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Free Radicals, Metal Ions, and Aβ Aggregation and Neurotoxicity

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Abstract

Two of the characteristic pathologic features present in the brains of Alzheimer's disease (AD) patients are the deposition of aggregated amyloid-β peptide (Aβ) and high levels of oxidative stress. Both these phenomena can be explained by Aβ's interactions with metal ions. When Aβ coordinates Zn, Cu, and Fe, the peptide aggregates. If the metals are redox active such as Cu and Fe, then reactive oxygen species (ROS) are generated. The generation of ROS has been implicated in the toxic mechanism of Aβ. A class of metal-protein attenuating compounds (MPACs) that are capable of inhibiting Aβ-metal interactions have been developed. The prototypic MPAC, clioquinol, has shown efficacy in cell and animal models of AD and promising results in a small-scale phase IIa clinical trial.

2.1. Introduction

Metal ions are essential for life, and approximately 30% of all proteins are metalloproteins. The unique chemical properties of the various metal ions allow the coordinating proteins to carry out vital cellular processes. Consequently, cells have developed highly elaborate means of regulating metal ion interactions; because the biochemical use of metal ions is not without hazard. The same properties that cells harness for beneficial means can become destructive when not properly regulated. This is particularly so for the most abundant redox-active metals copper and iron; the ability of these metal ions to occupy multiple valence states and activate molecular oxygen has been utilized by a variety of enzymes including those involved in cellular respiration. However, unregulated, redox-active metals will inappropriately react with oxygen to generate toxic ROS.

The brain is an organ that concentrates metal ions, and recent evidence suggests that a breakdown in metal homeostasis plays a critical role in a variety of age-related neurodegenerative diseases (Bush, 2003; Barnham *et al*., 2004b). Common features of these diseases include the deposition of misfolded protein (specific to the disease) and substantial cellular damage as a result of oxidative stress. As a general principle, the chemical origin of this oxidative damage comes from reactions of molecular oxygen with the redox-active metals copper and iron that are resident in the tissues (Halliwell and Gutteridge, 1999). We have noted that abnormal metal interaction with the target protein in several age-dependent degenerative diseases could contribute to the etiology (Bush, 2003; Barnham *et al*., 2004b). Hence, copper has been implicated in amyotrophic lateral sclerosis (the aggregating protein is superoxide dismutase 1) and

Creutzfeldt-Jakob disease (prion) while iron may play a significant role in Parkinson's disease (α-synuclein). Evidence is emerging that implicates copper, iron, and redox-silent zinc in the pathogenesis of AD, and modulating these interactions may have therapeutic potential.

Genetic evidence from cases of familial AD indicates that Aβ metabolism is linked to the disease (Hardy, 1997; Price *et al*., 1998). AD is characterized by the deposition of amyloid plaques; the major constituent of AD plaques is Aβ, which is cleaved from the membrane-bound amyloid precursor protein (APP) (Glenner and Wong, 1984; Masters *et al*., 1985; Kang *et al*., 1987).

The importance of Zn^{2+} in plaque formation has been highlighted by the finding that age and female sex-related plaque formation in Tg2576 transgenic mice was greatly reduced upon the genetic ablation of the zinc transporter 3 protein, which is required for zinc transport into synaptic vesicles (Lee *et al*., 2002). The amyloid plaques could be described as metallic sinks as remarkably high concentrations of Cu (400 μM), Zn (1 mM), and Fe (1 mM) have been found in amyloid deposits in AD-affected brains (Smith *et al*., 1997; Lovell *et al*., 1998).

In vitro studies have shown that low micromolar levels of Zn^{2+} are sufficient to induce protease-resistant aggregation and precipitation of Aβ (Huang *et al*., 1997). Cu^{2+} and Fe^{3+} also induce peptide aggregation that is exaggerated at acidic pH (Atwood *et al*., 1998). These metals are found in relatively high concentrations in the region of the brain most susceptible to AD neurodegeneration, where they play important roles in normal physiology. During neurotransmission, high concentrations of Zn (300 μM) and Cu (30 μM) are released, which may explain why Aβ precipitation into amyloid commences in the synapse (Terry, 1996). It has recently been shown that the *N*-methyl-D-aspartate (NMDA) receptor mediates copper homeostasis in neurons (Schlief *et al*., 2005) and conversely that copper has an inhibitory effect on NMDA receptors (Trombley and Shepherd, 1996).

Another factor that has been suggested to contribute to AD is a breakdown in the degradation/clearance mechanisms of Aβ with age. One of the genetic risk factors associated with AD is apolipoprotein E (Apo E) allotype ($\varepsilon 4 > \varepsilon 3 > \varepsilon 2$) (Strittmatter *et al*., 1993). Apo E is the principal cholesterol carrier in the brain and may be involved in the degradation/clearance of Aβ (Puglielli *et al*., 2003). It is not yet clear how the various Apo E isoforms act to impact upon AD pathophysiology. However, Apo E is also a metal chelator (Miyata and Smith, 1996). This property may contribute to the ability of Apo E to protect neuronal cell cultures against both H_2O_2 and Aβ-mediated toxicity, with the isoform ε2 giving greater protection than ε3, with ε4 giving the least protection (Miyata and Smith, 1996). The level of protection parallels the ability of the isoforms to coordinate metal ions and to inhibit Zn- or Cu-induced aggregation of Aβ (Moir *et al*., 1999).

The cause of the neuronal cell loss in AD may be related to increased oxidative stress from excessive free-radical generation (Martins *et al*., 1986; Smith *et al*., 1997; Sayre *et al*., 2000). The major source of free-radical production in the brain is from the reactivity of transition metals, Cu and Fe. These metals do not exist in a free ionic form but exert biochemical redox activity in the bound state. Despite being vital, if the redox activity of Cu and Fe is not strictly regulated, toxic ROS such as the hydroxyl radical (•OH) can be generated (Halliwell and Gutteridge, 1999). High oxygen consumption, relatively low antioxidant levels, and limited regenerative capacity results in brain tissue being particularly susceptible to oxidative damage from ROS. There is a large body of evidence indicating that the homeostasis of Zn, Cu, and Fe and their respective binding proteins are significantly altered in the AD brain (Atwood *et al*., 1999). Microparticle-induced x-ray emission analysis of the cortical and accessory basal nuclei of the amygdala indicated these metals accumulate in the neuropil of the AD brain in concentrations that are three- to fivefold increased compared with age-matched controls (Lovell *et al*., 1998). Importantly, these metal ions are normally concentrated in those regions of the brain most affected by AD pathology. Evidence for abnormal Cu

homeostasis in AD includes a 2.2-fold increase in the concentration of Cu in cerebrospinal fluid (Basun *et al*., 1991), and an accompanying increase of plasma Cu in AD (Squitti *et al*., 2002). There is an extensive literature describing abnormal levels of Fe and Fe-binding proteins in AD (Bishop *et al*., 2002). Importantly, the Fe that is found within the amyloid deposits of human brain and in amyloid-bearing APP transgenic mice is redox active (Smith *et al*., 1997, 1998). Raman spectroscopy studies have demonstrated that Zn^{2+} and Cu^{2+} are coordinated to the histidine residues of the deposited Aβ in the senile plaque cores from diseased brain tissue and that the sulfur atom of Met35 of Aβ is oxidized, indicative of a pro-oxidant environment (Dong *et al*., 2003).

2.2. APP/Aβ and Metal Homeostasis

To prevent transition metal-mediated oxidative stress, cells have evolved complex metal transport systems that deliver Cu and Fe to metalloenzymes and proteins. These include mammalian Cu chaperones that are involved in intracellular Cu trafficking to Cu/Zn superoxide dismutase and the Wilson's disease Cu ATPase (Waggoner *et al*., 1999). The chaperones direct the Cu atoms to specific intracellular proteins, which results in unbound Cu being essentially absent in the intracellular environment (Rae *et al*., 1999). Therefore, cuproproteins have an important role in maintaining cellular Cu metabolism (Andrews, 2001). APP is a ubiquitously expressed high-turnover protein, and data suggests that APP and Aβ may have some role in metal homeostasis. Using transgenic mouse models, it has been shown that overexpression of the carboxyl-terminal fragment of APP, containing Aβ, results in significantly reduced copper and iron levels in transgenic mouse brain; overexpression of APP in Tg2576 transgenic mice results in significantly reduced copper, but not iron (Maynard *et al*., 2002), while APP knockout mice have increased copper levels in the brain and liver (White *et al*., 1999). Consistent with this observation is the rise of intracellular copper in primary mouse cortical neurons and embryonic fibroblasts as a result of gene knockout of APP and APLP2 (Bellingham *et al*., 2004). Recently, gene expression profiling has been used to show that APP expression is increased in response to chronic copper overload (Armendariz *et al*., 2004), whereas copper deficiency leads to a downregulation of the APP gene (Bellingham *et al*., 2004b). Copper levels can modulate APP processing, such that higher copper levels result in a reduction in Aβ production and a consequential increase in the non-amyloidogenic p3 form of the peptide and an increase in the secretion of the APP ectodomain (Borchardt *et al*., 1999). Consistent with these cell-based findings are studies with APP transgenic mice that had their copper levels raised either by dietry supplement (Bayer *et al*., 2003) or genetic manipulation (Phinney *et al*., 2003), which showed lower levels of amyloid burden.

Independent copper binding sites have been identified on both Aβ and APP, which we hypothesize subserve a physiologic relationship with Cu. The molecular structure of the APP copper-binding domain has been solved and found to contain a novel copper-binding site that favors Cu(I) coordination (Barnham *et al*., 2003a). The solvent accessibility of this site, structural homology to copper chaperones, and the role of APP in neuronal copper homeostasis are consistent with APP acting as a neuronal metallotransporter. Additionally, a zinc-binding site has also been observed within this domain encompassing residues 181–198 (Bush *et al*., 1993).

It has recently been shown that soluble APP is able to stimulate NMDA receptor activation (Xiong *et al*., 2004); given the role that Cu plays in modulating NMDA receptor activity (Schlief *et al*., 2005) and APP plays in regulating Cu levels, it is possible that this APP activation of the NMDA is mediated through modulating Cu concentrations at the synapse.

2.3. Cu^{2+}- and Zn^{2+}-Induced Aggregation of Aβ

In vitro studies have shown that Aβ will coordinate Cu^{2+}, Zn^{2+}, and Fe^{3+} with high affinity (Bush *et al*., 1994b), explaining the presence of these metals in amyloid plaques. This study also showed stabilization by Cu^{2+} of an apparent Aβ1-40 dimer on gel chromatography. It has been shown that $^{65}Zn^{2+}$ is displaced from Aβ when co-incubated with excess Cu^{2+} (Clements *et al*., 1996), and that Cu^{2+} and Zn^{2+} share a common binding site (Yang *et al*., 2000). Atwood *et al*., 1998) found that Cu^{2+} was bound to soluble Aβ via histidine residues, and the precipitation of soluble Aβ by Cu^{2+} was reversibly modulated by pH with mildly acid conditions (pH 6.6) promoting Cu^{2+}-mediated precipitation, whereas raising the pH dissolved precipitated Aβ:Cu^{2+} complexes. Zn^{2+}-induced aggregation of soluble Aβ at pH 7.4 *in vitro* was totally reversible with chelation (Cherny *et al*., 1999). It has also been reported (Cherny *et al*., 1999) that marked Cu^{2+}-induced aggregation of Aβ1-40 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. Aβ1-40 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 Aβ1-40 and Aβ1-42 with submicromolar concentrations of Cu^{2+}. Unlike Aβ1-40, Aβ1-42 was precipitated by submicromolar Cu^{2+} concentrations at pH 7.4. Rat Aβ1-40 and histidine-modified human Aβ1-40 were not aggregated by Zn^{2+}, Cu^{2+}, or Fe^{3+}, indicating that histidine residues are essential for metal-mediated Aβ assembly. Subsequently, it was shown that Cu^{2+}- and Zn^{2+}-selective chelators enhanced the dissolution of amyloid deposits in postmortem brain specimens from AD subjects (Cherny *et al*., 1999) and from amyloid precursor protein overexpressing transgenic mice (Cherny *et al*., 2001), confirming the part played by these metal ions in cerebral amyloid assembly. In particular, Zn^{2+} efficiently induces aggregation of synthetic Aβ under conditions similar to the physiologic ones in the normal brain, that is, at nanomolar and submicromolar concentrations of Aβ and Zn^{2+}, respectively (Bush *et al*., 1994b). Recently, it has been demonstrated that Aβ does not precipitate when trace metal ions are rigorously excluded (Huang *et al*., 2004). The effect of Cu^{2+} on the aggregation of Aβ is ambiguous compared with Zn^{2+}· Cu^{2+} has been shown to be a strong inducer of Aβ aggregation under certain conditions (Garzon-Rodriguez *et al*., 1997). In contrast with the Zn^{2+}-induced Aβ aggregation that occurs over a wide pH range (5.5–7.5), the Cu^{2+}-induced aggregation occurs primarily at mildly acidic pH (Atwood *et al*., 1998).

2.4. The Metal Binding Site(s) of Aβ

In aqueous solution, Aβ undergoes rapid conformational exchanges making it difficult to determine the precise nature of the metal binding sites. The question has been approached using various spectroscopic techniques, including Raman, CD, and magnetic resonance. Raman spectroscopy has been used to study the binding modes of Zn^{2+} and Cu^{2+} to Aβ in solution and insoluble aggregates (Miura *et al*., 2000). Two different modes of metal-Aβ binding were reported, 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 Aβ 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 Aβ in the former mode, whereas the latter mode is predominant at neutral pH. From this data, it was proposed that the transition from one binding mode to the other explained the strong pH dependence of Cu^{2+}-induced Aβ aggregation (Miura *et al*., 2000). Raman microscopy was also

employed to study the metal binding sites in amyloid plaque cores, using the spectra-structure correlations for Aβ-transition metal binding (Dong *et al*., 2003). Again it was 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*. (1999a) used multifrequency electron paramagnetic resonance (EPR; S-, X-, and Q-band) to show that copper coordinates tightly to Aβ1-40 and that an approximately equimolar mixture of peptide and $CuCl_2$ produced a single Cu^{2+}-peptide complex. Computer simulation of the S band spectrum with an axially symmetrical spin Hamiltonian and the g and A matrices ($g_\|$, 2.295; $g_\perp$, 2.073; $A_\|$, 163.60; $A_\perp$ 10.0×10^{-4} 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 $g_\|$ and $A_\|$ values, together with Peisach and Blumberg plots (Peisach and Blumberg, 1974), are consistent with a fourth equatorial ligand binding to copper via an oxygen. Therefore, 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 Aβ1-40. 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, in agreement with the corresponding concentration of Cu^+ measured by bioassay. There was no evidence of free, uncoordinated Cu^{2+} remaining after addition of the peptide.

Using a combination of NMR and EPR spectroscopy, we (Curtain *et al*., 2001) 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 Aβ1-28 and Aβ1-40/2 indicated that both peptides were undergoing significant conformational exchange in aqueous solution. NMR and EPR spectra were also recorded for Aβ1-28 where the Nε2 nitrogens of the imidazole ring of the His residues 6, 13, and 14 were methylated (Me-Aβ1-28). The NMR spectra of Me-Aβ1-28 were virtually identical to Aβ1-28, 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 Aβ1-28 or Aβ in PBS. NMR spectra of the supernatant of Aβ1-28 treated with Zn^{2+} showed that peaks assigned to C2H and C4H of His6, His13, and His14 of Aβ1-28 had broadened significantly. However, there was little or no change in the rest of the spectrum compared with Aβ1-28 prior to the addition of Zn^{2+}. This broadening of the NMR peaks due to the histidine residues 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, and this result suggested that three of the ligands bound to Zn^{2+} 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 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 previously (Bush *et al*., 1994b). When Cu^{2+} or Fe^{3+} was titrated into an aqueous solution of Aβ1-28, 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. 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 definitive conclusions could be drawn from spectra of peptide remaining in solution. When Zn^{2+} was added to an aqueous solution of Me-Aβ1-28, the changes observed in the spectrum were identical to those observed for Zn^{2+} added to Aβ1-28, but there was no visible precipitate.

In aqueous solution and lipid environments, coordination of metal ions to Aβ 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, $g_{\|} = 2.28$ and $g_{\perp} = 2.03$, $A_{\|} = 173.8$ Gauss. A notable finding was that increasing the Cu^{2+} concentration above ~0.3 mol/mol of peptide induced 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. 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, Aβ coordinated a second Cu^{2+} atom cooperatively. The effects were abolished if the histidine residues were methylated at either Nδ1 or Nε2, suggesting that bridging histidine residues were being formed (Curtain *et al*., 2001; Tickler *et al*., 2005). One consequence of coordination by a metal ion to the Nδ1 of a histidine residue is a reduction in the pK_a of Nε2 NH, making this nitrogen more suitable for metal binding (Sundberg and Martin, 1974), resulting in a histidine residue that can bridge metal ions; a good example being His63 at the active site of superoxide dismutase (Parge *et al*., 1992). Similar bridging histidine residues have been proposed in the octarepeat region of the prion protein (Viles *et al*., 1999), which has been shown to possess significant superoxide dismutase (SOD) activity in the presence of Cu^{2+} (Brown *et al*., 1999). The line-broadening effects observed in the EPR spectra at Cu^{2+}/Aβ molar fractions up to 1.0 by us were not observed by Syme *et al*. (2004), or Antzutkin (2004). However, it is relevant that NaCl has a marked effect on metal-induced aggregation of Aβ (Huang *et al*., 1997; Narayanan and Reif, 2005). We (Curtain *et al*., 2001) obtained spectra from samples in phosphate-buffered saline at pH 7.4, Antzutkin (2004) adjusted the pH of the sample to pH 7.4 and dialyzed against distilled water, while Syme *et al*. (2004) used the nonphysiologic ethyl morpholine buffers. Recently, the existence of bridging Cu^{2+} dimers in Aβ1-28 in pH 7.4 PBS at Cu^{2+}/peptide ratios > 0.6 has been argued from the occurrence of *g* ~4 transitions in the EPR spectra (Curtain, private communication) with (Smith *et al*., 2006). Neither *g* ~2 line broadening nor the *g* ~4 transitions occurred in pH 7.4 ethyl morpholine buffer, further emphasizing the importance of using physiologic buffers in studying Aβ-metal ion interactions. Similar line-broadening phenomena to that observed by us have been observed in the EPR spectra of imidazole bridged-copper complexes designed as SOD mimetics (Ohtsu *et al*., 2000).

The bridging histidine may be responsible for the reversible metal-induced aggregation of Aβ. 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 (Curtain *et al*., 2001). 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 is quite possible that metal-induced precipitation of Aβ is quite different from that induced by prolonged incubation of monomeric peptide in the putative absence of metal. For example, Miura *et al*. (2000) strongly suggested that the metal-induced aggregation of Aβ was promoted by cross-linking of the peptides through metal-His{N_{τ}} bonds, most likely through His{N_{τ}}-metal-His{N_{τ}} bridges at three histidine residues.

Observations that rat Aβ by which differs from human Aβ by three substitutions, with Arg5, Tyr10, and His13 of human Aβ becoming Gly5, Phe10, and Arg13 (Shivers *et al*., 1988), does not reduce Cu^{2+} and Fe^{3+}, is not readily precipitated by Zn^{2+} or Cu^{2+}, does not produce ROS as strongly as the human sequence, and does not produce plaques highlight the importance of the three histidine residues (Atwood *et al*., 1998; Huang *et al*., 1999b). Rat Aβ forms a metal complex via two histidine residues and two oxygen ligands rather than three histidine residues and one oxygen ligand, compared with human Aβ where the side-chain of His13 of human Aβ is ligated to the metal ion. This was borne out by the EPR spectrum, which was typical of a square planar 2N2O Cu^{2+} coordination (Curtain *et al*., 2001).

Further advances in understanding the N coordination of Cu^{2+} will require more sophisticated EPR techniques than have been used so far, supported by input from other methods such as extended X-ray absorption fine structure (EXAFS). Equally, there remains uncertainty as to the nature of the potential O ligand. Raman data (Miura *et al*., 2000) suggest that the ligand was the O of the tyrosine hydroxyl, which plays an important role in Cu^{2+}-induced aggregation. They were able to assign the 1504 cm^{-1} band in the Raman spectra of insoluble Cu^{2+}-Aβ1-16 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 Aβ1-16. However, NMR (Syme *et al*., 2004) and EPR data combined with mutagenesis (Karr *et al*., 2005) has suggested that Tyr10 does not coordinate Cu^{2+}. Again, a variety of different solution conditions were used for these experiments, Miura *et al*. (2000) used phosphate-buffered saline, which might have had the effect of encouraging peptide association. In considering the issue of monomeric versus dimeric Cu^{2+}, it is important to remember that Aβ may form oligomers and multimers in a variety of ways, some more relevant to its neurotoxicity than others (Roher *et al*., 1996; Walsh *et al*., 2002; Barnham *et al*., 2004a; Cleary *et al*., 2005).

2.5. Aβ Redox Activity

Oxidative stress markers characterize the neuropathology both of Alzheimer's disease and of amyloid-bearing transgenic mice. The neurotoxicity of Aβ 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 Aβ coordinating redox active metal ions. Huang *et al*. (1999a,b) showed that human Aβ directly produces H_2O_2 by a mechanism that involves the reduction of metal ions, Fe^{3+} or Cu^{2+}. Spectrophotometry was used to show that the Aβ peptide reduced Fe^{3+} and Cu^{2+} to Fe^{2+} and Cu^{+} and that molecular oxygen was then trapped by Aβ and reduced to H_2O_2 in a reaction that is driven by substoichiometric amounts of Fe^{2+} or Cu^{+}. In the presence of Cu^{2+} or Fe^{3+}, Aβ produced a positive thiobarbituric-reactive substance, compatible with the generation of the hydroxyl radical (•OH). Tabner *et al*. (2002) used spin-trapping to identify the radical produced by Aβ in the presence of Fe^{2+}, concluding that it was •OH. However, they also found •OH was produced in the presence of Fe^{2+} by Aβ25-35, which does not contain a strong metal binding site.

Incubation with Cu^{2+} causes SDS-resistant oligomerization of Aβ (Atwood *et al*., 2004), which is also found in the neurotoxic soluble Aβ extracted from the AD brain. Atwood *et al*. (2004) found that Cu^{2+} induced SDS-resistant oligomers of Aβ gave a fluorescence signal characteristic of the cross-linking of the peptide's tyrosine 10. 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 Aβ1-28, Aβ1-40, and Aβ1-42, and Atwood *et al*. (2004) 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.

We have (Barnham *et al*., 2004a) used density functional theory calculations to elucidate the chemical mechanisms underlying the catalytic production of H_2O_2 by Aβ/Cu and the production of dityrosine. Here, tyrosine 10 (Y10) 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 (Whittaker, 2003).

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^{+}, and we proposed that the transfer could take place via a proton-coupled electron transfer (PCET) mechanism (Barnham *et al*., 2004a). Reactions involving PCET are being increasingly implicated in a range of biological systems, including charge transport in DNA and enzymatic oxygen production (Whittaker, 2003). In this system, the electron transfer involves both p- and d-orbitals on the ascorbate, Y10, and the copper ion, while proton transfer involves p-orbitals on the O_2-atom of ascorbate and the side-chain oxygen of Y10. 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 (Cukier and Nocera, 1998).

The Cu/tyrosinate hypothesis was tested using an Aβ1-42 peptide with tyrosine 10 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 $g_{\|}$ value of Y10A. The increase was probably due to some distortion of the coordination sphere because the oxygen ligand, which was possibly from Y10, was now derived from another oxygen donor (e.g., H_2O, phosphate, or carboxylate from the peptide). While wild-type Aβ1-42 rapidly reduces Cu^{2+} to Cu^{+} in aqueous solution, with near-complete reduction taking 80 min, the mutation of Y10 to alanine markedly decreased the ability of Aβ to reduce Cu^{2+}. Further, spin trapping studies also confirmed the DFT observation that Y10 acts as a gate that facilitates the electron transfer needed to reduce Cu^{2+} to Cu^{+}. When the spin trap 2-methyl-2-nitrosopropane was added to the reaction mixture *w.t*. Aβ1-42/Cu^{2+}/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.

2.6. The Effect of Metal Binding on the Interaction of Aβ with Membranes

Numerous reports have described the effects of Aβ on membranes and lipid systems and their possible roles in its neurotoxicity. Structural studies in different membrane-mimetic systems have demonstrated considerable variation in peptide conformation. There is much experimental evidence from far-ultraviolet circular dichroism spectroscopy and FT-IR spectroscopy that the Aβ peptides can be membrane associated in the β-configuration (Choo-Smith *et al*., 1997), although there are reports of membrane-associated α-helices being found in the presence of gangliosides (McLaurin *et al*., 1998), cholesterol (Ji *et al*., 2002), and Cu^{2+} or Zn^{2+} (Curtain *et al*., 2001, 2003).

This variability under different conditions can be understood because most of the amyloidogenic peptides have been identified as being exceptionally pleiomorphic in structure. As the cell membrane is a lipid mosaic, 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 cytoxicity 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.

We have used a combination of EPR and CD spectroscopy to study the effect of metal ions, pH, and cholesterol on the interaction of Aβ with bilayer membranes (Curtain *et al*., 2001; Curtain *et al*., 2003). 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 (Curtain *et al*., 2003). This population of lipids can be resolved in the EPR spectrum as a motionally restricted component distinct from the fluid bilayer lipids, 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 Aβ1-40 and Aβ1-42 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. When the peptide:lipid 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 lipid membrane was well defined. The lipid:peptide ratio is approximately 4:1. This stoichiometry can be satisfied by six helices arranged in a pore surrounded by 24 boundary lipids. In the presence of Zn^{2+}, Aβ1-40 and Aβ1-45 both inserted into the bilayer over the pH range 5.5–7.5, as did Aβ1-42 in the presence of Cu^{2+}. However, only Aβ40 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 upto 0.2 mole fraction of the total lipid inhibited insertion of both peptides under all conditions investigated. CD spectroscopy revealed that the Aβ peptides had a high α-helix content when membrane penetrant, but were predominately β-strand when not. It is also possible to gain an estimate of the secondary structure of the peptide from the degree of immobilization of the lipid in the shell; the more immobilized, the more likely the peptide is present as a β structure. Simulation of the spectra and calculation of the on-off rates suggested that the peptide was most likely penetrating as an α-helix (Curtain *et al*., 2003). 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 Cu^{2+}/peptide molar ratios > 0.3 in aqueous solution, line broadening was detectable in the EPR spectra, indicating that the peptide was coordinating a second Cu^{2+} atom in a highly cooperative manner at a site less than 6 Å from the initial binding site. So, there appear to be two switches, metal ions (Zn^{2+} and Cu^{2+}) and negatively charged lipids, needed to change the conformation of the peptide from β-strand nonpenetrant to α-helix penetrant.

2.7. Toxic Mechanism of Aβ

Synthetic Aβ is toxic to cells in the presence of Cu^{2+}, but this toxicity is inhibited by catalase implicating H_2O_2 in the toxic pathway (Behl *et al*., 1994; Opazo *et al*., 2002). A simple way of generating H_2O_2 and other ROS is the interaction between redox-active metal ions such as Cu and Fe with O_2, which is why cells have developed elaborate defense mechanisms for dealing with these metals. When Cu^{2+} or Fe^{3+} coordinate Aβ, extensive redox chemical reactions take place, reducing the oxidation state of both metals and producing H_2O_2 from O_2 in a catalytic manner (Huang *et al*., 1999a,b; Cuajungco *et al*., 2000; Opazo *et al*., 2002; Barnham *et al*., 2004a). The generation of H_2O_2 in the presence of the reduced form of the metal ion sets up conditions for Fenton chemistry where the generation of the highly toxic OH• radical can occur (Huang *et al*., 1999a).

The various forms of Aβ in the AD brain are usually oxidatively modified (Head *et al*., 2001), and reaction with Cu^{2+} can induce covalent cross-linking of Aβ yielding soluble oligomers and adducts on the side chains (Atwood *et al*., 2000a,b; Barnham *et al*., 2003; Atwood *et al*., 2004; Barnham *et al*., 2004a). We have hypothesized that such oxidative modification may

contribute to the release of abnormal soluble forms of Aβ from the membrane, which correlates with dementia (McLean *et al*., 1999). In the normal brain, most of the Aβ is found associated with membranes (Cherny *et al*., 1999). We hypothesize that when Aβ coordinates redox-active metal ions, subsequent oxidation releases soluble oxidized forms of the peptide that resist clearance. This may explain how zinc originating from the synapse becomes so enriched in amyloid in AD. Our model suggests that in health, soluble Aβ is not present in the cortical synapse. In AD, soluble oxidized Aβ accumulates within the synapse where the high Zn^{2+} concentrations precipitate the copper/iron coordinated Aβ, creating a reservoir of potentially toxic Aβ that is in dissociable equilibrium with the soluble pool. The Zn^{2+} in the amyloid mass partially quenches H_2O_2 production, which explains why plaque amyloid burden correlates poorly with clinical dementia (Cuajungco *et al*., 2000), whereas soluble Aβ levels correlate well with clinical severity (McLean *et al*., 1999).

One of the consequences of oxidative modification to Aβ is the formation of a sulfoxide on Met35, and Met(O)Aβ peptide has been isolated from AD amyloid brain deposits (Naslund *et al*., 1994; Kuo *et al*., 2001; Dong *et al*., 2003). A Raman spectroscopic study of senile plaque cores isolated from diseased brains has shown that much of the Aβ in these deposits contained methionine sulfoxide with copper and zinc coordinated to the histidine residues (Dong *et al*., 2003).

In vitro, the methionine at position 35 can act as an electron donor for the reduction of the metal ions bound to Aβ (Curtain *et al*., 2001). Although there are several potential electron donors such as GSH and ascorbic acid, *in vivo* it is likely that M35 occupies a privileged position being part of the Aβ sequence. Indeed, it has been shown using photoaffinity cross-linking experiments that in β-strand fibrils, M35 is cross-linked to the N-terminal region of Aβ (Egnaczyk *et al*., 2001), that is, within close proximity to the redox-active metal binding site. When it is missing as in Aβ(1-28), the addition of exogenous methionine permits redox reactions to proceed, but with slower kinetics. When M35 is sequestered within a lipid environment, there is also no metal reduction. M35 oxidation also alters the physical properties of the peptide. Met(O)Aβ is more soluble in aqueous solution, and there is a disruption of the local helical structure when the peptide is dissolved in SDS micelles (Watson *et al*., 1998). The formation of trimers and tetramers by Met(O)Aβ is significantly attenuated (Palmblad *et al*., 2002), and fibril formation is inhibited (Hou *et al*., 2002; Palmblad *et al*., 2002). We (Barnham *et al*., 2003a) showed by solid-state NMR that when Aβ coordinates and reduces Cu^{2+} to Cu^{+}, the M35 is oxidized. Although the Cu^{2+} coordination of the oxidized peptide is identical to non-oxidized Aβ and it will produce H_2O_2, it cannot penetrate lipid bilayers either in the presence or absence of Cu^{2+} or Zn^{2+}. On the other hand, Met(O)Aβ is toxic to neuronal cell cultures, a toxicity that is rescued by catalase and the metal chelator clioquinol. These results suggest that fibril formation and membrane penetration by Aβ could be epiphenomena and that the main requirement for cytoxicity is redox competence. In this connection, it is important to note that the oxidized M35 has the potential for further reduction to the sulfone and could thus still act as a Cu^{2+} reductant, acting *in vivo* in concert with agents such as ascorbic acid and GSH.

2.8. Aβ Membrane Association and Cytotoxicity

We (Ciccotosto *et al*., 2004) further probed the role of M35 by preparing Aβ1-42 in which it was replaced with valine (AβM35V). 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 wild-type Aβ1-42. When cortical cells were treated with the peptides for only a short 1 h duration 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 AβM35V compared with wild-type Aβ peptide bound to the cells after a 1-h cell exposure. It was suggested that the increased toxicity was related to the increased binding.

AβM35V bound Cu^{2+} with the same coordination sphere as *w.t.* Aβ and produced similar amounts of H_2O_2 as Aβ1-42 *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 Cu^{2+}-AβM35V EPR signal, which showed that the reduction of Cu^{2+} to the EPR silent Cu^{+} was much slower compared with *w.t.* Aβ1-42, confirming that the M35 residue in Aβ42 plays an important part in the redox behavior of this peptide in solution. Like *w.t.* Cu^{2+}-Aβ1-42, Cu^{2+}-AβM35V 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.* Cu^{2+}-Aβ1-42, 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 AβM35V had a higher affinity for the lipid bilayer compared with Aβ42. CD analysis showed that AβM35V had a higher proportion of β-sheet structure and random coil than Aβ1-42, which would also suggest a more flexible structure in the bilayer. In summary, these and the results described above tell us that the wild-type Aβ, its oxidized form, Met(O)Aβ, and the mutant peptide, AβM35V, induce cell death via similar pathways that are metal-dependent and can generate H_2O_2 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.

The important role that membrane association plays in the toxicity was emphasized by the observation that Aβ peptides where the histidine residues were methylated (Me-His Aβ) with the aim of inhibiting metal mediated histidine bridged oligomerization resulted in nontoxic forms of Aβ. Even though the modified peptides were four times more efficient than wild-type Aβ in generating H_2O_2, unlike wild-type Aβ they did not bind to the cell membrane (Tickler *et al*., 2005), indicating that the redox-associated toxicity of Aβ is a site-specific phenomena.

2.9. Therapeutic Potential of Inhibiting Aβ-Metal Interactions

Current U.S. FDA–approved drugs for AD provide modest functional improvement (e.g., acetylcholinesterase inhibitors) but do not retard the progression of the underlying disease. Hence, there is an urgent need to identify drugs that confront the disease in the central pathway of its pathogenesis.

Although we have identified metal-Aβ interactions as likely being central to AD pathogenesis, we are only beginning to understand the way by which the peptide coordinates up to 3.5 metal ions. Nevertheless, the efficacy of clioquinol (CQ; 5-chloro-7-iodo-8-hydroxyquinoline) *in vitro* and *in vivo* (see below) has encouraged us to proceed by empirical observation as we gather information about the qualities of the compounds that will attenuate metal-Aβ interactions. Broadly speaking, there are two potential classes of molecule capable of preventing such pathologic metal interactions. The first involves inhibiting Aβ-metal interactions by selectively occupying the metal-binding site on Aβ, thus preventing Cu/Zn/Fe coordination. The initial metal binding site coordinates His6, His13, and His14. However, these residues are natively unstructured in the absence of metal ions, and as a result the design of classic "lock and key" inhibitors is problematic. Interestingly, the epitopes of the vaccines that have been reported to inhibit Aβ cerebral accumulation in transgenic mice involve residues in this region of the peptide (1-15) (Spooner *et al*., 2002).

A second pharmacological approach is to identify a class of molecules that will effectively compete with the peptide for the metal ions, that is, a metal-protein attenuating compound (MPAC). Cherny *et al*. (1999) have shown using a range of commonly available metal chelators [tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN), ethylene glycol bis(2-aminoethyl ether)-*N,N,N'N'*-tetraacetic acid (EGTA), and bathcuporine] that such compounds with a reasonable affinity for Cu, Zn, and Fe can solubilize deposited Aβ from the postmortem brain tissue of AD patients. Conversely, chelation of Ca and Mg inhibited solubilization of Aβ. However, traditional hydrophilic chelators do not pass well through the blood-brain barrier (BBB).

Our requirement for a metal-attenuating compound as a possible therapeutic for AD should not be confused with the concept of "chelation therapy" for the treatment of AD. "Chelation therapy" is a term associated with the removal of bulk metals such as in Wilson's disease (Cu) and β-thallesemia (Fe). The breakdown in metal homeostasis in these diseases leads to tissue saturation of metal. The mechanism of action of such compounds is that metal is sequestered peripherally by the chelators and cleared by excretion. In AD, the metals become repartitioned into the amyloid mass. This is probably why treatment with CQ (see below) induced an increase (≈15%) in brain Cu and Zn levels in transgenic mice (Cherny *et al*., 2001) and also induced a similar increase in plasma Zn levels in AD subjects (+30%) (Ritchie *et al*., 2003). The intention of the metal-protein attenuating compound (MPAC) is to disrupt an abnormal metal-protein interaction to achieve a subtle repartitioning of metals and a subsequent normalization of metal distribution. Once the toxic cycle is inhibited, endogenous clearance processes can cope more effectively with the accumulated Aβ.

CQ was given via oral gavage over a 9-week period in a blind study to Tg2576 transgenic mice (Cherny *et al*., 2001). The results showed a 49% decrease in brain Aβ burden compared with nontreated controls, there was no evidence of any toxicity, and the general health and body weight parameters were more stable in the treated animals. Treatment with CQ did not lead to a systematic decrease in metal levels most likely due to its moderate binding affinities. The metal ions removed from Aβ are redistributed rather than excreted; as a result, Cu and Zn levels rose ~15% in the treated transgenic mice, possibly because the metals were no longer prevented from normal cellular uptake by being incorporated into the amyloid mass. This suggests that CQ is able to some extent to redress the balance in metal homeostasis that breaks down with age. Interestingly, in the same study, triethylenetetramine (TETA), a hydrophilic high-affinity metal chelator that is incapable of crossing the BBB and has been used to treat Wilson's disease, did not inhibit Aβ deposition, indicating that systemic depletion of metal ions ("chelation therapy") is not likely to be an effective therapeutic strategy for the treatment of AD.

The success of CQ in the transgenic mouse trials has encouraged the use of this molecule in clinical trials. The effects of oral CQ treatment in a randomized, double-blind, placebo-controlled pilot phase 2 clinical trial of moderately severe AD patients were evaluated (Ritchie *et al*., 2003). Thirty-six subjects were randomized (18 placebo and 18 CQ, with 32 completions) and stratified into more severely or less severely affected groups. The effect of treatment was statistically significant in preventing cognitive deterioration over 36 weeks in the more severely affected patients (baseline ADAS-cog ≥ 25). The performance of the less severely affected group (ADAS-cog < 25) deteriorated negligibly over this interval, so cognitive changes could not be discriminated in this stratum. Plasma Aβ42 declined in the CQ group but increased in the placebo group ($p < 0.001$). Plasma Zn levels rose significantly (~30 %) in the CQ group. These results reproduce the lowering of plasma Aβ and the paradoxical rise in brain zinc observed in APP transgenic mice treated with the drug. The drug was generally well tolerated by participants.

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