAS	X-ray Absorption Spectroscopy

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Figure 1.

Comparison among Cu K-edge XANES spectra. In each panel, we group samples prepared with equal concentration ratio (Al:Cu:Zn = 1:1:1, left panel; Al:Cu:Zn = 1:1:4, right panel) but different preparation procedures. The spectrum of S4_Cu and b_Cu on the left panel and S6_Cu and b_Cu on the right panel are almost perfectly superimposed.

Figure 2.

Fit of S2_Cu ($\text{A}\beta\text{:Cu:Zn} = 1:1:4$, simultaneously) XANES data as a weighted sum of sample S5_Cu ($\text{A}\beta\text{:Cu:Zn} = 1:1:4$, Cu first) and b_Cu data.

Figure 3. Comparison among Zn K-edge XANES spectra. In each panel we group samples prepared with the same concentration ratio but different preparation procedures.

Figure 4. Fit of S2_Zn ($\text{A}\beta\text{:Cu:Zn} = 1:1:4$, simultaneously) XANES data as a weighted sum of samples S6_Zn ($\text{A}\beta\text{:Cu:Zn} = 1:1:4$, Zn first) and b_Zn data.

Figure 5. Experimental EXAFS data of sample S3_Cu at the Cu K-edge (*dashed line*) and best-fit theoretical model (*full line*).

Figure 6. Experimental EXAFS data of sample S4_Zn at the Zn K-edge (*dashed line*) and best-fit theoretical model (*full line*).

Figure 7.

CW-ESR spectra illustrating the Cu(II)/Zn(II) metal binding competition for A β (1-16) peptide at physiological pH. The blue spectrum shows the ESR signal for a sample where Cu(II) is added first, with the subsequent addition of Zn(II) 60 seconds later. In this sample Cu(II) appears to be still bound to the peptide. Instead, when Zn(II) is added first, the successively added Cu(II) is unable to bind to the A β (1-16) peptide as shown by the flatness of the red spectrum.

Figure 8.

Schematic illustration of the effect of adding Cu(II) and Zn(II) in different order to the A β solution. Cu(II) and Zn(II) ions are drawn as *red* and *green* dots, respectively. Only the bonds between metals and His residues are drawn (as short blue segments).

Table 1List of measured samples^A.

Sample	A β :Cu:Zn	Metal addition order	Color
S1	1:1:1	Simultaneous	Green
S2	1:1:4	Simultaneous	Light Gray
S3	1:1:1	Cu(II) first	Orange
S4	1:1:1	Zn(II) first	Dark gray
S5	1:1:4	Cu(II) first	Red
S6	1:1:4	Zn(II) first	Burgundy
c_Cu	1:1:0	Only Cu	Purple
c_Zn	1:0:1	Only Zn	Light blue

Sample	A β :Cu:Zn	Metal addition order	Color
b_Cu	0:1.6:0	Cu in buffer	Blue
b_Zn	0:0:1.6	Zn in buffer	Blue

^A In the four columns we report the names of the measured samples, their concentrations in peptide equivalents (eq), the order in which the two metal ions are added and the color code we use in the plots below.

Table 2

The best fit structural parameters of the Cu(II) coordination site in Component I and II for the sample S3_Cu. Atoms falling within error bar during the first refinement steps were then linked together during the final optimization.

Atom	$r \pm \sigma$ (Å)	$\sigma^2 \pm \sigma^2$ (Å ²)
Component I - $w_I = (66 \pm 5)\%$		
O(H ₂ O)	1.94 ± 0.02	0.004 ± 0.001
O(Asp)	1.94 ± 0.02	0.004 ± 0.001
N(His)	1.96 ± 0.02	0.004 ± 0.001
N(His)	1.96 ± 0.02	0.004 ± 0.001
N(Asp)	2.33 ± 0.02	0.004 ± 0.001
Component II - $w_{II} = (34 \pm 5)\%$		
O(H ₂ O)	1.84 ± 0.02	0.003 ± 0.001
N(Ala)	1.84 ± 0.02	0.003 ± 0.001
N(His)	2.00 ± 0.03	0.003 ± 0.001
O(Asp)	2.00 ± 0.03	0.003 ± 0.001
N(Asp)	2.34 ± 0.02	0.003 ± 0.001
$E_0 = (3.9 \pm 0.5)$ eV		R-factor = 22%

Table 3

Best fit parameters of the Zn(II) coordination site in sample S4_Zn. Atoms falling within error bar during the first refinement steps were then linked together during the final optimization.

Atom	$r \pm \sigma$ (Å)	$\sigma^2 \pm \Delta\sigma^2$ (Å ²)
O	1.88 ± 0.01	0.003 ± 0.001
N(His)	2.01 ± 0.01	0.003 ± 0.001
N(His)	2.01 ± 0.01	0.003 ± 0.001
N(His)	2.01 ± 0.01	0.003 ± 0.001
$E_0 = (1.3 \pm 0.7) \text{ eV}$		R-factor = 28%