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pH-dependence of the specific binding of Cu(II) and Zn(II) ions to the amyloid-β peptide

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ABSTRACT

Metal ions like Cu(II) and Zn(II) are accumulated in Alzheimer's disease amyloid plaques. The amyloid-β (Aβ) peptide involved in the disease interacts with these metal ions at neutral pH via ligands provided by the N-terminal histidines and the N-terminus. The present study uses high-resolution NMR spectroscopy to monitor the residue-specific interactions of Cu(II) and Zn(II) with ^{15}N- and ^{13}C,^{15}N-labeled Aβ(1–40) peptides at varying pH levels. At pH 7.4 both ions bind to the specific ligands, competing with one another. At pH 5.5 Cu(II) retains its specific histidine ligands, while Zn(II) seems to lack residue-specific interactions. The low pH mimics acidosis which is linked to inflammatory processes *in vivo*. The results suggest that the cell toxic effects of redox active Cu(II) binding to Aβ may be reversed by the protective activity of non-redox active Zn(II) binding to the same major binding site under non-acidic conditions. Under acidic conditions, the protective effect of Zn(II) may be decreased or changed, since Zn(II) is less able to compete with Cu(II) for the specific binding site on the Aβ peptide under these conditions.

1. Introduction

Despite more than a decade of intense studies, the roles of metal ions in the process of aggregation and amyloid formation by the amyloid-β (Aβ) peptide involved in Alzheimer's disease are still not well understood. Early studies [1] showed that Cu(II), Zn(II) and Fe(III) ions give rise to increased pH dependent aggregation of Aβ(1–40), suggesting that aggregation is strongly induced by conditions representing physiological acidosis. Recent work [2] on the kinetics of fibril (amyloid) formation (determined from kinetic studies of thioflavin T fluorescence) have shown that low pH and Cu(II) ions could strongly accelerate the kinetics of fibril formation, whereas Zn(II) simply inhibited the formation of fibrils.

Other recent studies have shown distinct pathways for Aβ aggregation in the presence of Cu(II) ions at neutral pH for different metal-to-peptide ratios [3], whereas Zn-induced aggregation of Aβ has been proposed to give rise to new kinds of aggregated assemblies [4]. A recent review describes the present understanding of the Aβ peptide interactions with Cu(II) and Zn(II) and their effects on the fibrillation and toxicity at physiological conditions [5].

Given the proposed connection between metal ions and Aβ amyloid formation, metal chelation therapy has been tried and evaluated as a potential therapy against Alzheimer's disease [6]. However, the understanding is limited and the results are not conclusive, showing the need for further studies of the metal binding properties of the Aβ peptide [7].

Various specific ligands to the metal ions have been proposed [8,9], and our previous NMR studies of Aβ(1–40) at pH 7.4 have shown a major binding site, common for Zn(II) and Cu(II), with H6, H13, H14 and the N-terminus as ligands [10,11]. In the same study [10] we reported apparent dissociation constants in the micromolar range, with a somewhat higher Cu(II) affinity, 0.4 μM, than Zn(II) affinity, 1.1 μM (both values not corrected for ionic strength, cf. [5,12]). Both the histidine ligandation and the somewhat higher stability of the Cu(II) complex than the Zn(II) complex are supported by theoretical calculations [13].

Here we have studied the residue-specific interactions of Cu(II) and Zn(II) with Aβ(1–40) at three different pHs using high resolution NMR spectroscopy and ^{15}N- and ^{13}C,^{15}N-labeled Aβ(1–40) peptide. In agreement with our previous study, at pH 7.4 both ions bind to the specific ligands, competing with one another and displaying similar binding affinities. In contrast, at pH 5.5 only Cu(II) retains its specific histidine binding ligands, while Zn(II) seems to lose its residue specificity in the interactions. Since Cu(II) binding and acidosis linked to inflammatory processes *in vivo* have been suggested to enhance the neurotoxicity of Aβ [5], our observations suggest a rational molecular explanation for these effects: Under non-acidic conditions the toxic effects of redox active Cu(II) binding to Aβ

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(involving e.g. formation of Reactive Oxygen Species (ROS)) [14] may be attenuated by the protective activity of non-redox active Zn(II) binding to the same major binding site. In contrast, acidic conditions would decrease or change the protective effect of Zn(II), which is less able to compete with Cu(II) for the specific binding site on Aβ at low pH.

2. Materials and methods

2.1. Sample preparation

Unlabeled, ^{15}N-labeled and ^{13}C,^{15}N-labeled Aβ(1–40) were bought from AlexoTech (Umeå, Sweden) and prepared as previously described [15,16] with some modifications. First the peptide was dissolved in 10 mM NaOH to a peptide concentration of 1 mg/ml. After 1 min of sonication in ice bath, chilled double distilled water was added to half the final sample volume. After another sonication step sodium phosphate buffer (for samples of pH 7.4 and 6.5) or sodium acetate buffer (for samples of pH 5.5) was added to a final concentration of 10 mM. However, at pH 5.5 we are very close to the pI of the peptide (pI ≈ 5.5) [16], which made it difficult to obtain a stable sample, especially with the ^{13}C,^{15}N-labeled peptide. Thus, an alternative protocol was developed where the ^{13}C,^{15}N-labeled Aβ(1–40) peptide was dissolved in 5 mM DCl (Larodan AB, Malmö, Sweden), sonicated in an ice bath for 1 min and mixed with deuterated sodium acetate buffer (10 mM final buffer concentration) based on acetic-d_3 acid-d (Sigma, St. Louis, MO, USA). The pH was adjusted to 5.5 with 10 mM NaOD (Merck, Darmstadt, Germany). This protocol yielded a more stable Aβ sample during the preparation, and the deuterated solutions were used to avoid the water signal in the ^{1}H–^{13}C HSQC spectra. In order to minimize aggregation, sample preparation was always carefully performed on ice.

2.2. NMR spectroscopy

Bruker Avance 500 and 700 MHz spectrometers were used to record NMR spectra of 75 μM isotope-labeled Aβ(1–40) peptide at 5 °C. Both machines were equipped with triple-resonance cryogenically cooled probeheads, and all spectra were referenced according to the water signal and analyzed in nmrPipe and Sparky [17,18].

The 500 MHz NMR spectrometer was used to record ^{1}H–^{15}N heteronuclear single quantum correlation (HSQC) spectra when 10, 20, 30, 40 and 50 μM copper chloride or zinc acetate was titrated to ^{15}N-labeled Aβ(1–40) peptide dissolved in 10 mM phosphate buffer at pH 7.4 and 6.5, or dissolved in 10 mM sodium acetate buffer at pH 5.5 (90/10 H_2O/D_2O).

The 700 MHz NMR spectrometer was used to various assignment experiments, and to record ^{1}H–^{13}C HSQC spectra when 5, 10, 15, 20, 25 and 30 μM copper chloride was titrated to ^{13}C,^{15}N-labeled Aβ(1–40) peptide dissolved in 10 mM phosphate buffer at pH 7.4 and 6.5, or dissolved in 10 mM acetate buffer at pH 5.5 (100% D_2O).

We have previously published the assignment of the ^{1}H–^{15}N HSQC spectrum of Aβ(1–40) at pH 7.4 [19]. Using this known assignment it was straightforward to obtain the assignment of the ^{1}H–^{15}N HSQC cross-peaks of Aβ(1–40) at pH 6.5 and 5.5 by following the chemical shift changes at decreasing pH. To assign additional peaks, i.e. A2 and the histidines, NOESY–HSQC (^{1}H–^{15}N), TOCSY–HSQC (^{1}H–^{15}N), HNCA and HN(CO)CA experiments were carried out. ^{1}H–^{13}C cross-peaks at pH 5.5 were assigned by HNCA and HN(CO)CA together with the assignment at pH 7.4 communicated by Zagorski [20]. For the quantitation of spectra, the cross-peak amplitudes were evaluated.

2.3. CD spectroscopy

A Chirascan CD unit from Applied Photophysics was used to monitor each titration step when copper and zinc acetate was titrated to a 20 μM solution of Aβ(1–40) peptide dissolved in either 20 mM sodium phosphate buffer at pH 7.4 or 20 mM sodium acetate buffer at pH 5.5. The sample was held in a 2 mm pathlength quartz cuvette with 400 μl sample volume. CD spectra were recorded at 25 °C in the region 190–270 nm using a step size of 2.5 nm and a 1 nm slit size. Zinc and copper acetate was added in metal-to-Aβ-peptide molar ratios of 1:2, 2:2, 3:2, 5:2, 8:2, 13:2, 19:2, 30:2, and 50:2.

3. Results

Two-dimensional ^{1}H–^{15}N- and ^{1}H–^{13}C HSQC spectra were recorded at 5 °C for respectively ^{15}N- and ^{13}C,^{15}N-labeled Aβ(1–40) peptides at the three pH values 5.5, 6.5, and 7.4. Titrations with copper and zinc were found to reduce the peak intensities in the HSQC spectra, and the patterns of peak intensity reduction varied between the two metal ions and between the three different pH values.

Fig. 1A–C shows the ^{1}H–^{15}N HSQC spectra of 75 μM ^{15}N-labeled Aβ(1–40) alone and in the presence of 30 μM copper chloride at 5 °C at pH 7.4, 6.5 and 5.5. Fig. 2A–C shows the intensities of the NH cross-peaks along the peptide sequence at these pHs. At pH 7.4 the cross-peak intensities corresponding to the residues near the N-terminus of Aβ(1–40), i.e. residues 1–17, are around 20% percent of the peak intensities without copper. This is consistent with previous results indicating that metal ions bind to histidines 6, 13, and 14 of Aβ(1–40) [10]. Also the protons belonging to the central and C-terminal residues of Aβ(1–40) display reduced intensities, around 60–70% of the peak intensities without copper. There are no obvious chemical shift changes accompanying the loss of signal intensities. For pH 6.5 a similar pattern can be seen, although the intensity ratios of the amide protons are below 10% near the N-terminus and around 20–30% for the central and C-terminal residues. For pH 5.5 the same intensity pattern as for pH 6.5 is observed. Thus, for all three studied pH values there appears to be specific binding of copper ions near the N-terminus, resulting in specific loss of ^{1}H–^{15}N cross-peak intensities in this part of the sequence.

Similar results were obtained in the ^{1}H–^{13}C HSQC spectra for the cross-peaks from C^{α}–H in the ^{13}C,^{15}N-labeled Aβ(1–40) (Supplementary Figs. S1 and S2). At pH 7.4 a major reduction in peak intensity is seen for the N-terminal residues after addition of copper, while a smaller peak intensity reduction is seen for the central and C-terminal residues. At pH 5.5 all C^{α}–H cross-peak intensities are reduced to around 5% of their original intensity after addition of copper. It should be mentioned that the Aβ samples slightly aggregated during the titrations, and this aggregation was more pronounced at low pH. The ^{13}C,^{15}N-labeled sample was particularly prone to aggregate.

Fig. 1D–F shows the ^{1}H–^{15}N HSQC spectra of 75 μM ^{15}N-labeled Aβ(1–40) alone and in the presence of 30 μM zinc acetate at 5 °C at pH 7.4, 6.5 and 5.5. Fig. 2D–F shows the ^{1}H–^{15}N HSQC cross-peak intensities at these pHs. At pH 7.4 the results are similar to those of the copper addition – i.e. significant signal loss of amide cross-peak intensities for residues 1–16, and a smaller loss of signal – around 80% remaining – for the central and C-terminal residues 17–40. At pH 6.5 the specific intensity loss of cross-peaks from residues at the N-terminus is less pronounced. At pH 5.5 the cross-peak intensity for all residues is reduced to around 70% of the original intensity after zinc addition. This overall signal intensity ratio at pH 5.5 is much higher than the overall signal intensity ratio after addition of copper, which is about 15% at pH 5.5. At pH 6.5 the overall amide proton intensity ratio is approximately intermediate

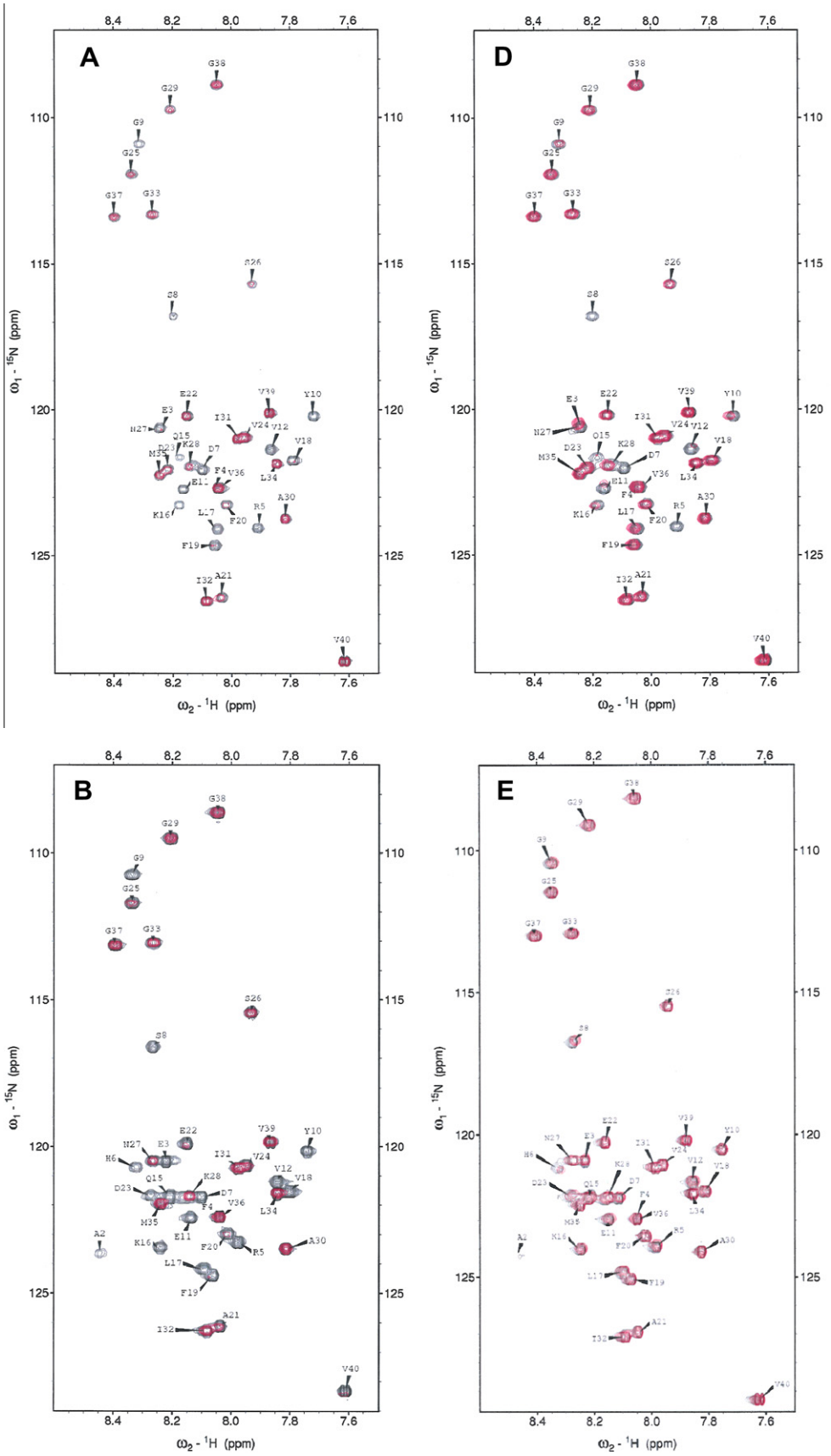

Fig. 1. $^{1}H–^{15}N$ HSQC spectra of 75 μM ^{15}N-Aβ(1–40) peptide alone (black peaks) and after addition of metal ions (red peaks, overlaid). The spectra were recorded at 5 °C after addition of 30 μM Cu(II) chloride at (A) pH 7.4, (B) pH 6.5 and (C) pH 5.5, or addition of 30 μM Zn(II) acetate at (D) pH 7.4, (E) pH 6.5 and (F) pH 5.5.

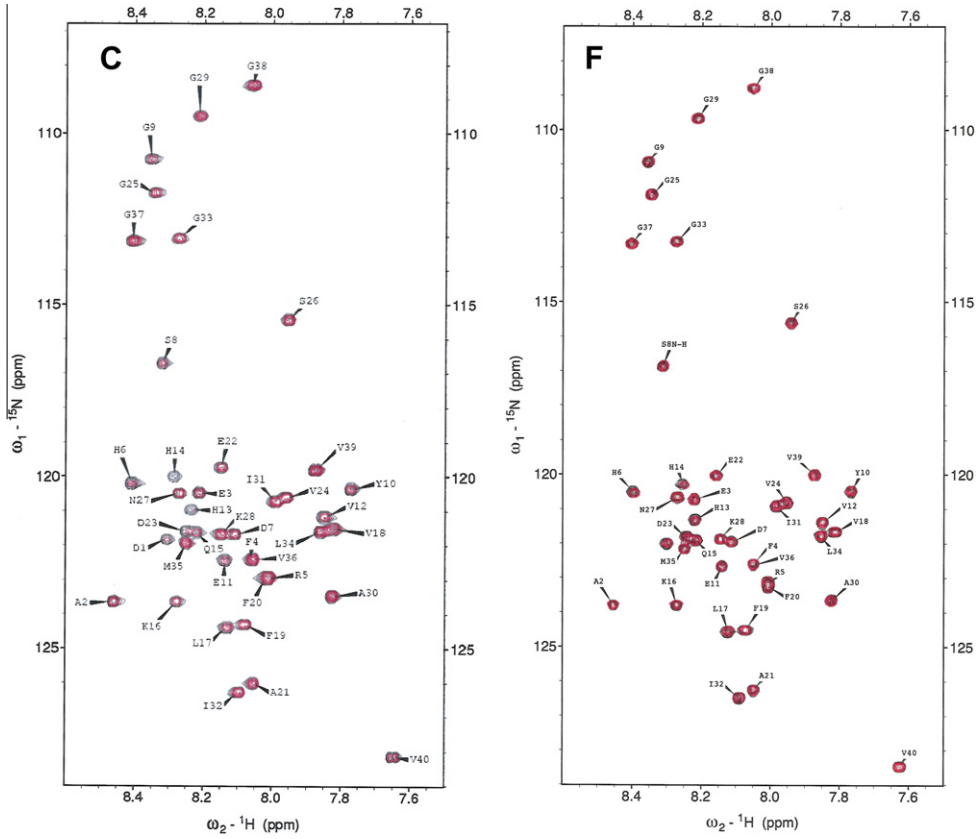

Fig. 1 (*continued*)

between the value for pH 7.4 (80%) and pH 5.5 (70%). We conclude that the sequence-specific interaction effects of the non-paramagnetic Zn(II) ion are best observed at pH 7.4 but not at pHs below 6.5.

CD spectroscopy was used to monitor structural changes in the Aβ(1–40) peptide upon titration with copper and zinc acetate, both at pH 5.5 and 7.4 at 25 °C. At pH 7.4, it is clear that addition of copper promotes a structural transformation from random coil to anti-parallel β-sheet (Fig. 3B). The same transition occurs at pH 5.5 at lower metal concentrations, suggesting a stronger effect of copper on the Aβ(1–40) structure at lower pH values (Fig. 3A). When zinc is added to Aβ(1–40) at pH 7.4 a similar structural transformation from random coil to anti-parallel β-sheet takes place, but even at a zinc-to-Aβ peptide ratio of 50:2 this transformation is only partial. The CD spectrum at the end of the titration displays a mixture of random coil and β-sheet structure (Fig. 3D). At pH 5.5 the zinc titration at the highest metal concentrations converts all Aβ(1–40) peptide into the anti-parallel β-sheet conformation, indicating that also the effect of zinc on Aβ structure is stronger at low pH values (Fig. 3C). For reference purposes a titration was carried out with sodium acetate at pH 7.4 (Fig. 3E). It is clear that in the absence of divalent ions, the kinetic effects on the peptide spectra are insignificant during the time of the titration. We conclude that the CD spectra show that both Cu(II) and Zn(II) ions promote the formation of anti-parallel β-sheet secondary structure, with pH 5.5 being somewhat more favorable than pH 7.4 for the structure transformation, and with Cu(II) being somewhat more potent than Zn(II) at both pHs.

4. Discussion

The overall loss of solution NMR signal coupled to no or minor chemical shift changes is a phenomenon observed in many studies of aggregating proteins and peptides. The simplest explanation is protein/peptide precipitation or formation of huge aggregates that are too large to be observed in solution NMR. However, in some cases where the signal loss is not complete, it has been possible to link it to formation of oligomeric states of the protein/peptides. It has been shown that the polymorphic oligomeric "dark" (NMR invisible) state of an aggregating species can be in slow chemical exchange with the free monomer and affect its relaxation properties [21]. In their study Fawzi et al. could show the presence of large heterogeneous oligomers of Aβ(1–40) via a monomer-to-oligomer exchange process on the 10 ms time scale which affects the NMR relaxation rates.

The present results can be discussed from a similar perspective, although it should be taken into account that whereas Cu(II) is paramagnetic, Zn(II) is not. Since both ions give similar results at pH 7.4 (compare Fig. 2A and D), paramagnetism may be only a partial cause of signal loss even for the Cu(II) sample. At pH 7.4 the overall signal reduction is not large in the C-terminus of the peptide. We therefore suggest that both metal ions bind to the N-terminus of the Aβ peptide, inducing an NMR-invisible metal-bound form of the peptide. This would explain the residue-specific loss of NMR signal from the N-terminal amino acids. The residue-specific effects remain observable in the Cu(II) sample also at the lower pHs, whereas they are partly lost for the Zn(II) sample at pH 6.5 and completely lost at pH 5.5.

Residue-specific loss of signals in a ^{1}H–^{15}N HSQC spectrum has previously been observed in an NMR study of α-synuclein in partial association with phospholipid vesicles [22]. In that case, the N-terminal residues lost part of their signal intensities, without any change in chemical shifts. The protein was proposed to be in slow exchange between a free form and multiple long-lived lipid-bound, and hence NMR invisible forms, involving the N-terminus of the protein [22]. In the present case, the NMR invisibility of the amide

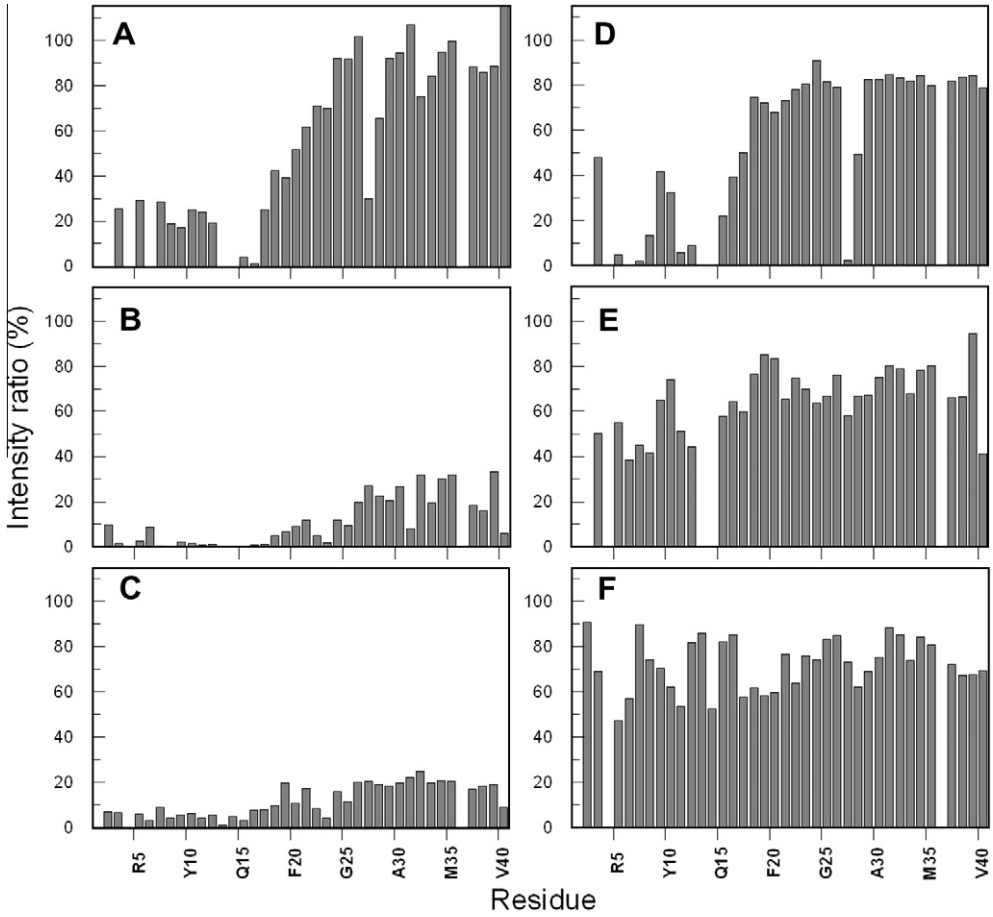

Fig. 2. $^{1}H–^{15}N$ HSQC cross-peak intensities evaluated as amplitudes of 75 μM ^{15}N-Aβ(1–40) peptide after addition of 30 μM Cu(II) chloride or Zn(II) acetate at 5 °C, given as ratios to the individual peak intensities without metal. (A) Cu(II), pH 7.4, (B) Cu(II), pH 6.5, (C) Cu(II), pH 5.5, (D) Zn(II), pH 7.4, (E) Zn(II), pH 6.5, (F) Zn(II), pH 5.5.

cross-peaks of the N-terminal residues involved in metal binding could possibly be due to structure heterogeneity around the metal binding site giving rise to a broad signal of low intensity. Alternatively the chemical exchange may be intermediate on the NMR time scale. The observation that signal attenuation by metal ions also is seen in the C^{α}–H cross-peaks (Supplementary Figs. S1 and S2) shows that the amide cross-peak attenuation effects cannot be related to increased amide proton exchange due to interactions.

The results also show that the overall signal intensities become reduced in the Cu(II) sample of Aβ(1–40) towards the lower pHs (Fig. 2). This suggests a situation similar to what was observed for the heterogeneous and large oligomeric forms of Aβ in the study by Fawzi et al. [21], with the difference that the aggregation phenomena observed here are due to metal ion interactions and not to environmental conditions like peptide concentration and temperature. For the paramagnetic Cu(II) ions, the great loss of signal intensities may partly be due to additional paramagnetic broadening. The CD results support the presence of an aggregated peptide state by showing metal-induced formation of β-structure. The more potent β-structure induction by Cu(II) ions compared to Zn(II) ions is also in line with a more extensive aggregation induction by Cu(II).

The striking difference between the NMR spectra observed for Aβ in the presence of Cu(II) and Zn(II) may reflect different pH-dependencies of their respective binding specificity for the Aβ peptide. At neutral pH the NMR observations can clearly identify the likely metal ligands for both ions, and they can compete for the same binding site (His 6,13,14 and the N-terminus). At the lower pH (5.5) only Cu(II) retains its specific binding pattern and ligands, whereas the residue specificity for Zn(II) is apparently lost or changed. These results are partially in line with Aβ/Cu(II) interaction studies with EPR spectroscopy, which suggest multiple binding sites with pH-dependent occupancies [23]. Although the reappearance at pH 5.5 of the signals from the N-terminal residues of the Zn(II) sample may partly be due to a different kinetic regime, the observations suggest that the Cu(II) and Zn(II) ions no longer compete for the same specific binding site.

The observed differences in pH-dependence of specific binding of Cu(II) and Zn(II) ions to Aβ(1–40) suggest a simple chemical background that may be partially responsible for the reported links between amyloid disease, chronic inflammation and physiological acidosis. Although there is evidence for Aβ oligomers being a "toxic species" [24], it is also clear that Cu(II) enhance Aβ toxicity [5], and that both Cu(II) and Zn(II) are accumulated in Alzheimer's disease amyloid plaques [25]. Most likely there are several mechanisms by which metal ions interact with the Aβ peptide at different stages of its aggregation, thereby affecting the toxicity of the peptide. Our current results show that Cu(II) and Zn(II) ions at neutral pH compete for the same specific binding site on the Aβ peptide. Towards lower pH the redox-active Cu(II) ion does not experience the same competition from Zn(II) for residue specific binding to Aβ. Cu(II) could therefore have a relatively higher peptide occupancy at low pH than at neutral pH. The Cu(II) ion bound to Aβ will be able to mediate increased formation of peptide aggregates and of cell toxic ROS in its environment, and thus make a larger contribution to the Aβ-related damage to the neighboring tissue.

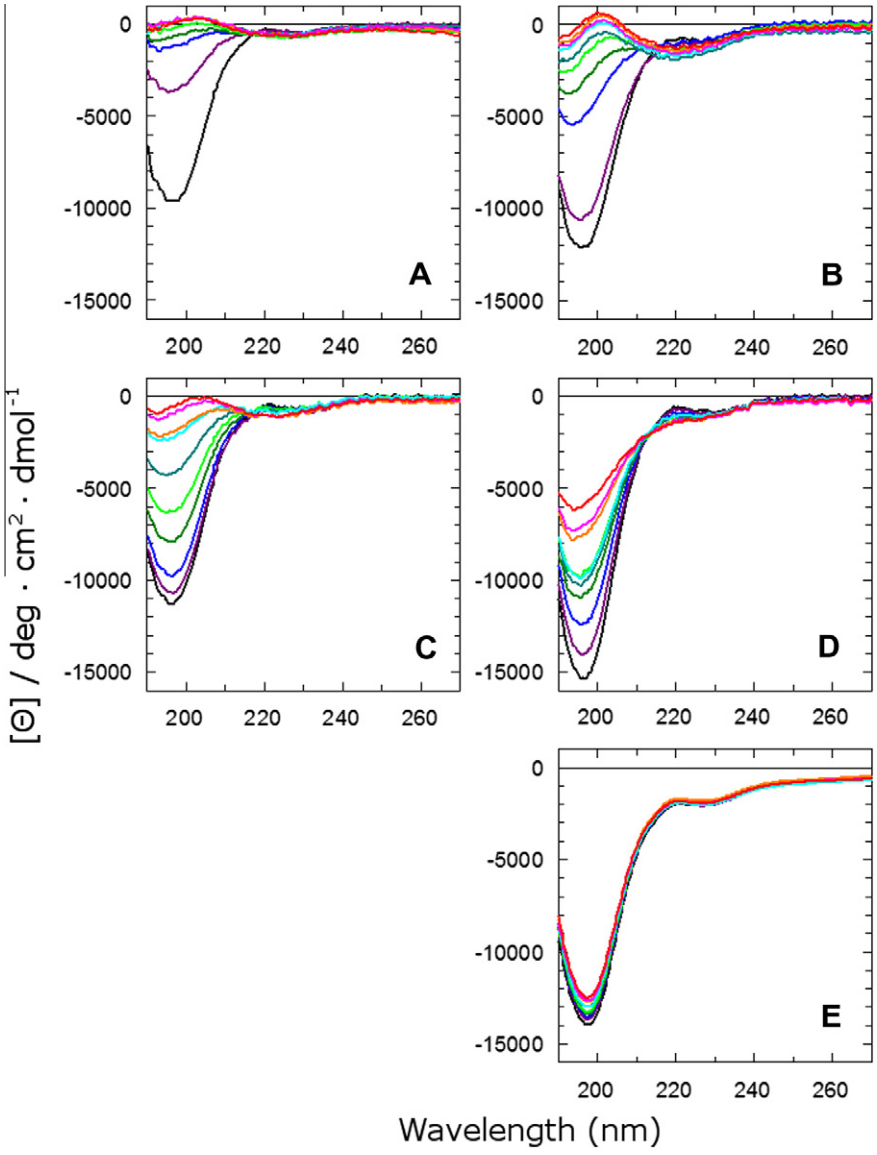

Fig. 3. CD titrations of Cu(II), Zn(II), and Na acetate to 20 μM Aβ(1–40) peptide in a 20 mM sodium phosphate buffer at 25 °C. (A) Cu(II) at pH 5.5; (B) Cu(II) at pH 7.4; (C) Zn(II) at pH 5.5; (D) Zn(II) at pH 7.4; (E) Na at pH 7.4. Metal-to-Aβ ratios were 0:2 (black); 1:2 (purple); 2:2 (dark blue); 3:2 (dark green); 5:2 (green); 8:2 (dark cyan); 13:2 (cyan); 19:2 (orange); 30:2 (magenta); and 50:2 (red).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.043.

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