8

Copper Coordination by β -Amyloid and the Neuropathology of Alzheimer's Disease

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8.1 Introduction

It is nearly two decades since high concentrations of the redox active transition metal ions Cu^{2+} and Fe^{3+} found in β -amyloid plaques were first proposed to play an important role in the pathology of Alzheimer's disease (AD) (see review by Bush [1]). Over this time, a new field of metallo-neurobiology relating to AD and other neurodegenerative diseases has arisen with approximately 250 original papers and more than 1000 references in secondary publications to date. At first, many neuroscientists failed to recognize the importance of this growing literature. However, a recent pilot Phase II clinical trial of a blood-brain barrier permeable metal protein attenuating compound (MPAC), clioquinol, in patients with moderately severe AD has shown promising results [2]. In a randomized sample of 36 subjects, the effect of treatment was significant in the more severely affected group, where those treated with clioquinol showed minimal deterioration in their cognitive scores (Alzheimer's disease Assessment Scale ≥ 25) compared with substantial worsening of the scores for the placebo group. Although subjected to the usual cautions applied to small-scale trials, this is an encouraging result that renders even more urgent the full elucidation of the possible role of transition metals, particularly Cu and Zn, in AD. It must be stressed that, although there is much experimental evidence on various aspects of the interaction between Cu, Zn, and the constituent of the amyloid plaques, the β -amyloid peptide ($\text{A}\beta$), the structural biology and elucidation of the neuropathological significance of metal binding are very much works in progress.

The naturally occurring $\text{A}\beta_{1-42}$, 1–41 and 1–39 peptides (sequence of $\text{A}\beta_{1-42}$ given in Fig. 8.1) represent part of the putative trans-membrane domain of the amyloid precursor protein, liberated from the membrane by proteolytic (secretase) action. Although its sequence is generally highly conserved, the rat sequence has Arg5, Tyr10, and His13 of human $\text{A}\beta$ replaced by Gly5, Phe10, and Arg13 (see highlighted residues in Fig. 8.1). Because the murine species do not develop amyloid plaques in the brain with aging, it was recognized that these substitutions could be an important pointer to mechanisms of plaque formation in human beings. The coordination of transition metals by $\text{A}\beta$ has been linked variously to their role in promoting peptide aggregation to form amyloid plaques, in the production of cytotoxic reactive oxygen species (ROS), and in promoting potentially cytotoxic interactions with cell membranes.

8.2 Cu^{2+} and Zn^{2+} Induced Aggregation of $\text{A}\beta$

Transition metal ion homeostasis is severely dysregulated in the AD brain [3, 4] and the role of these metals has been the subject of continuing study [5–11]. The transition metal ions Cu^{2+} , Fe^{3+} , and Zn^{2+} have been reported to occur at high concentrations in the neocortical parenchyma of healthy brain (total dry weight concentrations of 70, 340, and 350 μM , respectively). These concentrations may seem high but are not surprising when one considers the intense bioenergetics of the brain and the fact that the transition metal ions are an

Human	DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGGVIA
Rat	DAEFGHDSGF EV RRHQKLVFFAEDVGSNKGAIIGLMVGGGVIA

FIGURE 8.1. Sequence of human A β compared with that of the rat.

essential part of the redox systems involved. Their levels are far higher in the neuropil of the AD-affected brain, where they reach 0.4 and 1.0 mM for Cu and Fe/Zn, respectively in the amyloid plaque deposits [12]. It is of interest that these have been termed “trace metals,” an evident misnomer because their concentrations in the gray matter are of the same order of magnitude as Mg (0.1–0.5 mM).

Miller et al [13] have imaged the *in situ* secondary structure of the amyloid plaques in AD brain tissue. Using synchrotron Fourier transform infrared micro-spectroscopy and a synchrotron x-ray fluorescence microprobe on the same sample, they showed a strong spatial correlation between elevated β -sheet content in A β plaques and accumulated Cu $^{2+}$ and Zn $^{2+}$, emphasizing an association of metal ions with amyloid formation in AD. There was also a strong spatial correlation between the two ions. Higher Zn $^{2+}$ concentrations have also been seen histologically in plaque deposits [14], and the importance of Zn $^{2+}$ in plaque formation has been emphasized by the finding that age- and female sex-related plaque formation in APP2576 transgenic mice was greatly reduced upon the genetic ablation of the zinc transporter 3 protein, which is required for zinc transport into synaptic vesicles [15].

Bush et al. [16] found that A β coordinated Cu $^{2+}$, Zn $^{2+}$, and Fe $^{3+}$ with high affinity [17, 18], which would explain the presence of these metals in amyloid plaques. This study also showed stabilization of an apparent A β 1–40 dimer by Cu $^{2+}$ on gel chromatography suggesting an interaction between Cu $^{2+}$ and A β 1–40. Clements et al. [19] observed displacement of $^{65}\text{mZn}^{2+}$ from A β when co-incubated with excess Cu $^{2+}$, while Yang et al. [20] found that Cu $^{2+}$ and Zn $^{2+}$ shared a common binding site. Atwood et al. [21] found that Cu $^{2+}$ was bound to soluble A β via histidine residues and that the precipitation of soluble A β by Cu $^{2+}$ was reversibly modulated by pH with mildly acidic conditions (pH 6.6) greatly promoting Cu $^{2+}$ -mediated precipi-

tation, whereas raising the pH dissolved precipitated A β :Cu $^{2+}$ complexes. Cherny et al. [22] observed that Zn $^{2+}$ induced aggregation of soluble A β at pH 7.4 *in vitro*, which was totally reversible with chelation. They also found that marked Cu $^{2+}$ -induced aggregation of A β 1–40 occurred as the solution pH was lowered from 7.4 to 6.8 and that the reaction was completely reversible with either chelation or raising the pH. A β 1–40 was reported to bind three to four Cu $^{2+}$ ions when precipitated at pH 7.0. Rapid, pH-sensitive aggregation occurred at low nanomolar concentrations of both A β 1–40 and A β 1–42 with submicromolar concentrations of Cu $^{2+}$. Unlike A β 1–40, A β 1–42 was precipitated by submicromolar Cu $^{2+}$ concentrations at pH 7.4. Rat A β 1–40 and histidine-modified human A β 1–40 were not aggregated by Zn $^{2+}$, Cu $^{2+}$, or Fe $^{3+}$, indicating that histidine residues are essential for metal-mediated A β assembly. Cherny et al. [23] also showed that Cu $^{2+}$ - and Zn $^{2+}$ -selective chelators enhanced the dissolution of amyloid deposits in postmortem brain specimens from AD subjects and from amyloid precursor protein overexpressing transgenic mice, confirming the part played by these metal ions in cerebral amyloid assembly. In particular, Zn $^{2+}$ efficiently induces aggregation of synthetic A β under conditions similar to the physiological ones in the normal brain, that is, at nanomolar and submicromolar concentrations of A β and free Zn $^{2+}$, respectively [15–17].

Recently, it has been demonstrated that A β will not precipitate when trace metal ions are rigorously excluded [24]. On the other hand, the very strong precipitating effect of Zn $^{2+}$ implies that there are some factors protecting A β from Zn $^{2+}$ -induced aggregation in the normal brain. Certain metal ions such as Mg $^{2+}$ and Ca $^{2+}$, which do not exhibit a precipitating effect, have been hypothesized to have this protective effect [25]. However, the inhibition of Zn $^{2+}$ -induced A β aggregation by these metal ions has not yet been verified. The effect of Cu $^{2+}$ on the aggregation of A β is ambiguous compared with Zn $^{2+}$. Cu $^{2+}$ has been shown to be a strong inducer of

A β aggregation under certain conditions [24]. In contrast with the Zn²⁺-induced A β aggregation that occurs over a wide pH range (5.5–7.5), the Cu²⁺-induced aggregation occurs primarily at mildly acidic pH [21].

Atwood et al. [21] determined a half-maximal binding of Cu²⁺ for A β in the micromolar range (4.0 μ M for A β 1–40 and 0.3 μ M for A β 1–42) by indirect spectrophotometric analysis. However, this analysis of binding affinities was limited by the sensitivity of the spectrophotometric technique and the lack of competitive binding factors in the incubation that would emulate the physiological situation more closely. Garzon-Rodriguez et al. [26] used a more sensitive fluorescence technique and a single tryptophan (F4W) mutant of A β 1–40 to show that the relative affinities were Fe < Cu > Zn. Syme et al. [27] used the competitive effects of glycine and L-histidine to measure Cu²⁺ affinity for A β by fluorescence spectroscopy. Adding Cu²⁺ to A β 1–28 caused marked quenching of the tyrosine fluorescence signal at 307 nm. Added glycine competes with A β for the Cu²⁺, and the tyrosine fluorescence signal reappears at a sufficiently high glycine levels. Cu²⁺ coordinates to glycine via the amino and carboxylate groups with an apparent pH-adjusted K_a of $1.8 \times 10^6 \text{ M}^{-1}$, and two glycine residues will bind to a single Cu²⁺ ion [27]. It took more than 100 mol equivalents of glycine to cause the tyrosine fluorescence signal to completely return to its maximal strength. Half of the maximal quenching is achieved at approximately 18 ± 2 eq. of glycine. Finally, Huang et al. [24] had shown that binding of Cu²⁺ to A β 1–42 promoted precipitation with so high an affinity that it was hard to avoid aggregation unless buffers were most rigorously treated with chelating agents. Even then, it is difficult to remove the last traces of metal ion, which may account for many of the inconsistencies reported in the A β metal binding literature. Extremely small changes in free or exchangeable Cu²⁺ concentration are also likely to have a significant effect on A β solubility in vivo.

8.3 A β Structures

The structure of the metal binding site of A β must be considered in the context of the structure of the whole molecule. Because it has been widely held

that A β exerts its neurotoxic action via interactions with neuronal membranes additionally to or in concert with its redox activity, there have been many studies on its structure in a variety of membrane mimetic systems. A major obstacle to the determination of definitive structures is the difficulty of obtaining reproducibly a random-structured starting material or, alternatively, of mimicking its transmembrane conformation immediately after secretase cleavage. Furthermore, because aqueous solutions of A β accumulate significant amounts of aggregates within a few hours, NMR studies can be difficult. Nevertheless, early NMR studies of human A β 1–40 showed a random coil structure in aqueous solution (pH 4) at micromolar concentration [28]. The secondary structure of A β 40 peptide in 40% TFE buffered at pH 2.8 with 50 mM potassium phosphate was also studied by NMR. Under these conditions, there was aggregation only after a week and the NMR spectra were well resolved. Solution structures of A β 1–40 in perdeuterated sodium dodecyl sulfate (SDS- d_{25}) micelles obtained by Coles et al. [29] showed two α -helical segments. The helical arrangement of residues 15–25 and 29–37 was confirmed by intense NOE connectivity (3–4 residues) while medium-range NOE for residues 25–29 were either weak or not observed. The “break” between the two helices was suggested by D₂O exchange experiments, where protons on residues 25–29 were shown to exchange rapidly and, from quantitative structural and dihedral angle restraint calculation prediction, a kink was seen at residues 26–27 acting as a “hinge” for the two helices.

Shao et al. [30] showed two α -helical regions between Tyr10–Val24 and Lys28–Val36 for both A β 1–40 and A β 1–42 in SDS- d_{25} at pH 7.2. The data were supported by structural calculations indicating α -helices between residues 10–24 and 28–42 with the region Gly25–Asn27 as a connecting loop. Similar downfield shifts of A β 1–40 and A β 1–42 at Val39–Val40 and Val40–Ile41, respectively, suggested a structural preference for the peptides at their C-terminus. This may be related to conformational averaging between a micelle bound α -helical structure and β -sheet when the peptides leave the micelle surface.

Most NMR studies in solution were done in either trifluoroethanol (TFE) [31] or SDS- d_{25} /D₂O to mimic a membrane environment, although an

early study by Sorimachi and Craik [32] showed some α -helical structure in dimethyl sulfoxide (DMSO). The α -helical conformation found by NMR was further supported by far ultraviolet circular dichroism (CD) spectroscopy which showed that A β 1–28 in the presence of charged membrane-like surfaces, especially negatively charged SDS, preferred a helical structure. Other membrane-like species, zwitterionic dodecylphosphocholine (DPC) and dodecyltrimethylammonium chloride (DTAC), with heterogeneous amphiphilic environments similar to biological systems have been used. Fletcher and Keire [33] used solution NMR and CD to study the conformation of A β 12–28 in dodecylphosphocholine (DPC) and SDS micelles as a function of pH and lipid type. Interaction with micelles was weak but changed the conformation when compared with aqueous buffer alone. However, the peptide interacted strongly with anionic SDS micelles, where it was mostly bound, was α -helical from Lys16 to Val24, and aggregated slowly. The pH-dependent conformational changes of the peptide in solution occurred in the pH range at which the side-chain groups of Asp22, Glu23, His13, and His14 are deprotonated (pKs $\sim$ 4 and 6.5). The authors concluded that the interaction of A β 12–28 with SDS micelles altered the pH-dependent conformational transitions of the peptide whereas the weak interaction with DPC micelles caused little change.

These conformational changes indicate a relationship between peptide structure and electrostatic interactions involving protonation and deprotonation of the micelle lipid head groups at different pH. In experiments using A β 1–40 with the imidazole side chains of the histidine residues 6, 13, 14 methylated, Tickler et al. [34] found that the peptide-lipid interaction was modulated by the histidine residues and, therefore, would be pH sensitive. A β 1–28 appears to associate with the surface of the membrane based on an irregular pattern in the amide chemical shift temperature coefficient dependence, suggesting that the amide backbone is situated at the water and micelle interface. Narrower NMR line widths indicated conformational mobility at the micelle surface and the concentration of A β 1–28 not affecting CD and NMR data suggested that the α -helical structure is more likely to be stabilized by rapid exchange [33].

Jin et al. [35] used NMR spectroscopy to determine the solution structure of rat A β 1–28 (see Fig. 8.1) and its binding constant for Zn²⁺. They found that the three-dimensional solution structure of rat A β 1–28 was more stable than that of human A β 1–28 in DMSO-*d*₆ and that a helical region from Gln15 to Val24 existed in the rat A β 1–28. The affinity of Zn²⁺ for rat A β 1–28 was lower than that for human A β 1–28, and Arg13, His6, and His14 residues provide the primary binding sites for Zn²⁺. They also found that Zn²⁺ binding to rat A β 1–28 caused the peptide to change to a more stable conformation.

Gröbner et al. [36] have outlined a method for structure determination of A β in membrane systems. First, they used CD and ³¹P magic angle spinning (MAS) NMR spectroscopies to characterise the peptide in a dimyristoyl phosphatidyl choline/dimyristoyl phosphatidyl glycerol vesicle system. Their most notable finding was that they could get A β 1–40 to give an α -helical structure if the peptide were dialyzed from TFE solution into the vesicles. That is, it was given no opportunity to form β -structure inducing fibrils by contact with water. Second, they used rotational resonance ¹³C CP MAS NMR recoupling techniques to show that the membrane-penetrant part of the peptide was α -helical before major aggregation had occurred. To gain further insights, these authors concluded, future MAS studies would have to be made on multiple uniformly labeled peptides. Further advances in spectral resolution and sensitivity are vital, as is development of labeling methodologies. The development of pulse sequences and appropriate algorithms to extract multiple distance and torsion angle constraints from these systems would also be needed. Thus, the determination of the structure of A β by NMR in a membrane environment is still incomplete.

8.4 The Structure of A β in Fibrils

Conventionally, the supramolecular structure of β -sheet entities such as amyloid plaques can be considered to be either parallel or antiparallel. Which mode is likely to be important for determining the residues involved in the metal-bridged cross-links that occur in amyloid plaques and for the subsequent redox chemistry. ¹³C multiple quantum SS-NMR

has been used to probe the structure of the full-length A β peptide [37]. Internuclear distances of approximately 4.8 Å would be observed for ^{13}mC -labeled residues if the β -sheets form an in-register parallel structure. An antiparallel structure, on the other hand, would have nearest neighbor residues exhibiting far larger distances than 4.8 Å. Using these NMR techniques, A β 1–40 was shown to form a parallel β -structure [35]. This finding is similar to that of Benziger et al. [38] for A β 10–35. Comparison with their data shows evidence that A β 10–35 fibrils have parallel β -sheet organization beyond dimers. However, SS-NMR studies on A β 34–42 fibrils suggested an antiparallel β -structure, which was also observed for A β 16–22 capped at both ends [39]. Lansbury et al. [40] characterized fibrils made from the C terminal fragment A β 34–42. They found the alignment of A β 34–42 fibrils to be antiparallel and two residues out of register using rotational resonance experiments on doubly ^{13}C -labeled samples. Therefore, SS-NMR studies have presented evidence for both parallel and antiparallel alignments of A β fragments, depending on the peptide sequence studied and the methodology employed.

In a different approach, Egnaczyk et al. [41] used photo cross-linking. They synthesized a photoreactive A β 1–40 ligand by substituting *L-p*-benzoylphenylalanine (Bpa) for phenylalanine at position 4. This peptide was incorporated into synthetic amyloid fibrils and exposed to near-UV radiation. Analysis of the fibrils showed a Bpa4–Met35 intermolecular cross-link, which was consistent with an antiparallel alignment of A β peptides within amyloid fibrils. Together, the above results show that fibrils can adopt different supramolecular structures depending on the peptide length and properties of the residues present. The differences are of considerable significance. For example, the photo cross-linking data show that the Met35 could be very close to the metal binding site, thus favoring redox reactions with the Met as an electron donor. On the other hand, it is quite conceivable that parallel alignment would greatly favor metal-peptide cross-linking. It is possible that physiologically both kinds of alignment could occur, the proportions being affected by different environments, such as extracellular or membrane associated, the presence/absence of metal ions or differing ratios of Zn to Cu.

8.5 The Metal-Binding Sites and the Structure of A β

The randomness of the A β peptide in aqueous solution makes it difficult to determine the nature of the metal-binding sites. The problem has been approached using various spectroscopic techniques, such as Raman, CD, and magnetic resonance. Miura et al. [42] used Raman spectroscopy to study the binding modes of Zn^{2+} and Cu^{2+} to A β in solution and insoluble aggregates. They found two different modes of metal-A β binding, one characterized by metal binding to the imidazole N_τ atom of histidine, producing insoluble aggregates, the other involving metal binding to the N_π , but not the N_τ , atom of histidine as well as to main-chain amide nitrogens, giving soluble complexes. Zn^{2+} binds to A β only via the N_τ regardless of pH, while the Cu^{2+} binding mode is pH dependent. At mildly acidic pH, Cu^{2+} binds to A β in the former mode, whereas the latter mode is predominant at neutral pH. Miura et al. [42] proposed that the transition from one binding mode to the other explained the strong pH dependence of Cu^{2+} -induced A β aggregation. Dong et al. [43] also employed Raman microscopy to study the metal-binding sites in amyloid plaque cores, using the spectra-structure correlations for A β -transition metal binding. They observed that Zn^{2+} was coordinated to the histidine N_τ and the Cu^{2+} to the N_π , confirming that the metal binding mode was the same in both the synthetic peptide and its aggregates and the naturally occurring plaques.

Huang et al. [44] used multifrequency EPR (L-band, X- and Q-band) to show that copper coordinates tightly to A β 1–40 and that an approximately equimolar mixture of peptide and CuCl_2 produced a single Cu^{2+} -peptide complex. Computer simulation of the L-band spectrum with an axially symmetrical spin Hamiltonian and the g and A matrices ($g_\parallel$, 2.295; $g_\perp$, 2.073; $A_\parallel$, 163.60; $A_\perp$, $10.0 \times 10^{-4} \text{ cm}^{-1}$) suggested a tetragonally distorted geometry, which is commonly found in type 2 copper proteins. Expansion of the $M_I = -1/2$ resonance revealed nitrogen ligand hyperfine coupling. Computer simulation of these resonances indicated the presence of at least three nitrogen atoms. This and the magnitude of the $g_\parallel$ and $A_\parallel$ values, together with Peisach and Blumberg [45] plots, are

consistent with a fourth equatorial ligand binding to copper via an oxygen rather than a sulfur donor atom. Thus, the coordination sphere for the copper-peptide complex was considered to be 3N1O. These authors also used EPR spectroscopy to measure residual Cu^{2+} remaining after incubating stoichiometric ratios of CuCl_2 with A β 1–40. There was a 76% loss of the Cu^{2+} signal, compatible with peptide-mediated reduction of Cu^{2+} to diamagnetic Cu^+ , which is undetectable by EPR, agreeing with the corresponding concentration of Cu^+ measured by bioassay. There was no evidence of free, uncoordinated Cu^{2+} remaining after addition of the peptide, because unbound Cu^{2+} itself gives a different multiple resonance signal.

Using a combination of NMR and EPR spectroscopies, Curtain et al. [46] proposed a structure for the high-affinity site and drew some conclusions about the interaction of the peptide with lipids and its modification by Cu^{2+} , Zn^{2+} , and pH. NMR studies on A β 1–28 and A β 1–40/2 indicated that both peptides were undergoing significant conformational exchange in aqueous solution. NMR and EPR spectra were also recorded for A β 1–28 where the N ϵ 2 nitrogens of the imidazole ring of the His residues 6, 13, and 14 were methylated (Me-A β 1–28). The NMR spectra of Me-A β 1–28 were virtually identical to A β 1–28, 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. A precipitate formed when Zn^{2+} was added to the solutions of A β 1–28 or A β in PBS. NMR spectra of the supernatant of A β 1–28 treated with Zn^{2+} showed that peaks assigned to C2H and C4H of His6, His13, and His14 of A β 1–28 had broadened significantly. However, there was little or no change in the rest of the spectrum compared with A β 1–28 prior to the addition of Zn^{2+} . This broadening of the NMR histidine residue peaks is the result of the interaction of these residues with Zn^{2+} .

The histidyl side chain is a well-established ligand of zinc in proteins and peptides [47], so this result suggested that three of the ligands bound to Zn^{2+} were most likely to be the imidazole rings of the histidine residues [48]. Indeed, His13 had been established by Liu et al. [49] as a crucial residue in the Zn^{2+} -mediated aggregation of A β . The broadening of these peaks is the result of chemical exchange between free and metal-bound states or

among different metal-bound states. The extent of broadening of the peaks indicated intermediate exchange, which on the NMR timescale suggests that the metal-binding affinity is in the micromolar range, in agreement with the low-affinity site described by Bush et al. [16]. The absence of any change in the rest of the spectrum suggested that the metal-bound form of the peptide was monomeric and that there was little or no significant amount of soluble oligomer in solution, because higher order aggregates would have resulted in significantly broadened resonances.

When Cu^{2+} or Fe^{3+} was titrated into an aqueous solution of A β 1–28, 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 (Fig. 8.2). A slight broadening of all peaks in the spectrum (associated with the paramagnetism of Cu^{2+} and Fe^{3+}) was also observed, but there were no other major changes after the addition of the metal ions. Metal-induced precipitation blocked attempts to saturate the metal-binding site. The precipitate made the collection of NMR spectra difficult, and few conclusions could be drawn from spectra of peptide remaining in solution. When Cu^{2+} was added to an aqueous solution of Me-A β 1–28, the changes observed in the spectrum were identical to those

FIGURE 8.2. Amide and aromatic region of the 600 MHz ^1H NMR spectra of A β in aqueous PBS solution and following the addition of Cu^{2+} . Peaks caused by the C2H and C4H of histidines 6, 13, and 14 have been broadened beyond detection because of coordination to the copper. There is a generalized broadening of the rest of the spectrum due to the paramagnetism of the added Cu^{2+} . After Curtain et al. [46].

observed for Cu^{2+} added to A β 1–28, but there was no visible precipitate. In aqueous solution and lipid environments, coordination of metal ions to A β is the same, with His6, His13, and His14 all involved.

The X-band EPR spectrum of Cu^{2+} bound to the peptides had the unsplit intense $g_{\perp}$ resonance characteristic of an axially symmetric square planar 3N1O or 4N coordination, $g_{\parallel} = 2.28$ and $g_{\perp} = 2.03$, $A_{\parallel} = 173.8$ gauss. Similar parameters were found for Cu^{2+} coordination by A β 1–16, A β 1–40, and A β 1–42, indicating that the site was not affected by the size of the C-terminal regions of the peptides. A notable finding with peptides of all lengths was that increasing the Cu^{2+} above ~ 0.3 mol/mol peptide caused line broadening in the Cu^{2+} EPR spectra, over a pH range of 5.5 to 7.5, suggesting the presence of dipolar or exchange effects (Fig. 8.3). These would be observed if two or more Cu ions were within approximately 6 Å of each other. These effects could be explained if at Cu^{2+} /peptide molar ratios >0.3 , A β coordinated a second Cu^{2+} atom cooperatively. They were abolished if the histidine residues were methylated at either N δ 1 or N ϵ 2, suggesting that bridging histidine residues were being formed (Fig. 8.4) [32, 46].

FIGURE 8.3. EPR spectra (9.7 GHz) of A β 1–28 to which had been added respectively: A, 0.2/1 M/M; B, 0.4/1 M/M; C, 0.6/1 M/M; D, 0.8/1 M/M Cu^{2+} /peptide. All spectra recorded at 130 K in pH 7.4 phosphate-buffered saline. Spectra C and D show significant broadening of the $g_{\perp}$ line. All lengths of A β studied give identical spectra (Curtain et al. [46, 79]).

FIGURE 8.4. Model showing how two A β strands (A and B) could be linked by two copper atoms through a bridging histidine. The 6 Å distance between the copper atoms is within the range at which we would expect to see dipolar broadening of Cu^{2+} EPR spectra of the type seen in Figure 8.2.

One consequence of coordination by a metal ion to the N δ 1 of a histidine residue is a reduction in the pK_a of N ϵ 2 NH, making this nitrogen more suitable for metal binding [48], resulting in a histidine residue that can bridge metal ions; a good example being His63 at the active site of superoxide dismutase [50]. Similar bridging histidine residues have been proposed in the octarepeat region of the prion protein [51], which has been shown to possess significant SOD activity in the presence of Cu^{2+} [52]. The line-broadening effects observed in the EPR spectra at Cu^{2+} /A β molar fractions up to 1.0 by Curtain et al. [46] were not observed by Syme et al. [27], Huang et al. [44] or Antzutkin [52]. It is relevant that Huang et al. [54] along with Narayanan and Reif [55] have shown that NaCl has a marked effect on metal-induced aggregation of A β . Huang et al. [44] and Curtain et al. [46] obtained their spectra from samples in phosphate-buffered saline at pH 7.4, Antzutkin [53] adjusted the pH of his sample to pH 7.4 and dialyzed against distilled water, while Syme et al. [27] used ethyl morpholine buffers.

Similar line-broadening phenomena to that observed by Curtain et al. [46] have been observed in the EPR spectra of imidazole-bridged copper complexes designed as SOD mimetics [56]. The bridging histidine may be responsible for the

reversible metal-induced aggregation that is observed when A β is metallated with Cu²⁺ and Zn²⁺. The bridging histidine residues may also explain the multiple metal-binding sites observed for each peptide and the high degree of cooperativity evident for subsequent metal binding. With three histidines bound to the metal center, 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. It should be noted here that the bridging histidine hypothesis of peptide association would favor a parallel over an antiparallel β -sheet structure for the fibrils and plaques. It is quite possible that metal-induced precipitation of A β is quite different from that induced by prolonged incubation of monomeric peptide in the putative absence of metal. For example, Miura et al. [42] strongly suggested that the metal-induced aggregation of A β was promoted by cross-linking of the peptides through metal-His[N_T] bonds, most likely through His[N_T]-metal-His[N_T] bridges at three histidine residues.

Observations that rat A β , which differs from human A β by three substitutions (Fig. 8.1) [57], does not reduce Cu²⁺ and Fe³⁺, is not readily precipitated by Zn²⁺ or Cu²⁺, does not produce ROS as strongly as the human sequence, and does not produce plaques highlight the importance of the three histidines. Rat A β forms a metal complex via two histidine residues and two oxygen ligands rather than three histidine residues and one oxygen ligand, compared with human A β where the side chain of His13 of human A β is ligated to the metal ion. This was borne out by the EPR spectrum, which was typical of a square planar 2N2O Cu²⁺ coordination [44].

Syme et al. [27] and Antzutkin [53] both used X-band EPR to study the interaction of A β with Cu²⁺ in solution, confirming the axially symmetric binding site. Syme et al. [27] obtained EPR spectra at pH 7.4 and higher that showed heterogeneity attributed to a second high-affinity binding site. This site became much more prominent when the pH was raised to 10.0. The heterogeneity at pH 7.4 was not observed by Huang et al. [44], Curtain et al. [46], or by Antzutkin [53] and warrants further investigation. It is possible that the second binding site is a buffer ion effect. In order to define the binding site, Syme et al. [27] also prepared

mutants of A β 1–28 in which each of the histidine residues had been replaced by alanine or in which the N-terminus was acetylated, and their data suggested that the N-terminus and His13 and His14 are crucial for Cu²⁺ binding and that H6 also played a part. On this basis, they proposed a square planar model with the Cu²⁺ coordinated to His13, His14, His6, and the amino N of the N-terminus. Although a 4N model may be fitted to Syme et al.'s [27] X-band spectra, it is not compatible with the conclusions derived by Huang et al. [44] from L-band spectra and their superhyperfine structure that point to a 3N1O coordination.

Karr et al. [58] found that A β peptides lacking one to three N-terminal amino acids but containing His6, His13, and His14 and Tyr10 did not coordinate Cu²⁺ in the same environment as the native peptide, suggesting that these N-terminal residues are significant for Cu²⁺ binding. They also confirmed that the coordination is identical with any length of peptide (A β 1–16, A β 1–28, A β 1–40, A β 1–42) that contained the first 16 amino acids. These authors also showed [59] that the coordination of Cu²⁺ did not change during organization of monomeric A β into fibrils and that neither soluble nor fibrillar forms of A β 1–40 contained antiferromagnetically exchange-coupled binuclear Cu²⁺ sites in which two ions were bridged by an intervening ligand. The latter conclusion was based on a temperature-dependence study of the EPR spectra for Cu²⁺ bound to soluble or fibrillar A β showing that the Cu²⁺ center displayed normal Curie behavior, indicating that the site was mononuclear.

Further advances in understanding the N coordination of Cu²⁺ will require more sophisticated EPR techniques than have been used so far, supported by input from other methods such as XAFS. Equally, there remains uncertainty as to the nature of the potential O ligand. Proton NMR data obtained by Syme et al. [27] agreed with the findings of Huang et al. [44] and Curtain et al. [46] that histidine residues are involved in Cu²⁺ coordination, but they found that Tyr10 was not involved. Further, Karr et al. [58] found that the coordination of Cu²⁺ in the Y10F mutant of A β remained 3N1O with EPR spectra identical to the wild-type spectra. Isotopic labeling experiments showed that water was not the O-atom donor to Cu²⁺ in A β fibrils or in the Y10F mutant. However, the Raman data of

Miura et al. [42] suggest that the ligand was the O of the tyrosine hydroxyl. They were able to assign the 1504 cm^{-1} band in the Raman spectra of insoluble Cu^{2+} -A β 1–16 aggregates to Cu^{2+} -bound tyrosinate, and the high intensity of the 1604 cm^{-1} band was attributed to a contribution from the Y8a band of tyrosinate. Unlike Zn^{2+} , Cu^{2+} binds to tyrosine in the insoluble aggregates of A β 1–16. When the deprotonated phenolic oxygen of tyrosinate is bound to a transition metal ion such as Cu^{2+} and Fe^{3+} , the Y19a band shifts to about 1500 cm^{-1} and gains intensity through resonance with a δ (phenolate) $f d$ (metal) charge-transfer transition in the visible. Such charge transfer does not occur for Zn^{2+} , the d orbitals of which are fully occupied. It should be noted that in these experiments, Miura et al. [42] used phosphate-buffered saline, which might have had the effect of encouraging peptide association [54, 55].

In conclusion, although there is general agreement as to the nature of the monomeric binding site insofar as it is type two Cu^{2+} with a 3N10 coordination, varying buffer conditions, peptide concentration, and conformation make it difficult to compare one set of published data with another. There is a similarity here with the studies on the alignment of the peptide in fibrils. In considering the issue of monomeric versus dimeric Cu^{2+} , it is important to remember that A β may form oligomers and multimers in a variety of ways, some more relevant to its neurotoxicity than others [60–64].

8.6 A β Redox Activity and the Role of Metal Coordination

Oxidative stress markers characterize the neuropathology both of Alzheimer's disease and of amyloid-bearing transgenic mice. The neurotoxicity of A β has been linked to hydrogen peroxide generation in cell cultures by a mechanism that is still being fully described but is likely to be dependent on A β coordinating redox active metal ions. Huang et al. [65] showed that human A β directly produces hydrogen peroxide (H_2O_2) by a mechanism that involves the reduction of metal ions, Fe^{3+} or Cu^{2+} . They used spectrophotometry to show that the A β peptide reduced Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^+ and that molecular oxygen is then

trapped by A β and reduced to H_2O_2 in a reaction that is driven by sub-stoichiometric amounts of Fe^{2+} or Cu^+ . In the presence of Cu^{2+} or Fe^{3+} , A β produced a positive thiobarbituric-reactive substance, compatible with the generation of the hydroxyl radical [OH^*]. Tabner et al. [66] used the 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) spin-trap to identify the radical produced by A β in the presence of Fe^{2+} and concluded that it was OH^* . However, they also found OH^* was produced in the presence of Fe^{2+} by A β 25–35, which does not contain a strong metal binding site. Because Fe^{2+} with trace amounts of Cu^{2+} as low as 0.01 mol%, corresponding with the amount of adventitious Cu found in the average peptide preparation, will produce an OH^* adduct with the DMPO spin trap [Curtain et al., unpublished], Tabner et al.'s [66] results should be treated with caution even though they appear to confirm the findings of Huang et al. [65].

In the course of metal-catalyzed redox activity, A β may undergo under a number of changes. Atwood et al. [67] found that Cu^{2+} induced the formation of SDS-resistant oligomers of A β that gave a fluorescence signal characteristic of the cross-linking of the peptide's Tyr10. This finding was confirmed by directly identifying the dityrosine by electrospray ionization mass spectrometry and by the use of a specific dityrosine antibody. The addition of H_2O_2 strongly promoted Cu^{2+} -induced dityrosine cross-linking of A β 1–28, A β 1–40, and A β 1–42, and it was suggested that the oxidative coupling was initiated by interaction of H_2O_2 with a Cu^{2+} tyrosinate. The dityrosine modification is significant because it is highly resistant to proteolysis and would be important in increasing the structural strength of the plaques. Schoneich and Williams [68], however, were unable to find any evidence of tyrosine oxidation. They used ascorbate/ Cu^{2+} -induced oxidation and electrospray ionization-time-of-flight MS/MS analysis to study the oxidation products of A β 1–16, A β 1–28, and A β 1–40. Initial oxidation targets were His13 and His14, which were converted to 2-oxo-His, while His6 and Tyr10 were unchanged, although His6 was oxidized after longer oxidation times. The formation of 2-oxo-His suggests that a transient 2C centered His radical might have been formed. Such radicals have been described in a number of biological redox systems [69, 70], although not so far in any of neuropathological significance.

Schoneich and Williams [68] explained the insensitivity of His6 to initial oxidation by suggesting that histidine bridging of two Cu^{2+} -A β molecules lowered the electron density on His6, comparable with similar results on a Cu^{2+} - and Zn^{2+} -bridging His61 residue of bovine Cu,Zn superoxide dismutase.

Barnham et al. [71] used density functional theory calculations to elucidate the chemical mechanisms underlying the catalytic production of H_2O_2 by A β /Cu and the production of dityrosine. Here, Tyr10 was identified as the critical residue. This finding accords with the growing awareness that the O_2 activation ability of many cupro-enzymes is also coupled to the redox properties of tyrosine and the relative stability of tyrosyl radicals. The latter play important catalytic roles in photosystem II, ribonucleotide reductase, COX-2, DNA photolyase, galactose oxidase, and cytochrome-c oxidase [72].

With ascorbate as the electron donor, the first step in the catalytic production of H_2O_2 is the reduction of Cu^{2+} to Cu^+ . Barnham et al. [71] proposed that the transfer could take place via a proton-coupled electron transfer (PCET) mechanism.

Reactions involving PCET are being increasingly implicated in a range of biological systems, including charge transport in DNA and enzymatic oxygen production [73]. In this system, the electron transfer involves both p- and d-orbitals on the ascorbate, Tyr10, and the copper ion, while proton transfer involves p-orbitals on the O_2 -atom of ascorbate, and the side-chain oxygen of Tyr10 (Figs. 8.5A and 8.5B). The significant change in electron spin on the copper ion going from the ground state to the transition state suggests that the proton and the electron are transferred within different molecular orbitals, as is predicted to be necessary for PCET to occur [73]. The activation energy for this one electron reduction step was computed to be only 0.9 kcal/mol.

Barnham et al. [71] tested the Cu/tyrosinate hypothesis using an A β 1–42 peptide with Tyr10 substituted with alanine (Y10A). Both peptides gave rise to similar ^{65}Cu EPR spectra with the strong single $g_{\perp}$ resonance characteristic of an axially symmetric square planar complex, although there was a significant increase in the $g_{\parallel}$ value of Y10A. The increase was probably due to some distortion of the coordination sphere because the

FIGURE 8.5. (A) The transition state that is formed when a hydrogen atom is transferred from ascorbate to the side-chain oxygen of A β Y10, which acts as a gate, and passes an electron to Cu^{2+} reducing it to Cu^+ [71]. (B) An intermediate formed along the reaction path where Y10 has transformed into a tyrosyl radical giving up its side-chain hydroxyl hydrogen atom to $\text{O}_2^{\bullet-}$ via hydrogen atom transfer. Simultaneously, H_3O^+ has donated its proton to $\text{O}_2^{\bullet-}$ via proton transfer, whereupon H_2O_2 has formed. Formed tyrosyl radical and water molecule are hydrogen bonded to H_2O_2 . Ascorbyl radical anion coordinates via its O1-oxygen anion in an apical position to Cu^{2+} . Figures based on data of Barnham et al. [71].

oxygen ligand, which was possibly from Tyr10, was now derived from another oxygen donor (e.g., phosphate, or carboxylate from the peptide). While wild-type A β 1–42 rapidly reduces Cu²⁺ to Cu⁺ in aqueous solution, with near-complete reduction taking 80 min, the mutation of Tyr10 to alanine markedly decreased the ability of A β to reduce Cu²⁺. Further, spin trapping studies also confirmed the DFT observation that Tyr10 acts as a gate that facilitates the electron transfer needed to reduce Cu²⁺ to Cu⁺. When the spin trap 2-methyl-2-nitrosopropane (2MNP) [74] was added to the reaction mixture *w.t.* A β 1–42/Cu²⁺/ascorbate, a broad line triplet characteristic of a trapped carbon-centered radical bound to a peptide appeared in the EPR spectra. However, if Y10A peptide were substituted for the *w.t.*, formation of this triplet was inhibited (Fig. 8.6). Although this is not conclusive evidence that the radical is on Tyr10, the possibility that a His radical was trapped by the 2MNP can be discounted because the A_N value (15.5) of the spectrum in Figure 8.6 is closer to those found for Tyr adducts [75] than for C-centered His, which furthermore show marked superhyperfine structure [69]. It is likely that which transient radical is trapped in a given Cu:A β redox system will depend on a number of experimental variables only some of which may be biologically relevant.

FIGURE 8.6. X-band EPR spectra of adducts formed after the addition of 100 mM spin trap 2-methyl, 2-nitrosopropane to respectively wild-type A β (50 mM incubated at 20°C for 30 min with 25 mM Cu²⁺ in pH 7.4 PBS) and the Y10A mutant of A β at the same Cu/peptide ratio and pH. End-to-end width of spectrum, 100 gauss. Figure based on data of Barnham et al. [71].

8.7 The Effect of Metal Binding on the Interaction of A β with Membranes

An alternative explanation of A β neurotoxicity, not necessarily excluding the production of ROS, is based on the peptide's interaction with membranes and/or membrane proteins. Numerous reports have described the effects of A β on membranes and lipid systems and their possible roles in its neurotoxicity. The NMR studies cited earlier in this chapter showed considerable variation in peptide conformation in different membrane-mimetic systems. There is much experimental evidence from CD and Fourier transform infrared spectroscopies that the A β peptides can be membrane associated in the β -configuration [75], although there are reports of membrane-associated α -helices being found in the presence of gangliosides [76], cholesterol [77], and Cu²⁺ or Zn²⁺ [46]. This variability under different conditions can be understood because most of the amyloidogenic peptides have been identified, along with viral fusion peptides, as being exceptionally pleiomorphic in structure [78]. This identification was based on the high prevalence of alanine and glycine residues within a hydrophobic sequence.

As the cell membrane is a mosaic of lipids and protein segments, it is possible that the peptides will exhibit different structures with different properties in different parts of the mosaic. The pleiomorphism is highly relevant to the cytotoxicity of the peptide, because factors influencing it could act as switches to determine whether the peptide is a β -sheet with the potential to form amyloid or be membrane surface seeking, or a membrane-penetrant α -helix.

Curtain et al. [46, 79] used a combination of EPR and CD spectroscopies to study the effect of metal ions, pH, and cholesterol on the interaction of A β with bilayer membranes. EPR spectroscopy, using spin-labeled lipid chains or protein segments, has been used extensively to study translational and rotational dynamics in biological membranes. Lipids at the hydrophobic interface between lipid and transmembrane protein segments and peptides in their monomeric and oligomeric states have their rotational motion restricted [80]. This population of lipids can be resolved in the EPR spectrum as a motionally restricted component distinct from the

FIGURE 8.7. A: X-band EPR spectrum recorded at 305 K of the negatively charged spin probe 1-palmitoyl-2-(16-doxyl stearoyl) phosphatidyl serine in negatively charged LUV made from 50% palmitoyl oleoyl phosphatidyl serine and 50% palmitoyl oleoyl phosphatidyl choline (probe/lipid 1/300). B: X-band spectra of system A at the same temperature after the addition of Cu^{2+} A β 1–42 (peptide/lipid 1/50), showing a shoulder (marked with arrow) to the left of the low field line. This is typical of peptide penetration into the bilayer core [73, 75]. C: The difference spectrum $\times 5$ obtained when spectrum A is subtracted from spectrum B. This spectrum represents the motionally restricted lipid in the boundary. Original data given in Curtain et al. [46, 79].

fluid bilayer lipids (Fig. 8.7), which can be quantified to give both the stoichiometry and selectivity of the first shell of lipids interacting directly with membrane-penetrant peptides. The stoichiometric data can give an estimate of the number of subunits in a membrane-penetrant oligomeric structure. Using this approach, it was shown that A β 1–40 and A β 1–42 bound to Cu^{2+} or Zn^{2+} penetrated bilayers of negatively charged, but not zwitterionic lipid, giving rise to such a partly immobilized component in the spectrum (see Fig. 8.7 and its caption) [46, 79].

When the peptide:lipid ratio was increased, the relationship between the mole fraction of peptide and proportion of slow component was linear. Even at a fraction of 15%, all of the peptide was associated with the lipid, suggesting that the structure penetrating the membrane lipid was well defined, although at such a high peptide:lipid ratio further study would be needed to confirm whether

the lipid still retained a lamellar structure. Formation of non-lamellar structures in regions of the membrane associated with A β could well be the cause of the peptide's cytotoxicity. From the spin-label data, the first shell lipid:peptide was approximately 4:1. This stoichiometry can be satisfied by 6 helices arranged in a pore surrounded by 24 boundary lipids. This hypothetical structure gains credibility from atomic force microscopy studies of A β 1–42 reconstituted in a planar lipid bilayer that showed multimeric channel-like structures, many resembling hexamers, similar to that modeled in Figure 8.8 [81]. It was found [46] that in the presence of Zn^{2+} , A β 1–40 and A β 1–42 both inserted into the bilayer over the pH range 5.5–7.5, as did A β 1–42 in the presence of Cu^{2+} . However, A β 40 only penetrated the lipid bilayer in the presence of Cu^{2+} at pH 5.5–6.5; at higher pH, there was a change in the Cu^{2+} coordination sphere that inhibited membrane insertion. The addition of cholesterol up to 0.2 mole fraction of the total lipid inhibited insertion of both peptides under all con-

FIGURE 8.8. Animation of hexameric pore formed by A β 1–40 helices calculated from annular lipid stoichiometry as determined from the EPR data shown in Figure 8.5. Polar residues are shown as dark and nonpolar as light. View from N-terminus. Peptide coordinates (in SDS) obtained from Barrow and Zagorski [31]. Model prepared using Sculpt[®] by aligning hydrophobic contacts between helices and orienting nonpolar residues in sequence 21–40 to annular lipid.

ditions investigated. CD spectroscopy revealed that the A β peptides had a high α -helix content when membrane penetrant, but were predominantly β -strand when not. Simulation of the spectra and calculation of the on-off rates suggested that the peptide was most likely penetrating as an α -helix [82].

In membrane-mimetic environments, coordination of the metal ion is the same as in aqueous solution, with the three-histidine residues, at sequence positions 6, 13, and 14, all involved in the coordination, along with an oxygen ligand. As had been observed at Cu²⁺/peptide molar ratios >0.3 in aqueous solution, line broadening was detectable in the EPR spectra, indicating that the peptide was coordinating a second Cu²⁺ atom in a highly cooperative manner at a site 6 Å from the initial binding site. So, there appear to be two switches, metal ions (Zn²⁺ and Cu²⁺) and negatively charged lipids, needed to change the conformation of the peptide from β -strand nonpenetrant to α -helix penetrant. The closest parallel to this behavior is that observed with the B18 fusogenic sequence of the fertilization protein bindin [83] that, like A β , possesses three histidine residues strategically placed to coordinate metals. In the absence of Zn²⁺, this peptide forms nonfusing β -sheet amyloid fibrils. In the presence of Zn²⁺, an α -helical conformation is imposed on its backbone and it forms fusogenic oligomers.

8.8 The Relevance of Membrane Binding to A β Cytotoxicity: The Role of Methionine 35

In vitro, the methionine at position 35 can act as an electron donor, and its conversion to the sulfoxide form has been the subject of several studies, given that the Met(O)A β peptide has been isolated from AD amyloid brain deposits [84, 85]. Furthermore, the Raman spectroscopic study by Dong et al. [43] of senile plaque cores isolated from diseased brains has shown that much of the A β in these deposits contained methionine sulfoxide with copper and zinc coordinated to the histidine residues.

Although there are several potential electron donors such as GSH and ascorbic acid, in vivo it is likely that Met35 occupies a privileged position

being part of the A β sequence. When it is missing as in A β 1–28, the addition of exogenous methionine permits redox action to proceed, but with slower kinetics [46]. When Met35 is sequestered within a lipid environment, there is also no metal reduction. Its oxidation also alters the physical properties of the peptide. Met(O)A β is more soluble in aqueous solution, and there is a disruption of the local helical structure when the peptide is dissolved in SDS micelles [86].

The formation of trimers and tetramers by Met(O)A β is significantly attenuated and fibril formation is inhibited [87, 88]. Barnham et al. [89] showed by solid-state NMR that when A β coordinates and reduces Cu²⁺ to Cu⁺, the Met35 is oxidized. Although the Cu²⁺ coordination of the oxidized peptide is identical to nonoxidized A β and it will produce H₂O₂, it cannot penetrate lipid bilayers either in the presence or absence of Cu²⁺ or Zn²⁺. On the other hand, Met(O)A β is toxic to neuronal cell cultures, a toxicity that is rescued by catalase and the MPAC clioquinol. These results suggest that fibril formation and membrane penetration by A β could be epiphenomena, and that the main requirement for cytotoxicity is redox competence. In this connection, it is important to note that the oxidized M35 has the potential for further reduction to the sulfone [90] and could thus still act as a Cu²⁺ reductant, acting in vivo in concert with agents such as ascorbic acid and GSH.

It might be legitimately asked whether Met35 could act as a Cu²⁺ ligand. After all, there are many instances of copper proteins where the ion is coordinated to a thioether, giving in most cases a type 1 binding site [91]. Such coordination involving two nitrogens and an oxygen in addition to the sulfur is generally distorted tetrahedral rather than square planar and would favor Cu⁺ over Cu²⁺. Because the former is EPR silent, the possibility of this coordination might have been overlooked. However, in their Raman spectroscopic studies, Miura et al. [42] were unable to detect any Cu-S bonds.

Ciccotosto et al. [92] further probed the role of Met35 by preparing A β 1–42 in which it was replaced with valine (A β M35V). The neurotoxic activity on primary mouse neuronal cortical cells of this peptide was enhanced, and this diminished cell viability occurred at a much faster rate compared with A β 1–42. When cortical cells were

treated with the peptides for only a short 1-h duration so as to minimize the incidence of cell death, and the amount of peptide bound to cortical cell extracts was quantitated by Western blotting, it was found that twice as much A β M35V compared with wild-type A β peptide bound to the cells after a 1-h cell exposure. It was suggested that the increased toxicity was related to the increased binding.

A β M35V bound Cu²⁺ with the same coordination sphere as *w.t.* A β and produced similar amounts of H₂O₂ as A β 1–42 *in vitro*. The neurotoxic activity was rescued by catalase. The redox activity of the mutated peptide was followed by measuring the decline in time of the strength of the Cu²⁺-A β M35V EPR signal, which showed that the reduction of Cu²⁺ to the EPR silent Cu⁺ was much slower compared with A β 1–42, confirming that the M35 residue in A β 42 plays an important part in the redox behavior of this peptide in solution. Like Cu²⁺-A β 1–42, Cu²⁺-A β M35V inserted into a spin-labeled lipid bilayer gave a partially immobilized component in the EPR spectrum. This component had a narrower linewidth than that found for the similar component obtained with *w.t.* Cu²⁺-A β 1–42, suggesting that the valine substitution made the mutant peptide less rigid in the bilayer region and possibly easier to insert, thus explaining the increased cell membrane binding. The on- and off-rate constants estimated from the simulation experiments showed that A β M35V had a higher affinity for the lipid bilayer as compared with A β 42. CD analysis showed that A β M35V had a higher proportion of β -sheet structure and random coil than A β 1–42, which would also suggest a more flexible structure in the bilayer [80, 82]. In summary, these and the results described above tell us that the wild-type A β , its oxidized form, Met(O)A β , and the mutant peptide, A β M35V, induce cell death via similar pathways that are metal-dependent and can generate H₂O₂ in the absence of a methionine residue. Fibril formation as a toxic species is not responsible for cell death. Membrane association *per se* may play a part in localizing the peptide, perhaps in domains particularly susceptible to oxidative damage. It follows, therefore, that elucidating the metal ion binding site of A β may provide a promising new therapeutic target for AD.

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