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Amyloid-β Binds Cu^{2+} in a Mononuclear Metal Ion Binding Site

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Abstract: Amyloid-β (Aβ) peptide is the principal constituent of plaques associated with Alzheimer's disease and is thought to be responsible for the neurotoxicity associated with the disease. Metal ions have been hypothesized to play a role in the formation and neurotoxicity of aggregates associated with Alzheimer's disease (Bush, A. I.; et al. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 11934). Elucidation of the chemistry through which transition-metal ions participate in the assembly and toxicity of Aβ oligomers is important to drug design efforts if inhibition of Aβ containing bound metal ions becomes a treatment for Alzheimer's disease. In this paper, we report electron paramagnetic resonance (EPR) spectroscopic characterization of Cu^{2+} bound to soluble and fibrillar Aβ. Addition of stoichiometric amounts of Cu^{2+} to soluble Aβ produces an EPR signal at 10 K with observable Cu^{2+} hyperfine lines. A nearly identical spectrum is observed for Aβ fibrils assembled in the presence of Cu^{2+}. The EPR parameters are consistent with a Type 2 Cu^{2+} center with three nitrogen donor atoms and one oxygen donor atom in the coordination sphere of Cu^{2+}: $g_{\|} = 2.26$ and $A_{\|} = 174 \pm 4$ G for soluble Aβ with Cu^{2+}, and $g_{\|} = 2.26$ and $A_{\|} = 175 \pm 1$ G for Aβ fibrils assembled with Cu^{2+}. Investigation of the temperature dependence of the EPR signal for Cu^{2+} bound to soluble Aβ or Cu^{2+} in fibrillar Aβ shows that the Cu^{2+} center displays normal Curie behavior, indicating that the site is a mononuclear Cu^{2+} site. Fibrils assembled in the presence of Cu^{2+} contain one Cu^{2+} ion per peptide. These results show that the ligand donor atom set to Cu^{2+} does not change during organization of Aβ monomers into fibrils and that neither soluble nor fibrillar forms of Aβ(1−40) with Cu^{2+} contain antiferromagnetically exchange-coupled binuclear Cu^{2+} sites in which two Cu^{2+} ions are bridged by an intervening ligand.

A central, unresolved question in the pathophysiology of Alzheimer's disease (AD) relates to the role of metal ions in plaque formation and neurodegeneration. AD plaques, containing fibrils composed of the 39−42-residue amyloid-β (Aβ) peptide, are thought to be linked to neurodegeneration in AD.[1] Metal ions have been proposed to play a significant role in the assembly and neurotoxicity of AD fibrils.[2,3] Transition-metal ions can contribute to the neuropathology associated with Aβ fibrils by affecting the rate of fibril formation,[2−4] by modifying fibril morphology,[5,6] and by direct chemical reaction with Aβ.[7−9] Administration of a metal ion chelator decreases deposition of Aβ in the brains of transgenic mice[10] and releases soluble Aβ from preformed amyloid deposits,[11] supporting the hypothesis that metal ions are incorporated in AD plaque architecture in vivo. Advances in selective chelation therapy to combat AD[12] require that details of the binding sites of relevant metal ions be known and that the mechanism through which metal ions participate in fibrillization events be better understood.

In vitro study of Aβ with Cu^{2+} and Zn^{2+} continues to be used as a model for the role of metals in fibril formation in vivo.[5−7,13,14] The Cu^{2+} coordination environment in Aβ has been

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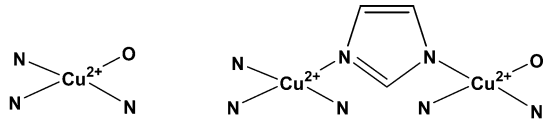
Chart 1. Mononuclear and Binuclear Cu^{2+} Ion Sites[a]



Mononuclear Type 2 Cu^{2+} | Imidazolate-bridged binuclear Cu^{2+}

[a]The mononuclear Cu^{2+} site has three nitrogen donor atoms and one oxygen donor atom (3N1O). A binuclear Cu^{2+} site could form when the oxygen atom donor on one Cu^{2+} is replaced by a deprotonated histidine residue, which bridges two Cu^{2+} ions.[7]

probed by Raman and electron paramagnetic resonance (EPR) spectroscopies.[8,15] EPR spectroscopy is an accepted method for determining the ligand donor atom identities for Cu^{2+} sites through measurement of the magnitude of the Cu^{2+} hyperfine coupling constant, $A_{||}$, and the corresponding g value, $g_{||}$.[16] When 1 equiv of Cu^{2+} is added to soluble Aβ(1−40), the Cu^{2+} EPR spectrum indicates that three nitrogen donor ligands and one oxygen donor ligand (3N1O) are in the Cu^{2+} coordination sphere.[8] Additional EPR spectroscopic studies of Cu^{2+} with soluble Aβ(1−28), a shortened version of Aβ(1−40) that also forms aggregates, suggest the existence of multiple soluble copper−peptide species as a function of the Cu^{2+}:peptide ratio.[7] In contrast to the EPR results, Raman measurements of Cu^{2+} bound to soluble Aβ suggest that the Cu^{2+} ligands are histidine and deprotonated amides.[15] Characteristic Raman bands for Cu^{2+} bound to histidine were observed for aggregates of Aβ(1−40) assembled in the presence of Cu^{2+}, suggesting that Cu^{2+} cross-links β-sheets via histidines from multiple peptides. Together, these data indicate that Aβ oligomers containing histidine-bridged Cu^{2+} centers are possible precursors to metal-cross-linked β-sheets.[5] A binuclear imidazolate-bridged Cu^{2+} site (Chart 1), similar to that found in copper, zinc superoxide dismutase (Cu,Zn-SOD), has been proposed as a possible structure for soluble Aβ(1−28) in the presence of stoichiometric amounts of Cu^{2+}.[7] Controversy about the coordination environment of Cu^{2+} in soluble vs fibrillar forms of Aβ requires that further work be undertaken to firmly establish the Cu^{2+} coordination environment in Aβ.

We have used low-temperature EPR spectroscopy to determine the ligand donor atoms to Cu^{2+} in fibrillar Aβ(1−40). Electron microscopy images show that our samples, assembled from Aβ(1−40) in the presence of Cu^{2+}, are Aβ fibrils. By inductively coupled plasma mass spectrometry (ICP-MS) and amino acid analysis, 1.1 Cu^{2+} ions bind per Aβ(1−40) peptide in our fibrils. The Curie temperature dependence of the EPR signal for Cu^{2+} bound to Aβ(1−40) in both soluble and fibrillar forms is consistent with Cu^{2+} in a mononuclear metal ion binding site in the peptide.

Materials and Methods

Commercially available Aβ(1−40) peptide was obtained from rPeptide (Athens, GA) or Bachem (King of Prussia, PA). Biological grade glycerol, Tris, and sodium chloride were purchased from Fisher. Quartz EPR tubes (4 mm o.d.) were purchased from Wilmad (Buena, NJ). Solutions were prepared in MilliQ water (resistivity >18 mΩ, total organic content <35 ppb).

Sample Preparation. Peptide was monomerized with hexafluoro-2-propanol (HFIP) according to literature procedures and stored at −80 °C in HFIP.[17] The peptide in HFIP was removed with a Hamilton gastight syringe that had been washed with multiple volumes of HFIP. Immediately prior to use of peptide, HFIP was removed using a spin-vacuum system. Preparation of samples by dissolution in HFIP followed by removal of HFIP produces homogeneous solutions of monomeric peptide.[18]

Samples of soluble Aβ(1−40) were made by dissolving dried peptide in buffer containing 100 mM Tris, 150 mM NaCl, pH 7.4, with 50% glycerol (v/v). Inclusion of glycerol as a cryoprotectant for biological samples is an accepted method for protecting sample fidelity.[19,20] We have attempted to use lower concentrations of glycerol in our samples, but find that the samples do not make good glasses, a prerequisite for EPR spectral collection at low temperatures. After the peptide was resuspended in buffer, an aliquot of the sample was removed for peptide concentration determination. The appropriate concentration of a Cu^{2+} stock solution in buffer was added to the remainder of the peptide sample. The sample was vortexed, transferred to a quartz EPR tube,[7,8] and frozen to 77 K prior to data collection. The time between addition of Cu^{2+} to the peptide and freezing of the sample is not sufficient to generate a substantial concentration of β-sheets as detected by thioflavin T fluorescence assay.[21]

For fibrillar samples, the peptide as prepared above was incubated at 37 °C for 7−14 days without agitation in the presence of Cu^{2+}. Samples were then assayed for fibril formation by transmission electron microscopy and thioflavin T fluorescence. Fibrils for EPR experiments were formed in 100 mM Tris, 150 mM NaCl, pH 7.4, with (Figures 2 and 3) or without (Figure 4) 50% glycerol (v/v), separated by centrifugation (30 min, 16000 rcf), washed once with 100 μL of buffer containing 50% glycerol (v/v), and resuspended in 100 μL of the same buffer. Fibrils for ICP-MS experiments were formed in 100 mM Tris, 150 mM NaCl, pH 7.4, separated by centrifugation, washed with 20 μL of water to remove salts, and then dried in a spin-vacuum system. ICP-MS was performed by the ICP-HEX-MS Laboratory in the Department of Geological Sciences at Michigan State University.[22]

Peptide concentrations were determined on the basis of the absorbance at 214 nm using a calibration curve generated with BSA standards from Sigma. The concentration of peptide in the stock solution also was measured by amino acid analysis (AAA Service Laboratory, Inc., Portland, OR). Amino acid analysis of fibrils without Cu^{2+}, but assembled from the same peptide stock solution used to prepare fibrils with Cu^{2+}, also was performed (AAA Service Laboratory, Inc.).[22]

The $^{63,65}Cu^{2+}$ stock solution was generated by dissolution of cleaned copper wire in nitric acid and water. The Cu^{2+} concentrations for samples in 100 mM Tris, 150 mM NaCl, pH 7.4, with 50% glycerol (v/v) were determined on the basis of a calibration curve generated from Cu^{2+} standards in the same buffer. The concentration of Cu^{2+} in the Cu^{2+} EPR standards was assayed by chelation with bath-

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(22) The Cu^{2+} concentration determined by ICP-MS for fibrils assembled in the presence of Cu^{2+} was 750 ± 75 ppb in 1 mL. By amino acid analysis, the concentrations of peptide determined for three injections of a 100 μL initial volume sample of fibrils were 105.17, 106.55, and 107.47 μM. The Cu^{2+} to peptide ratio is an estimate because standard amino acid analysis equipment is incompatible with high concentrations of metal ions.

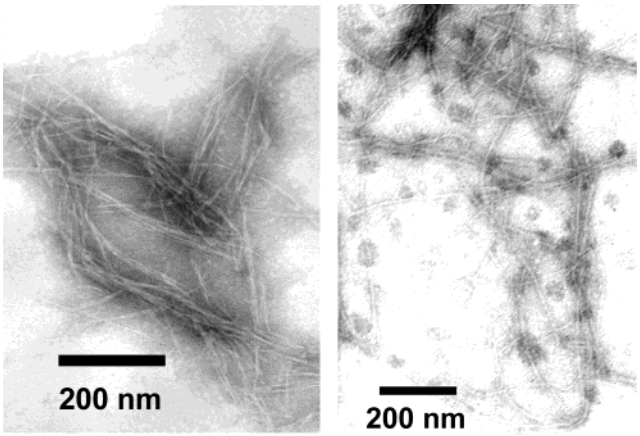

Figure 1. Negatively stained TEM images of aged solutions of Aβ(1−40) containing Cu^{2+}. Samples were prepared as described in the Materials and Methods and stained with 1% phosphotungstic acid (left) or 1% uranyl acetate (right). The magnification is 25000×.

ocuproinedisulfonic acid (BC) and reduction with ascorbate.[23] The quantity of total copper as $[Cu(BC)_2]^{3-}$ was quantified using ϵ_{483} = 12500 M^{-1} cm^{-1}.[23] The 0, 25, and 100 μM Cu^{2+} standards contained 0.0 ± 0.7, 26.9 ± 0.6, and 108.4 ± 0.6 μM Cu^{2+}, respectively.

Electron Microscopy. Electron microscopy images were collected at the University of Maryland, Baltimore Dental School with a JEOL JEMEX II transmission electron microscope. Samples were placed on 300-mesh Formvar films and stained with 1% phosphotungstic acid or 1% uranyl acetate.

EPR Spectroscopy. EPR spectra were collected on a Bruker EMX 6/1 spectrometer equipped with a frequency meter and an Oxford Instruments ESR900 liquid He cryostat system. Spectral collection parameters are given in the figure captions. Errors given for the peak widths at half-height are based on relative errors for the peak heights. Relative errors were calculated by dividing the average noise height in each spectrum by the measured peak height. Errors for the doubly integrated spectra were calculated from the maximum variation in peak height as a function of field position.

Results and Discussion

Characterization of Aβ(1−40) Fibrils Assembled in the Presence of Cu^{2+}. In our experiments, the pH of the buffers promotes the formation of fibrils over the formation of amorphous aggregates.[17] Electron microscopy clearly shows the presence of protofilaments and fibrils in aged solutions of Aβ-(1−40) peptide with Cu^{2+} (Figure 1). The width of the protofilaments is 6 ± 1 nm, which is similar to the width of protofilaments assembled in the absence of Cu^{2+}.[24,25] No amorphous aggregates were observed, confirming that EPR spectra of aggregates isolated by centrifugation and resuspended in buffer are of fibrils that contain Cu^{2+}.

To determine the Cu^{2+} to peptide ratio in fibrils, ICP-MS and amino acid analyses were performed on matched samples. By ICP-MS, fibrils assembled with Cu^{2+} contained 0.0118 ± 0.0013 μmol of Cu^{2+}. By amino acid analysis, fibrils contained 0.0106 ± 0.0001 μmol of peptide, giving an estimated ratio of 1.1 mol of Cu^{2+}/mol of peptide.[22] This result is consistent with the 1:1 stoichiometry for Cu^{2+} bound to soluble Aβ published by Garzon-Rodriguez et al.[26] Other Cu^{2+} to peptide ratios have been reported: approximately two Cu^{2+} ions per precipitated Aβ(1−40) and essentially none per soluble peptide.[13] These stoichiometries were determined by UV−vis spectrophotometric detection of soluble peptide (Micro BCA assay) and metal ions.[13] The ratio obtained by ICP-MS and amino acid analysis reported here is derived from direct measurements on fibrils. A possible explanation for the discrepancies in Cu^{2+}:peptide ratios could be the existence of multiple types of peptide precipitates, a common occurrence with amyloid peptides.[17,18,27] In our case, EM confirms that the solid samples assayed by ICP-MS and amino acid analysis are Aβ fibrils.

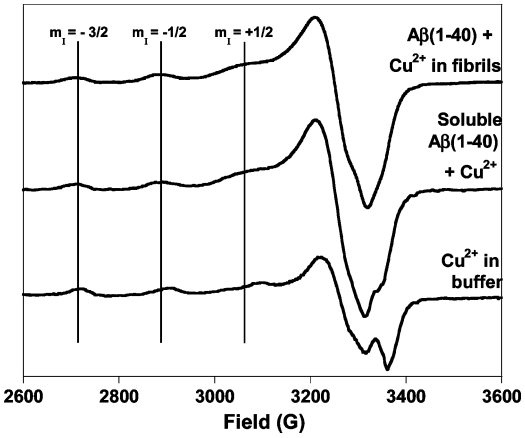

Figure 2. EPR spectra of Cu^{2+} in Aβ(1−40) fibrils, Cu^{2+} with soluble Aβ(1−40), or 25 μM Cu^{2+} in buffer. Hyperfine lines arising from the I = 3/2 ^{63,65}Cu nucleus are identified by the m_I value. Aβ(1−40) fibrils (37 μM initial peptide concentration) were assembled in the presence of 48 μM Cu^{2+}, separated by centrifugation, and washed to remove excess Cu^{2+}: $g_{||}$ = 2.26, $A_{||}$ = 175 ± 1 G. Soluble Aβ(1−40) with Cu^{2+} contains 50 μM Aβ and 50 μM Cu^{2+}: $g_{||}$ = 2.26, $A_{||}$ = 174 ± 4 G. All samples are in 100 mM Tris, 150 mM NaCl, pH 7.4, buffer with 50% glycerol (v/v). EPR conditions: T = 10 K; modulation amplitude 10 G; power 0.5 mW; gain 5 × 10^4; frequency 9.38 GHz; time constant 40.96 ms; conversion time 40.96 ms; four or eight scans.

Spectroscopic Measurements on Cu^{2+} Bound to Aβ(1−40). The coordination environment of Cu^{2+} in soluble or fibrillar forms of Aβ was monitored by EPR spectroscopy. EPR spectra collected at 10 K of soluble Aβ(1−40) with stoichiometric amounts of Cu^{2+} show Cu^{2+} EPR spectra with distinguishable hyperfine lines arising from the $^{63,65}Cu^{2+}$ I = 3/2 nucleus (m_I labels in Figure 2). The magnitudes of the $A_{||}$ and $g_{||}$ values are consistent with a Type 2 Cu^{2+} center with mostly nitrogen donor atoms (Figure 2).[16] Our results are in agreement with the previously proposed 3N1O coordination environment for Cu^{2+} bound to soluble Aβ(1−40).[8] The major differences in the EPR spectra for a Cu^{2+} center ligated to four N donor atoms rather than 3N1O coordination are in the magnitudes of the $A_{||}$ and $g_{||}$ values. On average, $A_{||}$ values are slightly higher and $g_{||}$ is slightly lower for 4N coordination than for 3N1O coordination.[16] When fibrils assembled in the presence of Cu^{2+} and glycerol are separated by centrifugation and washed with buffer that does not contain Cu^{2+}, the EPR spectrum of Cu^{2+} bound to fibrils is nearly identical to that observed for Cu^{2+} with soluble Aβ(1−40). The major difference between the spectra for the soluble and fibrillar forms of Aβ(1−40) containing Cu^{2+} is in the $g_\perp$ region of the spectrum. This region of the spectrum is sensitive to the sequence order of ligand donor atoms. Thus, even when the donor atom composition is 3N1O in both samples, $g_\perp$ varies

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depending on where a nitrogen atom donor such as histidine appears in the sequence.[28]

Our results show that the ligand donor atom set for Cu^{2+} does not change whether Cu^{2+} is bound to soluble Aβ or incorporated into Aβ fibrils. The $g_{||}$ and $A_{||}$ values for Cu^{2+} bound to soluble or fibrillar Aβ are most consistent with a donor atom set of 3N1O.[8,16] These results alone cannot be used to determine the identities of the ligating groups for Cu^{2+} bound to soluble and fibrillar Aβ(1−40). However, if ligands to Cu^{2+} do not change during fibrillogenesis, fibrils assembled in the presence of Cu^{2+} could contain distinct structural regions: the N-terminal segment of the peptide could bind Cu^{2+}, while the C-terminus could participate in β-sheet formation. This hypothesis is consistent with the demonstration that the N-terminal region of the peptide is not confined rigidly in mature fibrils.[29] The amino acids that have been proposed to bind Cu^{2+} in Aβ are between residues 6 and 14,[7,15,30] suggesting that this region of the peptide might be available to bind metal ions in fibrils without disrupting fibril formation.

Solid-state NMR structural studies on Aβ fibrils have shown that fibrils contain parallel β-sheets.[25,31] The interstrand distance in the parallel β-sheets is approximately 5 Å.[32] If a parallel β-sheet model holds for fibrils with approximately one Cu^{2+} per peptide, Cu^{2+} ions could be in close enough proximity in the fibrils to interact via dipolar and/or exchange interactions. Dipolar interaction between paramagnets can cause spectral broadening. The peak widths at half-height for the discernible Cu^{2+} hyperfine peaks in soluble Aβ(1−40) are 57 ± 5 (m_I = −3/2, Figure 1), 65 ± 8 (m_I = −1/2), and 118 ± 34 G (m_I = +1/2). In fibrillar Aβ(1−40), the corresponding Cu^{2+} hyperfine peak widths at half-height are 58 ± 5, 60 ± 5, and 116 ± 27 G. Thus, significant broadening is not apparent in the spectrum of Cu^{2+}-containing fibrils in Figure 1. To our knowledge, the peptide orientation in fibrils containing metal ions has not been determined. Therefore, it is possible that the strands in fibrils containing Cu^{2+} are oriented in a parallel β-sheet arrangement that does not produce observable dipolar broadening between Cu^{2+} ions or that the strands are aligned in a configuration other than a parallel β-sheet. It should be noted that Aβ fibril structural assignments are not always in agreement, with the exact composition of the peptide playing a major factor in controlling fibril morphology.[33]

If an antiferromagnetically exchange-coupled binuclear Cu^{2+} site exists in soluble or fibrillar Aβ, it should have a diamagnetic ground state. The magnetic ground state of a paramagnetic system can be determined by EPR spectroscopy.[20] The EPR signal intensity is temperature dependent, with the largest intensity observed when a single paramagnetic state is fully populated.[20] We detect a spectrum of Cu^{2+} bound to Aβ at temperatures as low as 6 K, indicating that the ground state of Cu^{2+} bound to soluble Aβ or bound to fibrillar Aβ is paramagnetic. Magnetic susceptibility data for imidazolate-bridged binuclear Cu^{2+} model complexes indicate a diamagnetic ground state, so these complexes should not display an EPR signal below 50 K.[34] Antiferromagnetically coupled Cu^{2+} ions in Cu,Cu-SOD show a 90 K EPR spectrum that is diminished relative to that for Cu,Zn-SOD because it arises from the S = 1 excited state.[35] Because an EPR signal for Cu^{2+} bound to soluble Aβ is detectable at temperatures below 60 K and we detect no half-field transition (not shown), exchange-coupled binuclear Cu^{2+} centers in Aβ are unlikely.

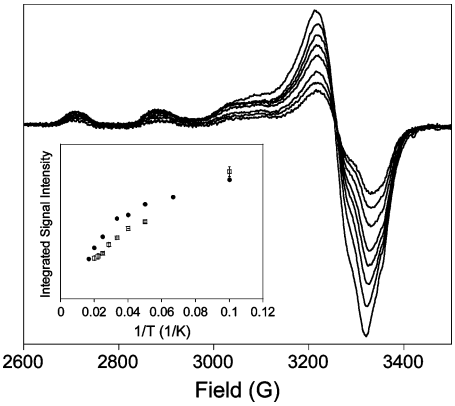

Figure 3. Effect of temperature on the EPR spectrum of Cu^{2+} in Aβ(1−40) fibrils. EPR parameters are as in Figure 2 except that T = 10−60 K. Microwave power saturation measurements were collected at all temperatures to ensure that spectral intensities are not decreased through saturation effects. Inset: Curie plot of doubly integrated Cu^{2+} EPR spectral intensities for Cu^{2+} with soluble Aβ(1−40) (squares) or Cu^{2+} in Aβ(1−40) fibrils (circles). Samples are the same as in Figure 2.

To support the interpretation that Cu^{2+} bound to Aβ is a mononuclear Cu^{2+} site, the temperature dependence of spectra for Cu^{2+}-bound peptide samples was monitored. The spectrum of Cu^{2+} bound to Aβ(1−40) decreases and broadens as the temperature is raised (Figure 3). A Curie plot shows that doubly integrated EPR spectral intensities for Cu^{2+} bound to Aβ fibrils or Cu^{2+} bound to soluble Aβ have Curie temperature dependences, characteristic of mononuclear Cu^{2+} (Figure 3, inset). The binuclear imidazolate-bridged Cu^{2+} model for 1:1 Cu^{2+}/Aβ-(1−28) complexes is based on experiments performed at pH 7.4 in the presence of Cu^{2+}, which are the same conditions under which our experiments were performed.[7]

The spectra in Figure 3 are for fibrils assembled in the presence of glycerol. Glycerol has been shown to affect the rate

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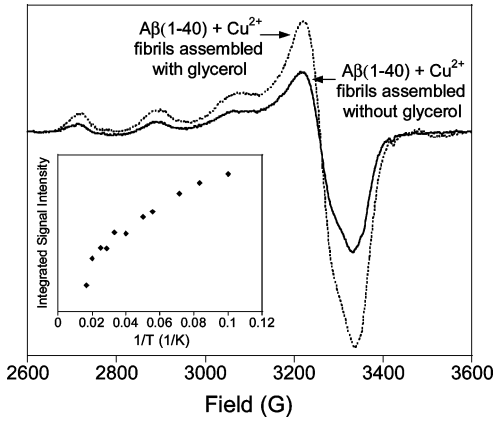

Figure 4. EPR spectra of Cu^{2+} in Aβ(1−40) fibrils assembled in buffer with (dashed line) or without (solid line) glycerol. Both sets of fibrils were separated by centrifugation, washed with the appropriate buffer (100 mM Tris, 150 mM NaCl, pH 7.4, buffer with or without 50% glycerol (v/v)), and resuspended in buffer containing 50% glycerol (v/v) immediately prior to data collection. The spectra have different overall intensities because the amount of fibrils collected differs between samples. EPR conditions: $T = 20$ K; modulation amplitude 10 G; power 0.5 mW; gain 5×10^4; frequency 9.38 GHz; time constant 40.96 ms; conversion time 40.96 ms; eight scans. Inset: Curie plot of doubly integrated Cu^{2+} EPR spectral intensities for Cu^{2+}-containing Aβ(1−40) fibrils assembled without glycerol. EPR parameters are as above except that $T = 10-60$ K.

of fibril formation.[36] In this work, the rate of fibril formation in the presence of Cu^{2+} was not monitored; spectra were collected of soluble peptide containing Cu^{2+} (the initial time point) or fibrils containing Cu^{2+} (the end point). Nevertheless, it is important to demonstrate that fibrils containing Cu^{2+}, but assembled in the absence of glycerol, exhibit Cu^{2+} EPR spectra and temperature dependences identical to those of Cu^{2+}-containing fibrils formed in the presence of glycerol. Figure 4 shows that the EPR spectrum of Cu^{2+}-containing fibrils assembled without glycerol is very similar to that for Cu^{2+}-containing fibrils assembled in the presence of glycerol. For fibrils assembled with Cu^{2+} but without glycerol, the Cu^{2+} $g_{||}$ value is 2.25 and $A_{||}$ is 174 ± 2 G. Like Cu^{2+} bound to fibrils assembled in the presence of glycerol, these fibrils display a normal Curie temperature dependence of the Cu^{2+} EPR signal (Figure 4, inset). Thus, although glycerol affects the random coil to β-sheet transition rate of Aβ,[36] it has little effect on the EPR properties of Cu^{2+} bound to Aβ fibrils.

The temperature dependence of the EPR signal of Cu^{2+} bound to soluble Aβ can be interpreted in several ways. One possibility is that our samples contain both Cu^{2+} and Zn^{2+} and form an imidazolate-bridged Cu, Zn binuclear center in which the Cu^{2+} behaves like a mononuclear center. This explanation would require that samples contain high levels of contaminating Zn^{2+} prior to the addition of Cu^{2+} and that a significant fraction of added Cu^{2+} be detected as free Cu^{2+}. In Tris buffers, both bound and unbound Cu^{2+} ions are detectable. We observe no free Cu^{2+} when a stoichiometric amount of Cu^{2+} is added to soluble Aβ. It has been demonstrated that Cu^{2+} can displace Zn^{2+},[7] so Cu^{2+} should replace Zn^{2+} in our samples if Zn^{2+} is bound to the Cu^{2+} site. These observations rule out a situation in which Cu^{2+} sites are occupied by Zn^{2+} prior to the addition of exogenous Cu^{2+} to Aβ.

A second possibility is that both a Type 2 Cu^{2+} site, observable at temperatures below 60 K, and a binuclear Cu^{2+} site, which is not observable at temperatures below 60 K, exist in soluble Aβ. If this situation is applicable, addition of Cu^{2+} to soluble Aβ should load both the binuclear site and the Type 2 site, resulting in only a fraction of the added Cu^{2+} (bound in the Type 2 site) being observed in our spectra. Quantification of the Cu^{2+} concentration by double integration of the Cu^{2+} EPR signal for Cu^{2+} bound to soluble Aβ typically deviates by less than 10% from the expected concentration of added Cu^{2+} on the basis of comparison to a calibration curve,[37] making the existence of both a Type 2 and a binuclear site in soluble Aβ unlikely.

A final possibility is that, at stoichiometric ratios of Cu^{2+} and Aβ, Cu^{2+} is bound in a mononuclear site and is not antiferromagnetically coupled through a histidine bridge to another Cu^{2+} ion. The EPR spectrum for stoichiometric ratios of Cu^{2+} bound to soluble Aβ(1−40) broadens as the temperature increases. The same trend is observed for spectra of Cu^{2+} incorporated into fibrillar Aβ. Observation of the broadened EPR spectrum for Cu^{2+} with soluble Aβ(1−28) as reported by Curtain et al. might be a function of the temperature (110 K) at which the spectrum was collected, rather than evidence for the existence of antiferromagnetically exchange-coupled Cu^{2+} ions in Aβ.

Our results have implications for the formation and global structure of fibrils assembled in the presence of Cu^{2+}. The ligand donor atoms to Cu^{2+} in soluble and fibrillar Aβ(1−40) are the same, potentially indicating that the Cu^{2+} ligands also are the same. A mechanism for metal-induced Aβ aggregation involving imidazolate-bridged binuclear Cu^{2+} centers is not supported by our results, even though such a model might explain the increased rate of Aβ aggregation observed in the presence of metal ions.[3,5,38] Similarly, Aβ(1−40) fibrils assembled in the presence of Cu^{2+} do not contain binuclear Cu^{2+} sites in which Cu^{2+} ions are antiferromagnetically coupled, even though such an arrangement might be possible given the 5 Å interstand distance in parallel β-sheets. Finally, the one Cu^{2+} ion per peptide ratio and 3N1O coordination environment for Cu^{2+} in fibrils exclude a model for Aβ fibrils in which one Cu^{2+} ion is ligated to four histidine residues from multiple peptides to form cross-links between β-sheets as has been proposed for Zn^{2+}.[5,15,39]

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(37) For example, in Figure 1, the calculated Cu^{2+} concentration for Cu^{2+} bound to soluble Aβ(1−40) is 54 μM, whereas the expected value is 50 μM. Microwave power saturation data for Cu^{2+} bound to Aβ revealed that the power saturation behavior differed from that for the Cu^{2+} standards, even though all samples were in the same buffer. None of the spectra was collected at saturating powers. For quantification purposes, double integrations of baseline-corrected spectra for Cu^{2+} bound to soluble Aβ were corrected to account for the $P_{1/2}$ difference.

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