Alzheimer’s Disease Amyloid-β Binds Copper and Zinc to Generate an Allosterically Ordered Membrane-penetrating Structure Containing Superoxide Dismutase-like Subunits

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Amyloid β peptide (Aβ) is the major constituent of extracellular plaques and perivascular amyloid deposits, the pathognomonic neuropathological lesions of Alzheimer’s disease. Cu2+ and Zn2+ bind Aβ, inducing aggregation and giving rise to reactive oxygen species. These reactions may play a deleterious role in the disease state, because high concentrations of iron, copper, and zinc have been located in amyloid in diseased brains. Here we show that coordination of metal ions to Aβ is the same in both aqueous solution and lipid environments, with His2, His3, and His4 all involved. At Cu2+/peptide molar ratios >0.3, Aβ coordinated a second Cu2+ atom in a highly cooperative manner. This effect was abolished if the histidine residues were methylated at Nε2, indicating the presence of bridging histidine residues, as found in the active site of superoxide dismutase. Addition of Cu2+ or Zn2+ to Aβ in a negatively charged lipid environment caused a conformational change from β-sheet to α-helix, accompanied by peptide oligomerization and membrane penetration. These results suggest that metal binding to Aβ generated an allosterically ordered membrane-penetrating oligomer linked by superoxide dismutase-like bridging histidine residues.

The amyloid β peptide (Aβ) is a normally soluble 4.3-kDa peptide found in all biological fluids, but it accumulates as the major constituent of the extracellular deposits that are the pathologic hallmarks of Alzheimer’s disease (AD) (1). Genetic evidence from early onset cases of AD indicates that Aβ metabolism is linked to the disease (2). Aβ peptides are neurotoxic (3, 4), but the mechanism of toxicity and the species of Aβ responsible have not been clearly defined.

There is mounting evidence that oxidative stress causing cellular damage is central to the neurodegeneration of AD (5, 6). There is an increase in oxidation of proteins as well as nuclear and mitochondrial DNA in AD brains (7–9). Aβ has the ability to enhance the generation of reactive oxygen species in cells of neural origin as well as in cell-free media (10–12). Aβ in vitro binds metal ions, including Zn2+, Cu2+, and Fe3+, inducing peptide aggregation that may be reversed by treatment with chelators such as EDTA (13, 14). Furthermore, extensive redox chemical reactions take place when Aβ binds Cu2+ and Fe3+, reducing the oxidation state of both metals and producing H2O2 from O2 in a catalytic manner (12). Because elevated levels of copper (400 μM), zinc (1 mM), and iron (1 mM) are found in amyloid deposits in AD-affected brains (15, 16), the oxidative stress observed in AD may be related to the production of reactive oxygen species by metal-bound forms of Aβ. This hypothesis is supported by the recent observation that senile plaques and neurofibrillary tangles isolated from AD brains were capable of generating reactive oxygen species and that copper and iron were essential (17). Moreover, our studies (18) have shown that the solubilization of Aβ from post-mortem brain tissue of AD patients was increased in the presence of metal chelators such as N,N′,N′,N′′-tetraakis(2-pyridyl-methyl) ethylene diamine and bathocuproine. Recently, the dramatic inhibition of amyloid deposition in transgenic mice treated orally with a Cu2+/Zn2+-selective chelator has been reported (19).

The discovery that metal binding to Aβ may be responsible for some of the pathological effects of AD makes characterization of the metal-binding site of interest as a potential therapeutic target. The aim of the present study was to characterize the structural consequences of Aβ binding to Cu2+ and Zn2+ in solution and to identify amino acid residues involved in metal binding.

MATERIALS AND METHODS

Peptides were synthesized as described previously (20) or obtained from Auspep (Melbourne, Australia) and from the W. M. Keck Laboratory (Yale University, CT). H2O, TFE-2H2O, Na2O2, SDS-2H2O, and HCl were obtained from Cambridge Isotope Laboratories (Andover, MA). The spin-labeled zwitterionic phospholipid 1-palmitoyl-2(16-doxyl-stearoyl) phosphatidyl choline was obtained from Avanti Polar Lipids Inc. (Pelham, AL). The acidic phospholipid spin label 1-palmitoyl-2(16-doxyl-stearoyl) phosphatidyl serine was synthesized according to Hubbell and McConnell (21). Both spin probes were checked for...
purity and to ensure that the number of spins/mol were >90% of theory (22). Synthetic palmitoyloleoyl phosphatidyl choline (POPC) was purchased from Sigma, and palmitoyloleoyl phosphatidyl serine (POPS) was purchased from Avanti Polar Lipids Inc. LUV were prepared by the method described by Mayer et al. (23). Peptides were added to the desired concentration to a suspension of LUV in PBS, and the mixture was vortexed under N₂ for 10 min at 305 K. Negatively charged LUV were made with 50% POPS and 50% POPC. Twiterionic LUV were made with 100% POPC. Because the longer Aβ peptides are prone to aggregation in solution, all experiments were carried out with freshly prepared samples. The samples for NMR varied depending on the solution conditions used; in aqueous PBS with 10% 2H₂O added, samples containing Aβ28 had a peptide concentration of 1 mM, whereas those containing Aβ40 were run at 0.3 mM. When the peptide is made up fresh in metal-free conditions and the undissolved aggregates are spun out before use, the peptide aggregates only very slowly (days). Some studies were performed in SDS solution (50 mM phosphate, 200 mM SDS-2H₂O, pH 5.3. 10% 2H₂O) where all peptide concentrations were 1.5 mM. NMR spectra were recorded on Bruker DRX-600 and AMX-500 spectrometers as described previously (24). Ultracentrifugation measurements were carried out on Aβ28 and Aβ40 in PBS at 1 and 0.3 mM, respectively, as described previously (24).

**EPR Spectroscopy**—X-band continuous wave EPR spectra of the Cu²⁺-peptide complexes were obtained using a Bruker EC106 spectrometer. Samples were loaded into hematocrit capillary tubes and inserted reproducibly into the cavity using a Kornberg holder (22). The sample temperature was maintained at 110 K using a flow-through cryostat. The microwave frequency was measured using a Bruker EIP 548B frequency counter, and the magnetic field was calibrated with an α,α’-diphenyl-beta-cyclohexyl hydroxyl sample. The exact peptide concentrations were determined by amino acid analysis of total copper by inductively coupled plasma mass spectrometry. EPR of peptide/spin-labeled LUV was carried out as above, except that the temperature was kept at 305 K, above the liquid crystal/gel transition temperature of the lipids. Analysis of EPR spectra of peptide/lipid mixtures was carried out using the spectral subtraction and addition methods described by Marsh (25).

To check the validity of these procedures, the lipid spin label spectra were simulated using modified Bloch equations, as described in model I of Davoust and Devaux (26).

**CD Spectroscopy**—The CD spectra of peptides in the LUV were obtained using a CD spectropolarimeter model 62DS (AVIV, Lakewood, NJ). The peptide concentrations were determined using the molar extinction of the UV absorption from the tyrosine residue. CD was obtained for each solution both neat and after a 1:4 dilution with MilliQ® water. CD spectra were obtained using a 1-mm-pathlength quartz cell, acquired at 297 K in 0.5-nm steps over a 185–250-nm-wavelength range. The base line acquired in the absence of peptide was subtracted, and the resulting spectra were smoothed and analyzed for the percentages of α-helix, β-strand, and disordered structures using the K2d Kohonen neural network program (27, 28).

### RESULTS

**NMR Spectroscopy**—Sedimentation equilibrium measurements in aqueous solution at pH 6.9 (100 mM NaCl, 50 mM phosphate, PBS) indicated that under the conditions used for our NMR experiments, all peptides were monomeric (Table I). Tseng et al. (29) also report that synthetic Aβ40 freshly dissolved in aqueous solution is monomeric. In aqueous solution, there was little chemical shift difference between the backbone amide and C⁵H resonances of Aβ28 (Aβ1–28) and those of the corresponding residues of Aβ40 (Aβ1–40), suggesting that both peptides were in a similar conformation. Indeed, Aβ28 and Aβ40 backbone chemical shifts deviated little from random coil values. This, coupled with the lack of both inter- and intra-residue nuclear Overhauser enhancement connectivities in the nuclear Overhauser enhancement spectroscopy spectra, indicated that both peptides were undergoing significant conformational exchange in aqueous solution. NMR spectra were also recorded for Aβ28 where the N⁵⁵ nitrogens of the imidazole ring of the His residues 6, 13, and 14 were methylated, hereafter referred to as Me-Aβ28. This peptide (purchased from Auspep) was prepared by incorporating histidine residues that were already methylated at the N⁵⁵ nitrogens of the imidazole ring into the synthesis of Aβ28, and its identity was verified by mass spectrometry and NMR. The spectra of Me-Aβ28 were virtually identical to Aβ28, the only significant differences being three strong singlets in the ¹H spectrum at 3.80, 3.82, and 3.83 ppm from the methyl groups attached to the His imidazole rings.

**Metal Binding**—When Zn²⁺ was added to the solutions of Aβ28 or Aβ40 in PBS, a precipitate formed. NMR spectra of the supernatant of Aβ28 treated with Zn²⁺ showed that peaks assigned to C2H and C4H of His⁶, His¹³, and His¹⁴ of Aβ28 had broadened significantly. However, there was little or no change in the rest of the spectrum compared with Aβ28 prior to the addition of Zn²⁺ (Fig. 1). This broadening of the NMR peaks caused by histidine residues is the result of the interaction of these residues with Zn²⁺. The histidyl side chain is a well established ligand of zinc in proteins and peptides (30), and this result suggested that three of the ligands bound to Zn²⁺ were most likely the imidazole rings of the histidine residues. The broadening of these peaks is the result of chemical exchange between free and metal-bound states or among different metal-bound states. The broadening of peaks is indicative of intermediate exchange that on the NMR time scale suggests that the metal binding affinity is in the micromolar range, in agreement with the low affinity site described by Bush et al. (31). The absence of any change in the rest of the spectrum suggests that the metal-bound form of the peptide is monomeric and that there is little or no significant amount of soluble oligomer in solution, because higher order aggregation would result in significantly broadened resonances. When Cu²⁺ or Fe³⁺ was titrated into an aqueous solution of Aβ28, similar changes were observed in the ¹H spectrum, with the peaks assigned to the C2H and C4H of His⁶, His¹³, and His¹⁴ disappearing from the spectrum. A slight broadening of all peaks in the spectrum (associated with the paramagnetism of Cu²⁺ and Fe³⁺) was also observed, but there were no other major changes following the addition of Cu²⁺ or Fe³⁺. The metal-induced precipitation prevented the addition of enough metal to saturate the metal-binding site. Addition of Zn²⁺ or Cu²⁺ to an aqueous solution of Aβ40 (0.3 mM) at pH 6.9 caused the formation of large amounts of precipitate, as previously observed (13, 14, 31). The precipitate made the observation of NMR spectra problematic, and few conclusions could be drawn from spectra of the peptide that remained in solution. When Cu²⁺ was added to an aqueous solution of Me-Aβ28, the changes observed in the spectrum were identical to those observed for Cu²⁺ added to Aβ28, but there was no visible precipitate.

Copper ions also induced aggregation in the rat Aβ28 peptide in aqueous solution. Rat Aβ28 differs from human Aβ by three substitutions, with Arg⁶, Tyr¹⁰, and His¹³ of human Aβ becoming Gly⁶, Phe¹⁰, and Arg¹³ (32). In vitro it has been shown that, compared with human Aβ, rat Aβ binds Zn²⁺ and Cu²⁺ less avidly (14, 33), that the coordination of Cu²⁺ or Fe³⁺ does not

### Table I

| Molecular weight | Ultracentrifugation | NMR |
|------------------|--------------------|-----|
| Aβ28             | 3263               | 1.50×10⁻¹⁰ m²/s | 8.10×10⁻¹¹ m²/s |
| Aβ40             | 4330               | 1.70×10⁻¹⁰ m²/s | 8.10×10⁻¹¹ m²/s |
induce redox chemical reactions, and that limited reactive oxygen species are generated (11). 1H NMR spectra of the supernatant showed that peaks from His6 and His14 had broadened beyond detection, indicating that these residues were involved in copper binding. Apart from some general broadening associated with paramagnetic Cu2+, no other significant changes were observed in the spectra.

We next studied metal binding to Aβ28 and Aβ40 in SDS-micelle solution to investigate the metal binding properties of Aβ in membrane-mimicking environments. Chemical shift differences between Aβ28 and Aβ40 were very small, again suggesting that both peptides adopt similar conformations in solution. In SDS-micelle solution we determined that Aβ adopted a well-defined α-helical structure from residue 15 to the C terminus. For Aβ40 the helix was kinked near residue 31. These results are similar to those recently described (34–36).

At pH <6.5 no interaction was observed between Zn2+ and Aβ in SDS-micelles. The addition of Zn2+ to Aβ40 in SDS-micelles at pH 6.5 broadened resonances caused by C2H and C4H of all three histidine residues such that they were not observed, indicating that the zinc was in exchange with these residues, but precipitation was not observed. Addition of more Zn2+ (~10-fold) in an attempt to saturate binding did not make the resonances observable. Raising the pH to 7 sharpened the resonances slightly, although they were still broad, suggesting that Zn2+ binding was slightly stronger at this pH. Raising the pH further did not measurably increase the affinity for Zn2+ by the peptide. Further addition of large quantities of Zn2+ (up to 200 equivalents) failed to produce sharper resonances attributable to the histidine residues, suggesting that binding was not saturated and is weak under these conditions.

When Cu2+ was added to Aβ40 in SDS-micelles at pH 5.5, resonances caused by residues in the 6–14 region were broadened as a result of their proximity to bound paramagnetic copper. This region of the peptide contains the three histidine residues previously implicated in copper binding, and peaks attributable to the side chains of these residues disappear completely from the spectrum. This region also contains residues Asp7, Tyr10, and Glu12 that could act as ligands for Cu2+, and peaks caused by the side chains of these residues were also broadened beyond detection. The rest of the spectrum was largely unaffected, suggesting that there is no major conformational change by the peptide in SDS-micelles upon copper binding and that the helical C terminus is unaffected. Precipitation was not observed when Cu2+ bound to Aβ40 in SDS-micelle solution.

**EPR Spectroscopy: Cu2+ Studies**—The EPR spectra of Cu2+ complexed with Aβ28 peptide in PBS over a metal/peptide molar fraction range of 0.2–1.0 are shown in Fig. 2. By the criteria of Peisach and Blumberg (37), the A1 (15.9 millikaisers) and g∥ (2.295) values for spectrum 2A suggest a square planar configuration for Cu2+ with, most probably, a 3N1O coordination sphere about the metal. Spectra of Cu2+ complexed at a 0.2 molar fraction with Aβ42 peptide in 35 mM SDS in PBS were identical for those of Aβ28 in PBS at the same Cu2+ molar fraction. On the other hand, the A1 (15.8 millikaisers) and g∥ (2.341) for the rat Aβ42 peptide in PBS, which has one histidine less, fell clearly within the criteria for a 2N2O coordination sphere. The concentration of Cu2+ in each sample was determined by double integration of the spectra of the complexes and of a pure standard solution of CuCl2. The concentration of Cu2+ determined in this way in each sample was within 95% of the total copper determined by inductively coupled plasma mass spectrometry, suggesting that, within the limits of experimental error, reduction of Cu2+ to the EPR-silent Cu+ had not occurred in the presence of Aβ28 or rat Aβ28, in agreement with the findings of Huang et al. (11), nor did reduction occur for Aβ42 bound to Cu2+ in SDS-micelles.

When the Cu2+/peptide molar fraction was increased above 0.2, line broadening was observed with Aβ28 that was attributed to Heisenberg exchange effects brought about by the metal beginning to occupy sites adjacent to those initially occupied at lower molar ratios. As can be seen from Fig. 2, the broadening was more pronounced with the increasing molar fraction until the spectrum was apparently completely broadened at 1.0 mole equivalent of Cu2+, giving a line identical to that found by Ohtsu et al. (38) for the exchange broadened spectrum in Cu2+-bridged imidazolate complexes. The amount of broadened line in each spectrum was determined by subtracting from it the spectrum obtained at the 0.1 molar fraction until the remaining line was identical to that of spectrum 1 in
that this ratio did not change when peptide:lipid ratios or tide did not increase with the increasing mole fraction. The fact associated with the lipid and that the aggregation of the pep-
mole fraction and proportion of slow component was linear,
Cu²⁺ against added Cu²⁺, indicating that the peptide was not forming exchange broadening was observed in the EPR spectra up to a 0.9 molar fraction, indicating that the peptide was not forming multimers. It appears that metal ions have to coordinate both nitrogens on the imidazole ring of the His residues before aggregation can occur.

EPR Studies of the Interaction of Aβ Peptide with Spin-labeled LUV—Fig. 4 shows spectra of 1-palmitoyl-2-(16-docyl stearoyl) phosphatidyl serine in the negatively charged LUV in the absence (spectrum A) and the presence (spectrum B) of a 0.05 mole fraction of Aβ42, coordinated by 0.3 mole equivalents of Cu²⁺. Spectrum C was obtained by subtracting the broad Cu²⁺ line from spectrum B and shows that there is a relatively immobilized component in the spectra. Increasing amounts of spectrum A of the control LUV preparation were subtracted from spectrum C until a spectrum with a clear end point was obtained (Fig. 4, spectrum D). Attempts were made to determine whether the presence of the peptide extensively perturbed the bulk lipid, as suggested by McIntyre et al. (39), by subtracting the spectrum of the control LUV recorded at 300 K. This assumes that the lower temperature spectrum would simulate that of bulk lipid, the motion of which had been restricted by long range effects arising from the presence of the peptide. Using the lower temperature spectrum did not lead to a clear end point, and it was impossible to obtain a well behaved first integral with no negative excursions below the baseline (40). It was concluded, therefore, that long range effects of the type described by McIntyre et al. (39) were absent in our system. It was also possible to simulate the spectrum as described by Davoust and Devaux (28). The proportion of the slow motion component in spectrum C was therefore calculated by double integration of spectrum D. Spectra were then run over a range of Cu²⁺/Aβ42 ratios, from 0.025 to 0.15 mole fraction, and the proportion of slow motion component at each mole fraction is plotted in Fig. 4 (bottom panel). The relationship between the mole fraction and proportion of slow component was linear, suggesting that even at a fraction of 15%, all of the peptide was associated with the lipid and that the aggregation of the peptide did not increase with the increasing mole fraction. The fact that this ratio did not change when peptide:lipid ratios or peptide:metal ratios changed suggested that the structure that penetrated the lipid membrane was well defined. The lipid:peptide ratio of ~4:1 is much lower than the value of 10:1 usually associated with a single α-helix penetrating the hydrophobic region of the membrane yet higher than the expected ratio for β-sheet conformation, which is 1–2 lipids/strand, depending on the tilt of the structure in the bilayer (41).

The Cu²⁺ Aβ42 peptide/negatively charged phospholipid mixtures were examined at 110 K to determine the nature of the coordination sphere about the copper bound to Aβ. They gave spectra characteristic of those illustrated in Fig. 2, except that a small amount of broadening was evident at the high field end of the line caused by the contribution of frozen phospholipid spin label. The copper spectra taken at a 0.3 mole fraction of Cu²⁺ showed no trace of exchange broadening, but at Cu²⁺ mole fractions above 0.5 there was exchange broadening characteristic of the spectra shown in Fig. 2. Measurement of the parameters of the copper EPR signal at a 0.3 mole fraction showed that the coordination sphere about the copper ions in each case was 3N1O.

A similar relatively immobilized component was found when
Zn$^{2+}$ coordinated to Aβ42 was added to the negatively charged LUV, except that much higher concentrations of metal (up to a molar ratio of 4) were required. This reflects the lower affinity of zinc for the metal-binding sites of Aβ, suggesting that the metal is playing a structural role and that redox chemistry is not involved in this process.

These experiments were repeated in the presence of the chelating agent diethylenetriaminepentaacetic acid at a 2:1 molar ratio relative to Cu$^{2+}$. No sign of peptide penetration of the bilayer was then evident in the EPR spectra, establishing that Aβ42 penetration of the membrane was a consequence of metal binding. No bilayer penetration was observed over a range of mole fractions from 0.025 to 0.1 for Cu$^{2+}$/Aβ28. Similarly, no penetration over this mole fraction range was observed with the zwitterionic phospholipid DOPC LUV and Cu$^{2+}$/Aβ42 probed with 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidyl choline, indicating that Aβ penetration only occurs with negatively charged membranes.

**CD Spectroscopy**—CD spectra were obtained on freshly prepared solutions of Aβ42 with one molar equivalent of CuCl$_2$, in the presence of negatively charged LUV so that the peptide: LUV mole fraction was 0.15. Spectra were also obtained of 0.15 mole fraction Aβ42 in 1:1 DOPC:DPPS LUV and 1 mM DTPA. In the absence of Cu$^{2+}$ in a membrane environment (Fig. 5, curve A), Aβ42 was mainly β-sheet, as indicated by the minimum of 220 nm. There is a small inflection in the spectrum at 228 nm. This has been observed previously in CD spectra of lipid-bound peptides and was believed an artifact caused by anomalous light scattering. The structures of these peptides were confirmed as β-sheets by Fourier transform infrared spectroscopy in the same lipid (44).

Aβ42 in the presence of Cu$^{2+}$ was significantly more α-helical, as indicated by a double minimum at 208 and 222 nm (Fig. 5, curve B). The helix content was calculated to be 57% for the Cu$^{2+}$-containing system and 8% in the absence of the metal, indicating that it is involved in converting Aβ42 from predominantly β-sheet to predominantly α-helix in this membrane-mimetic environment. This is consistent with Cu$^{2+}$ promoting Aβ42 membrane insertion as helical multimers.

**Reduction of Cu$^{2+}$ in the Presence of Phospholipids**—Double integration of the copper EPR spectrum of aliquots taken at 30-s intervals of the Cu$^{2+}$/Aβ42 peptide/acidic phospholipid mixtures at 110 K determined the amount of Cu$^{2+}$ in each sample. We assumed that the diminution in the EPR signal was due to Cu$^{2+}$ reduction to Cu$^{-}$ and not due to the formation of antiferromagnetically coupled (S = 0) dicopper because it has already been shown by other assays that Aβ42 reduces Cu$^{2+}$ (11, 12). As shown in Fig. 6A, there was a 25% reduction of Cu$^{2+}$ to Cu$^{-}$ by Aβ42 in the presence of acidic phospholipid, as compared with <10% for the peptide in SDS and almost 100% reduction in aqueous buffer. In neutral lipids there was almost 90% reduction of Cu$^{2+}$.

**Role of Methionine in the Reduction of Cu$^{2+}$ by Aβ**—The decrease in the reduction of Cu$^{2+}$ to Cu$^{-}$ by Aβ42 in the presence of acidic phospholipids and SDS might have been due to the seclusion of the hydrophobic Met$^{35}$ residue in the hydrocarbon core lipid bilayer of the LUV or the interior of the SDS-micelles. Therefore, we studied the effect of adding soluble methionine to the metal-peptide mixtures. Cu$^{2+}$/Aβ28 was also studied, because it lacks the hydrophobic region containing the Met residue. As shown in Fig. 6B, adding methionine (mole fraction 2.0) to the metal-peptides dramatically increased the rate of reduction of Cu$^{2+}$ in each case, supporting a critical role for Met$^{35}$ in the redox activity of Aβ.
proposed model explaining the aggregation, cooperative binding, and a fourth ligand for Zn$^{2+}$ tides as proposed by Miura. A metal could bridge between histidine residues of different peptides, indicating that metal-induced aggregation is not mediated by metal but were not observed for Cu$^{2+}$ and Aβ. It is important to note that the Heisenberg exchange broadening observed at Cu$^{2+}$ mole fractions >0.3 with Aβ was eliminated when the N$^\circ$ of three imidazole rings of the His residues were methylated, thereby preventing the formation of histidine-bridged complexes. This evidence provides definitive proof that the imidazole rings of the His residues of Aβ are able to bridge between metal centers under the conditions studied here. Fig. 7B shows how the histidine could act as a bridge between metal centers; the distance between copper ions in this model is 6 Å, and exchange phenomena would be evident in the EPR of such a complex, matching those observed (Fig. 2). It is the bridging histidine that is probably responsible for the reversible metal-induced aggregation that is observed when Aβ is metallated with Cu$^{2+}$ and Zn$^{2+}$. Bridging histidine residues would also explain the multiple metal-binding sites observed for each peptide (43) and the high degree of cooperativity evident for subsequent metal binding (Fig. 3). With three histidines bound to the metal center there are several potential sites for further coordination of metal ions such that a large scope exists for metal-mediated cross-linking of the peptides leading to aggregation, which will be reversible when the metal is removed by chelation. This type of metal binding with bridging histidine residues would result in complexes very similar to the active site of SOD. The occurrence of exchange broadening in the Cu$^{2+}$ EPR spectra of Aβ28 and its modulation by Zn$^{2+}$, which has also been observed in model SOD imidazolate-bridged dinuclear complexes (38), further suggests the occurrence of structured complexes that are not merely random aggregates of peptide.

Cu$^{2+}$ and Zn$^{2+}$ Induce Aβ to Form Allosterically Ordered Multimers That Penetrate Lipid Membranes—Zn$^{2+}$ and Cu$^{2+}$ bound to the same site on Aβ in SDS-micelles as in aqueous solution, although there were significant differences in the effects of metal binding. Metal-induced aggregation and reduction of Cu$^{2+}$ to Cu$^+$, as observed for Aβ in aqueous solution (11, 12, 14), were not observed when copper bound to Aβ40/42 in SDS-micelles or LUVs. One of the effects of SDS is to drive the C-terminal part of the peptide (residues 15–42) into an α-helical conformation, preventing the formation of aggregated β-sheet structures. The α-helical region of the peptide starts just after the metal-binding site. This result suggests that the peptide may need to be in a β-sheet form before it becomes redox-active, which would be consistent with previous observations that the neurotoxicity associated with Aβ also requires the presence of aggregated β-sheet structures (49–51). However, results with the addition of exogenous methionine that are discussed below show that β-sheet/ aggregation are not necessary for Cu/Aβ to produce redox-active species.

We found that as a consequence of Zn$^{2+}$ and Cu$^{2+}$ binding, Aβ42 forms allosterically ordered, α-helical structures that penetrate negatively charged membranes. These results suggest that the Aβ42- and Aβ43-induced decrease in fluidity in the hydrocarbon core of human cortex membranes as measured by fluorescence polarization (52) was most likely due to association of the membrane lipids with the hydrophobic face of the rigid peptide. There have also been reports of cation channel formation (53, 54) by Aβ40 and Aβ42 that indicate penetration of the bilayer. Many previous studies have indicated that Aβ peptides form β-sheet structures in lipid environments (55–57), although the structure appears to be dependent on the composition of the lipid. For example, it was shown that Aβ1–40 was
α-helical in phosphatidyl glycerol vesicles but adopted a β-sheet conformation when GM1 ganglioside was added (58). Further, the physical state of the lipid systems used varied widely, with small unilamellar vesicles being the most common. In many cases, the temperature at which the measurements were made was not reported, leaving some doubt as to whether the lipids were in the liquid crystal or gel state. It is highly probable that the Aβ peptides are pleomorphic, able to adopt different conformations in different lipid environments. Because the cell membrane is a mosaic of different lipid environments, it is even possible that the peptides will exhibit different structures with different properties in different parts of the mosaic.

We found that approximately four lipids are associated with each membrane-penetrating peptide subunit (Fig. 4, bottom panel), which is a low ratio for lipid association with a single α-helix and suggests the presence of oligomers penetrating the lipid. Preliminary modeling showed that 24 lipid molecules would fit around an oligomer of six helices. Taken with the value of 5.9 for the Hill coefficient calculated from the data in Fig. 3 (curve A), the modeling suggests that Aβ42 forms an allosterically induced hexamer in the presence of metal ions. The metal ions do not penetrate the membrane but form structures very similar to the active site of SOD on the surface of the membrane.

Earlier studies of Aβ peptide-membrane interaction were not controlled for the presence of metal ions, ubiquitous trace contaminants, the concentration of which was minimized in our controls by use of excess chelator. It is possible, therefore, that the other reported results of Aβ penetration into the membrane, measured by sensitive methods, might have been initiated by the peptide binding trace Cu²⁺ or Zn²⁺.

The failure of Cu²⁺:Aβ28 to perturb the vesicle bilayer shows that, as is the case with the redox activity of the peptide (11, 12), the hydrophobic C terminus is essential for the interaction. The absence of line broadening, which is characteristic of Heisenberg exchange between free radicals and transition metals (59), in the phospholipid nitroxide label spectrum in the LUV interacting with the Cu²⁺/Aβ42 also indicates that the Cu²⁺-binding site is outside the bilayer. The role of Cu²⁺ in the interaction may indicate that the peptide must be oligomerized for it to penetrate the bilayer. It has been suggested recently (60) that aggregation of Aβ peptides might be a prerequisite for penetration of the lipid bilayer.

The Role of Methionine in Aβ Redox Reactions—It has been reported that Met₃⁵ is essential for the toxicity and induced oxidative stress of Aβ (61, 62). The lack of redox activity associated with the copper interactions with Aβ28 suggested that the Met of Aβ42 incubated in SDS or negatively charged LUV was buried in the hydrophobic core of the micelles and vesicle bilayers and therefore unavailable as a cofactor in the metal reduction. Addition of exogenous methionine restored redox activity, albeit with a much slower rate of reaction. These results suggest that Met₃⁵ participates in the reduction of copper by Aβ. Although Aβ28 and Aβ42 in LUV with bound copper have multiple copper-binding sites resulting in peptide aggregation, Aβ42 in SDS is monomeric in the presence of bound Cu²⁺ with a single metal-binding site as determined by the lack of Heisenberg interactions in the EPR spectra. The coordination sphere of Cu²⁺ under these conditions includes the three histidine residues and an oxygen ligand, probably Tyr₁₀, and this is the initial copper-binding site of Aβ. The effects of the addition of methionine (Fig. 6) showed that this is potentially a redox-active site.

Pathological versus Functional Metal Binding to Aβ: a Model for Alzheimer’s Disease—We have shown previously that Cu²⁺ and Zn²⁺ binding to Aβ modulate the toxicity of the peptide through the generation of H₂O₂ by electron transfer to O₂ (9, 12). Cu²⁺ and Zn²⁺ binding to Aβ also induces the precipitation of the peptide (13, 14, 31, 42). Increased binding of these metals to Aβ is evident in AD (18), and we have found recently that the metal-mediated redox activity and aggregation of Aβ, as well as amyloid deposition in APP2576 transgenic mice, are inhibited by treatment with a bioavailable zinc/copper-selective chelator, clioquinol (19). Therefore, the structural characterization of the Cu²⁺- and Zn²⁺-binding sites on Aβ may be essential for elucidating the pathogenesis of AD, as well as for developing new therapeutics.

In light of our current findings that Cu²⁺ and Zn²⁺ binding induce monomeric Aβ to form allosterically ordered SOD-like metallopeptide complexes that insert into negatively charged membranes, it is interesting to speculate on the likelihood that this redox-inert Aβ assembly may be biologically relevant and subsume some function. We have recently reported that Cu²⁺/Zn²⁺-bound Aβ possesses significant SOD catalytic activity (69). Our current structural data support the possibility of such SOD-like activity. Physiologically, the combination of Aβ with a lipid vesicle occurs in high density lipoproteins (HDLs), which are found in plasma and cerebral spinal fluid (63, 64). HDLs possess antioxidant properties (65–67), and we hypothesize that copper/zinc-Aβ complexes inserted into HDL membranes may play a role in superoxide clearance by HDLs. Supporting a role for HDLs in nullifying the adverse redox activity of soluble Aβ, HDLs are able to decrease Aβ toxicity in cortical cell culture (68). In the heuristic model that we contemplate, the perturbation of Zn²⁺ or Cu²⁺ homeostasis that is associated with AD (15) may interfere with Aβ insertion into membranes, liberating increased amounts of neurotoxic, redox-active Aβ.

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