Peptide Aggregation

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The Aggregated State of Amyloid-β Peptide In Vitro Depends on Cu^{2+} Ion Concentration**

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Despite significant progress, there is still uncertainty about the molecular basis for the onset of Alzheimer's disease. The misfolding of amyloid-β peptide (Aβ), wherein soluble peptide aggregates form plaques, is central to this process. Recent research indicates that intermediates of Aβ formed at the early stages of aggregation of Aβ are more toxic than the fully formed fibrils.[1] A leading hypothesis for the toxicity is the generation of hydrogen peroxide during the early stages of aggregation.[1b] Results in vitro indicate that metal ions like Zn^{2+} and Cu^{2+} ions affect the rate of formation of fibrils.[2] Even more intriguingly, it is believed that Cu^{2+} ions stabilize the toxic early-stage aggregates of Aβ.[3] The high concentrations of metal ions in plaques found in the brains of Alzheimer's patients suggest that the results in vitro have real significance in vivo.[4] Herein, we show that the copper-binding geometry in aggregated Aβ changes with copper concentration. The overall morphology of the aggregates also depends on the concentration of Cu^{2+} ions. Together, the data suggest that microscopic metal–Aβ interactions play a major role in dictating the aggregated state of Aβ. Our results also indicate that the misfolding mechanism of Aβ is dependent on the concentration of Cu^{2+} ions.

Figure 1 shows the continuous wave (CW) electron spin resonance (ESR) spectra of Cu^{2+} ions bound to Aβ(1-40) aggregates at three different metal/peptide ratios. To prepare aggregates, Aβ(1-40) was incubated at 37 °C for 4–5 days in the presence of an appropriate concentration of Cu^{2+} ions. All experiments were performed at a pH value of 7.4 to minimize the ESR signal owing to aqueous Cu^{2+} ions.[5] The CW-ESR spectra provide information on strong interactions between the Cu^{2+} electron and neighboring nuclei. The magnitude of $g_{\|}$ (the parallel component of the axially symmetric g tensor) and $A_{\|}$ (the parallel component of the hyperfine interaction) are dependent on, among many factors, the ligand environment of Cu^{2+} ions.[6] At low [Cu^{2+}]:[Aβ] ratios (shown in Figure 1a), the spectrum has the value, $g_{\|} = 2.27$ and $A_{\|} =$ 160G, which suggests that the Cu^{2+} ion has a three nitrogen and one oxygen coordination environment.[5,6] With increasing amounts of Cu^{2+} ions, a new set of hyperfine lines, shown by the gray lines in Figure 1, is observed. These lines have a $g_{\|} = 2.24$ and $A_{\|} = 190$G (Figure 1b and c), which corresponds to four nitrogen ligands.[5,6] These data suggest that the Cu^{2+} ion is coordinated to the peptide in a nitrogen-rich site and suggests that another binding mode may be present at higher Cu^{2+} ion concentrations. The second Cu^{2+} binding site may be due to coordination with the ^{14}N of the neighboring amide bonds.[7]

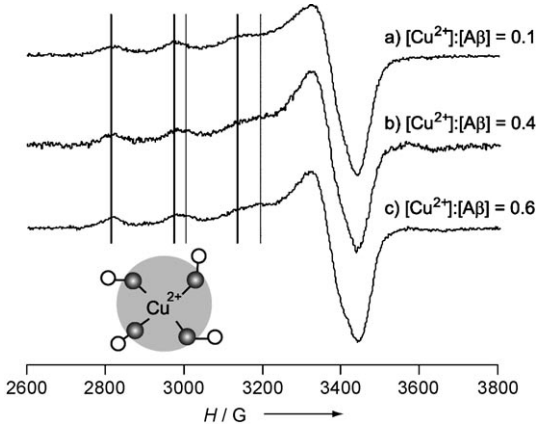

Figure 1. CW-ESR spectra of Cu^{2+} ions in aggregates of Aβ(1-40). The spectra are at a [Cu^{2+}]:[Aβ] mole equivalent ratio of a) 0.1, b) 0.4, and c) 0.6. All spectra were collected at 80 K, with microwave power = 1.993 mW, modulation amplitude = 1 G, time constant = 40.96 ms, conversion time = 81.92 ms, and a sweep width of 1200 G. The data indicate a nitrogen-rich coordination environment. H = magnetic field.

The CW-ESR results are complemented by pulsed electron spin echo envelope modulation (ESEEM) ESR spectroscopy, which probes the interactions of the electron spin with more-distant nuclei. Figure 2 shows the three-pulse ESEEM spectra of three different Cu^{2+} ion concentrations on Aβ(1-40). In Figure 2a, the ESEEM spectrum of [Cu^{2+}]:[Aβ] at a ratio of 0.1 contains peaks below 5 MHz. The three-pulse ESEEM experiment detects the modulation of the Cu^{2+} spin echo from the ^{14}N nuclei ($I = 1$) of surrounding amino acids, and the remote nitrogen of the histidine imidazole provides characteristic frequencies in the ESEEM spectrum.[5,8] In Figure 2a, the peaks below 2 MHz can be assigned to a nuclear quadrupole interaction and the peak around 4 MHz is due to a double quantum transition of the remote nitrogen of the histidine imadazole.[5,8] This is direct evidence that the Cu^{2+} ion is bound to the N-terminal region of Aβ(1-40), which includes three histidines (H6, H13, and H14), as has been proposed before by the work of Syme et al. and Karr et al.[7,9]

The presence of spectral features between 2–4 MHz indicates that electron spin of the Cu^{2+} ion interacts with

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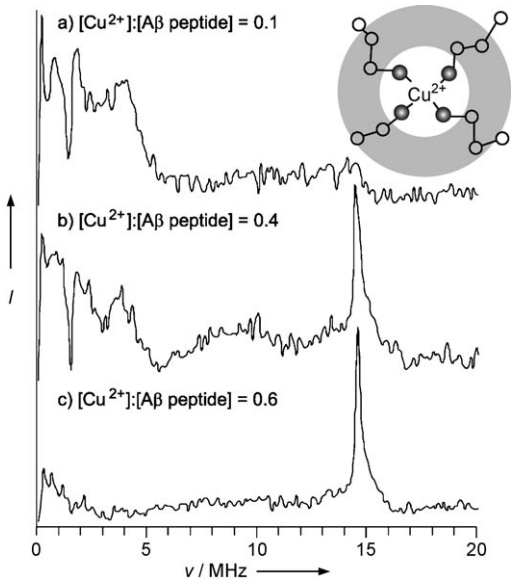

Figure 2. Three-pulse ESEEM spectra of aggregated Aβ(1-40). The data are at a Cu^{2+} ion to peptide ratio of a) 0.1, b) 0.4, and c) 0.6. At low $[Cu^{2+}]$:[Aβ] ratios, the peaks below 5 MHz indicate that the Cu^{2+} ion is coordinated to at least one histidine of the Aβ peptide. This coordination weakens as the $[Cu^{2+}]$:[Aβ] ratio increases. The experiments were performed at 20 K by using a $\pi/2$–τ–$\pi/2$–T–$\pi/2$ pulse sequence with a $\pi/2$ pulse width = 16 ns, τ = 120 ns, T increment = 16 ns, and 100 averages per point.

more than one nitrogen, which is consistent with interaction with multiple histidines.[10] The Cu^{2+} ion coordination might also be associated with the ^{14}N of the neighboring amide bonds.[5b] Indeed, NMR experiments suggest that the amide bond between E3-V18 may be involved in metal binding.[11] The large line widths indicate that the Cu^{2+} ion coordination environment is heterogeneous.

Systematic ESEEM experiments indicate that the peaks below 5 MHz become broader as the Cu^{2+} ion concentration increases, and the peaks therefore have increasingly lower amplitudes (see the Supporting Information). Furthermore, another peak at approximately 14 MHz emerges. Figure 2b shows the results at a $[Cu^{2+}]$:[Aβ] ratio of 0.4. The peak at 14 MHz, in addition to the peaks below 5 MHz, is clear. In Figure 2c, where the $[Cu^{2+}]$:[Aβ] ratio is 0.6, the peak at approximately 14 MHz is dominant. The 14 MHz peak is characteristic of electron–nuclear interactions between the unpaired electron and proton spins (note that ESEEM does not probe directly coordinated nuclei).[12] The 14 MHz proton ESEEM peak in Figure 2c is eliminated when the sample is prepared in D_2O, indicating that the peak is due to the interaction with hydrogen from the solvent or from exchangeable protons (see the Supporting Information). Together, the CW-ESR and ESEEM results suggest that the 3N–1O coordination becomes more heterogeneous and water accessible as the $[Cu^{2+}]$:[Aβ] ratio increases.

TEM images of the aggregated state of Aβ also demonstrate a transition with an increasing Cu^{2+} ion concentration, which is in accord with the ESR results. In Figure 3a, at low concentrations of Cu^{2+} ions, the TEM image shows that Aβ(1-40) forms fibrillar precipitates that have an extensive linear

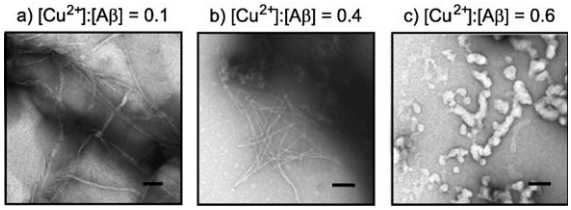

Figure 3. Negatively stained TEM images of aggregated Aβ(1-40). The scale bars correspond to 100 nm. The images are for aggregates prepared at a $[Cu^{2+}]$:[Aβ] ratio of a) 0.1, b) 0.4, and c) 0.6 at 37 °C for 4–5 days. The overall morphology changes as the concentration of the Cu^{2+} ions increases.

shape. At high Cu^{2+} ion concentrations (Figure 3c), short and granular aggregates result, and this morphology resembles that of previously described early-stage intermediates.[1b,13] At 0.4 mol equivalents of Cu^{2+} to the peptide (see Figure 3b) both linear fibrils and granular aggregates are observed.

The changes in morphology of aggregated Aβ with Cu^{2+} ions are different from those observed for the case of Zn^{2+} ions. Lynn and co-workers showed that the fibril morphology changes from a thin-linear shape to thick ribbons as the $[Zn^{2+}]$:[Aβ] ratio increases.[14] These differences in morphology might be due to the different coordination environments of the two metals. At a pH value of 7.4, the Zn^{2+} ion binding site involves interpeptide interactions and is believed to lie between two different peptide sheets.[3a,15] The Cu^{2+} ion binding geometry is largely intrapeptide in nature,[3a,15] especially at low Cu^{2+} ion concentrations as demonstrated by the ESR results (Figures 1 and 2).

Our ESR and TEM results demonstrate a correlation between specific Cu^{2+} ion coordination and the overall morphology of aggregates. At low $[Cu^{2+}]$:[Aβ] ratios, the Cu^{2+} ion is coordinated to the histidine(s) of the Aβ peptide. As the $[Cu^{2+}]$:[Aβ] ratio increases, this coordination becomes more heterogeneous and the metal becomes exposed to water or to exchangeable protons on the peptide. Images obtained by using TEM indicate that at low concentrations of Cu^{2+} ions, the aggregates are mature fibrils, whereas at high Cu^{2+} ion concentrations, granular aggregates result. Together, these data suggest that the misfolding mechanism is dependent on Cu^{2+} ion concentration. In future, a systematic understanding of the atomic-level structural details of Cu^{2+}–Aβ interactions will be generated by isotope labeling and pulsed ESR to shed light on the mechanism of Aβ aggregation.

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