Amyloid β−Cu^{2+} Complexes in both Monomeric and Fibrillar Forms Do Not Generate H_2O_2 Catalytically but Quench Hydroxyl Radicals[†]

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ABSTRACT: Oxidative stress plays a key role in Alzheimer's disease (AD). In addition, the abnormally high Cu^{2+} ion concentrations present in senile plaques has provoked a substantial interest in the relationship between the amyloid β peptide (Aβ) found within plaques and redox-active copper ions. There have been a number of studies monitoring reactive oxygen species (ROS) generation by copper and ascorbate that suggest that Aβ acts as a prooxidant producing H_2O_2. However, others have indicated Aβ acts as an antioxidant, but to date most cell-free studies directly monitoring ROS have not supported this hypothesis. We therefore chose to look again at ROS generation by both monomeric and fibrillar forms of Aβ under aerobic conditions in the presence of Cu^{2+} with/without the biological reductant ascorbate in a cell-free system. We used a variety of fluorescence and absorption based assays to monitor the production of ROS, as well as Cu^{2+} reduction. In contrast to previous studies, we show here that Aβ does not generate any more ROS than controls of Cu^{2+} and ascorbate. Aβ does not silence the redox activity of $Cu^{2+/+}$ via chelation, but rather hydroxyl radicals produced as a result of Fenton−Haber Weiss reactions of ascorbate and Cu^{2+} rapidly react with Aβ; thus the potentially harmful radicals are quenched. In support of this, chemical modification of the Aβ peptide was examined using ^{1}H NMR, and specific oxidation sites within the peptide were identified at the histidine and methionine residues. Our studies add significant weight to a modified amyloid cascade hypothesis in which sporadic AD is the result of Aβ being upregulated as a response to oxidative stress. However, our results do not preclude the possibility that Aβ in an oligomeric form may concentrate the redox-active copper at neuronal membranes and so cause lipid peroxidation.

Along with the accumulation of extracellular amyloid plaques, Alzheimer's disease (AD)[1] is characterized by marked oxidative injury. Numerous studies have shown evidence of increased levels of oxidative damage in the brain tissue, as well as in the cerebrospinal fluid (CSF) and plasma, of patients with AD. Observed oxidative damage includes protein oxidation (*1*), DNA and RNA oxidation (*2*), and also lipid peroxidation (*3*) (as reviewed in ref *4*). Indeed, much of the Aβ isolated from plaques contains oxidized methionine (Met^{35}) (*5, 6*). Intriguingly, some studies have shown that oxidative stress occurs before the appearance of amyloid plaques (*7, 8*).

The principal component of amyloid plaque deposits is a small peptide, amyloid β peptide (Aβ), of between 39 and 43 residues in length. Soluble Aβ40 and Aβ42 are found in all humans in the blood plasma, bound principally to serum albumin (*9*), and the CSF where Aβ40 has a concentration of 5 nM (*10*). One of the most compelling pieces of evidence for Aβ being the primary culprit in AD comes from genetic mutations underlying familial AD. These mutations are within Aβ itself or are associated with increased production of Aβ42 in the brain, which promotes more rapid fibril growth (*11*). Small oligomers or pore-like protofibrils rather than mature amyloid fibers may be the neurotoxic form of Aβ (*12*). The mechanism by which Aβ is toxic to neurons remains unclear, but it is suggested the cause may be ROS production (*13, 14*), perturbation in Ca^{2+} regulation, or interaction of Aβ oligomers with the membrane causing cell depolarization.

In vivo, copper ions are a major source of reactive oxygen species (ROS) generation via Fenton−Haber Weiss reactions. Under aerobic conditions oxidation of Cu^+ ions results in the generation of hydrogen peroxide, superoxide, and hydroxyl radicals (*15*). The redox-active metal ion Cu^{2+} is found bound to Aβ in isolated senile plaque cores (*5*). Total copper content within the brain increases with age, as do oxidative processes. Due to the potentially toxic nature of copper ions they are carefully regulated and compartmentalized. However, the dyshomeostasis of redox-active metal ions occurs in AD, with copper being found in abnormally high concentrations, 340 μM, in cerebral amyloid deposits (*16*). Controversially, trace amounts of copper in a cholesterol-high diet were found to induce amyloid β plaques and learning deficits in a rabbit model of Alzheimer's disease (*17, 18*).

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[1] Abbreviations: Aβ, amyloid β; AD, Alzheimer's disease; APP, amyloid precursor protein; BC, bathocuproinedisulfonic acid; 3-CCA, coumarin-3-carboxylic acid; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; His, histidine; H_2O_2, hydrogen peroxide; 7-OHCCA, 7-hydroxycoumarin-3-carboxylic acid; 8-OHG, 8-hydroxyguanine; LDL, low-density lipoprotein; Met, methionine; Met^{ox}, methionine sulfoxide; Msr, methionine sulfoxide reductase; NMR, nuclear magnetic resonance; ODAD, *o*-dianisidine; 2-oxo-His, 2-oxohistidine; PBS, phosphate-buffered saline; ROS, reactive oxygen species.

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As such, antioxidants and copper chelators represent possible therapeutics for AD (*19*).

The most widely accepted model of Cu^{2+} bound to Aβ indicates Cu^{2+} ions bind to histidine residues within the N-terminal portion (His^6, His^{13}, His^{14}) in a square-planar complex (*20*). The nature of the fourth ligand is still debated, with the N-terminus (*20*−*22*), tyrosine (*23*), and glutamic or aspartic acid (*24*) proposed. Reported Cu^{2+} affinities for monomeric Aβ vary widely between micromolar (μM) (*25*), nanomolar (nM) (*20, 26*), and the much higher attomolar, $K_d \sim 10^{-18}$ affinity for Aβ42 (*27*). Cu^{2+} ions have a plasma concentration of ∼15−20 μM and a lower concentration of ∼250 nM in the CSF (*28*); however, when released at the synapse concentrations can be as high as 250 μM (*29*).

Both antioxidant and prooxidant roles for Aβ have been suggested. As a prooxidant, the neurotoxicity of Aβ peptides has been linked to oxidative ROS generation (*30*−*32*). Neuronal cell viability studies indicate that reactive oxygen species mediate the toxicity of Aβ, with the neurotoxicity of Aβ and Cu^{2+} being abolished by the addition of catalase or anti-Aβ antibodies (*33*) or attenuated through the use of metal chelators (*34*). In contrast, others have highlighted an antioxidant activity of Aβ (*35, 36*). In particular, monomeric Aβ40 and Aβ42 were shown to inhibit neuronal death caused by transition metal-induced oxidative damage (*37*). A dual role for Aβ has also been suggested (*38*−*40*).

A number of groups have published studies monitoring the generation of ROS in the presence of Aβ and Cu^{2+} in a cell-free system. Early studies suggested spontaneous peptide free radical generation (*41*). However, this was later disputed by a number of groups (*42, 43*). It was then suggested that Aβ in fact required the presence of transition metal ions (e.g., Cu^{2+} and Fe^{3+}) to produce reactive oxygen species via Fenton−Haber Weiss-type reactions (*44*−*47*). However, redox potentials recently determined for the Cu−Aβ complex suggested that this is unlikely (*48*). Initial studies by Huang et al. suggested that Cu^{2+}-bound Aβ was capable of generating H_2O_2 without the presence of an additional reducing agent (*44, 45*). However, subsequent studies by the same laboratory indicated that the initial experiments were flawed, and the method used to detect the H_2O_2 contained a reagent (TCEP) capable of acting as a reducing agent for Aβ40 and Aβ42 but not the controls (*33*). This study highlighted the additional requirement of biological reductants (such as ascorbic acid, cholesterol, L-DOPA, or dopamine) to act as substrates for the sequence of electron transfer reactions (*33, 49*). A model proposed by Opazo et al. for catalytic hydrogen peroxide production by Aβ and Cu^{2+} hypothesizes that reduction of the metal ion occurs through the transfer of an electron from the peptide backbone, with the peptide radical then being reconstituted through recruitment of a biological reducing agent (*33*). However, in contrast more recently studies have indicated H_2O_2 depletion in a Cu^{2+}/ascorbate system by Aβ (*31*) and similarly hydroxyl radical depletion (*50*).

The aim of this study is to characterize the anti- and/or prooxidant nature of the Aβ peptide, in both monomeric and fibrillar form. To date there have been many inconsistencies in the literature on this subject, and the role of Aβ in relation to the observed oxidative stress in AD is still highly disputed. Cu−Aβ samples were studied in the presence and absence of a physiological reductant, ascorbate, in a cell-free system. ROS generation was monitored using absorption and fluorescence assays. Cu^{2+} reduction was monitored using the BC assay, and effects of redox cycling on Aβ itself were monitored using ^{1}H NMR.

MATERIALS AND METHODS

Peptide Syntheses and Purification. Peptides representing various fragments of the amyloid β peptide were synthesized by employing solid-phase F-moc chemistry (ABC; Imperial College, London). After removal from the resin and deprotection, the samples were purified using reverse-phase HPLC and characterized using mass spectrometry.

Peptides synthesized were as follows: Aβ(1−42), Aβ(1−40), Aβ(1−28), and Aβ(1−16), designated as Aβ42, Aβ40, Aβ28, and Aβ16 in the text. In addition, the neurotoxic fragment Aβ(25−35) was synthesized. Aβ42 is DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA.

Full-length Aβ peptides (Aβ42 and Aβ40) were synthesized with the N-terminus left as the native amino group and a carboxyl group at the C-terminus, while Aβ fragments were synthesized with an amino group at the N-terminus, and the truncated C-terminus was blocked as the corresponding ethyl ester. Although Aβ(25−35) is not a naturally occurring peptide, its N-terminus was not acetylated as previous studies into this fragment's toxicity used a free N-terminal amino group.

The peptide concentrations were determined using the extinction coefficient at 280 nm of the single tyrosine residue at position 10, specifically 1280 M^{-1} cm^{-1}. Typically, the freeze-dried peptides contained ∼20% moisture by weight.

Solubilization of Aβ and Amyloid Fibril Formation. Lyophilized peptide stocks were solubilized in water, with additional processes being needed for full-length Aβ stocks to prevent aggregation/oligomerization. Briefly, Aβ40 and Aβ42 were dissolved at ∼0.7 mg/mL concentration at pH 10.5 and kept on ice (0−5 °C) to keep aggregation/oligomerization to a minimum. Samples described in this paper as monomeric Aβ were freshly prepared at pH 7.4, with ThT and CD measurements suggesting minimal fibrillar content. However, even freshly prepared Aβ may contain some dimers and low molecular weight oligomers.

Amyloid fibrils of Aβ40 and Aβ42 were generated by incubation of full-length Aβ (50 μM) at 37 °C in 10 mM phosphate buffer at pH 7.4 with 300 mM NaCl (PBS) and 0.05% sodium azide. Fibrils were generated over a period of 120 h with gentle agitation on a rocker. Fibril formation was confirmed by a variety of techniques including far-UV circular dichroism (CD), thioflavin T (ThT) fluorescence assay (see Supporting Information Figure S1), seeding experiments, and electron microscopy (EM) imaging.

Materials. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich at the highest purity available. Deuterium oxide was obtained from GOSS Scientific Instruments Ltd. Water used was of ultrahigh quality (>18 Ω cm^{-1} resistivity) level. All studies were performed under aerobic conditions.

H_2O_2 Detection: o-Dianisidine (ODAD) and Peroxidase Assay. A modified version of the *o*-dianisidine (ODAD) and peroxidase assay (*51, 52*) was utilized to monitor hydrogen peroxide production. A Thermo Labsystems Multiskan

Ascent photometric microplate reader fitted with a 450 nm filter was used to detect hydrogen peroxide with this assay, using 96-well culture plates (Corning Costar). Stock solutions were prepared freshly for each experiment, and each 50 μL reaction mixture typically contained 10 μM peptide, 10 μM $CuCl_2$, and 30 μM ascorbate in 10 mM phosphate buffer, pH 7.4, with 300 mM NaCl (PBS). Samples were left to incubate for 1 h at 37 °C, and then a volume of 250 μL of assay reagents was added to each reaction mixture. The assay reagents consisted of 240 μL of ODAD (0.066 mg in 100 mL of phosphate buffer) and 10 μL of peroxidase (1 mg/mL in H_2O). Following reagent addition, the samples were subjected to a period of shaking (30 s at 60 rpm) using the inbuilt shaker to mix the sample and reagents, and the absorbance readings were measured. An extensive number of different sample/control conditions were tested, with up to 24 repeats being performed of each sample condition and then an average calculated. Absorbance readings of phosphate buffer plus assay reagents were recorded to be used later for baseline subtraction. The net changes in absorbance (ΔA) were determined by multiplying the absorbance by 6 to return it to original concentrations (50 μL of sample was diluted by 250 μL of assay reagent) and then subtracting the baseline absorbance readings of phosphate buffer plus reagents. The amount of H_2O_2 produced was quantified by comparing resultant absorbance readings with a linear calibration curve acquired with known concentrations of H_2O_2 (see Supporting Information Figure S2).

Hydroxyl Radical Detection: Coumarin-3-carboxylic Acid (3-CCA) Assay. A variety of different peptide fragments/forms were studied in the presence and absence of ascorbate and $CuCl_2$ and monitored for hydroxyl radical production. A modified version of the coumarin-3-carboxylic acid (3-CCA) assay (*52, 53*) was used to quantify hydroxyl radical production. Upon reaction with hydroxyl radicals the 3-CCA forms a fluorescent product, 7-OHCCA, with characteristic emission at 450 nm and excitation maximum at 388 nm. Samples typically contained 1 mM 3-CCA, 10 μM peptide, 10 μM $CuCl_2$, and 30 μM ascorbate, in 40 mM phosphate buffer (pH 7.4). Prior to recording spectra samples were incubated for 1 h at 37 °C. At least three replicates were performed of each sample condition. Emission readings were measured on a Hitachi F-2500 fluorescence spectrophotometer, and fluorescence spectra were collected over the wavelength range 410−550 nm at 37 °C. A very slight baseline fluorescence of 3 AFU was observed from the unreacted 3-CCA reagent. Phosphate buffer plus reagent was used as a baseline sample and subtracted from all other fluorescence values.

Cu^+ Detection: Bathocuproinedisulfonic Acid (BC) Assay. For monitoring the reduction of Cu^{2+} ions the specific Cu^+ chelator bathocuproinedisulfonic acid (BC) was used, by observing the characteristic purple color produced by the complex $Cu^+(BC)_2$ ($\epsilon_{483nm} = 12250\ M^{-1}\ cm^{-1}$) which has an absorbance maximum at 483 nm (*52, 54*). Typically, a 1 mL sample containing 10 μM Aβ, 10 μM $CuCl_2$, 10 mM phosphate buffer, pH 7.4, and 300 mM NaCl (PBS) was allowed to incubate with 360 μM BC. Absorbance readings were monitored at two time intervals, after 60 s and 60 min, with samples being incubated and recorded at 37 °C. The capacity of BC to detect Cu^+ is rapid (less than 30 s). UV−visible absorption spectra were collected using a Hitachi U-3010 spectrophotometer. An array of control samples were recorded for baseline readings to be subtracted from resultant spectra. Three replicates were performed for each sample condition. This assay uses optical absorption and therefore may be influenced by light scatter from turbidity in the solution which should be considered. So, further control samples of peptide plus $CuCl_2$, and peptide plus BC, were performed to check absorbance contributions from light scatter. We see from the controls of Aβ that the addition of BC (or $CuCl_2$) alone to Aβ40 does not cause an increase in apparent absorption due to light scatter (see Figure 3b), while the less soluble Aβ42 fragment does produce slight light scatter indicated by very weak absorbance of ∼0.003 at 483 nm. However, the effect is small and does not alter the interpretation of the data. Phosphate buffer plus BC reagent was therefore used as the baseline condition to be subtracted.

^1H Nuclear Magnetic Resonance (NMR) Spectroscopy. Experiments were carried out on a Bruker Avance spectrometer, operating at a 600 MHz ^1H frequency. Water was suppressed using the excitation sculpting with gradients method. NMR experiments were recorded at either 30 or 37 °C, pH 7.4. Samples contained 100 μM peptide incubated and monitored over time for up to 60 h with varying amounts of $CuCl_2$ and ascorbate. A lack of NaCl and agitation mean that fibrillization is minimal over the period. Alternatively, samples of Aβ25−35 at 140 μM were incubated at 4 °C and then monitored after a 7 day incubation. NMR samples contained 10% D_2O and 90% H_2O or 100% D_2O. The pH of NMR samples was recorded before and after acquisition. An exponential line broadening of 3.0 Hz was applied to the data before Fourier transformation. Chemical shifts were typically referenced to water, or formate, which produces a singlet at 8.495 ppm. Typically, ^1H NMR spectra were obtained with either 256 or 512 transients with a recycle time of ∼4 s. For analysis purposes, individual spectra were summed to compare the results of spectra acquired over ∼40 min duration with results acquired over several hours of incubation. Assignments of His ring protons and other resonances are based on 2D TOCSY (total correlation spectroscopy) studies and the use of His/Ala analogues described elsewhere (*20, 55*).

RESULTS

Influence of Aβ on H_2O_2 Production by Copper and Ascorbate. In the course of $Cu^{2+/+}$ redox cycling via Fenton−Haber Weiss reactions, the reactive oxygen species H_2O_2 is generated. Thus detection of H_2O_2 is an effective method of monitoring Fenton−Haber Weiss reactions. The ability of Aβ to generate H_2O_2 in the presence of Cu^{2+} ions was studied using the ODAD and peroxidase assay, monitoring the absorbance value at 450 nm.

Reaction mixtures of $CuCl_2$ (10 μM) with/without various Aβ peptide fragments (10 μM) were incubated at 37 °C for 60 min prior to measurement under aerobic conditions. The recorded absorbance measurements for these samples were converted into concentration of H_2O_2, based on H_2O_2 calibration curve data (see Supporting Information Figure S2). Figure 1 shows the results of a selection of control and peptide samples. As expected, the buffer or Cu^{2+} ions on their own do not generate any H_2O_2. A solution of ascorbate

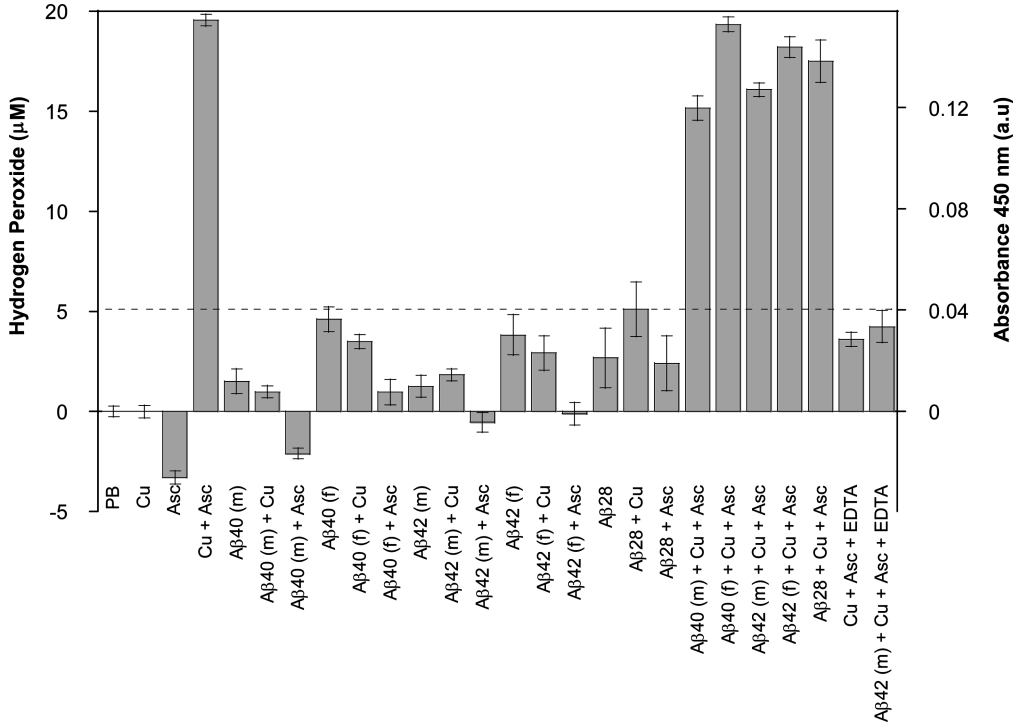

FIGURE 1: H_2O_2 production by Cu^{2+} and ascorbate in the presence of monomeric and fibrillar Aβ, monitored using ODAD and peroxidase assay. Aβ28/40/42 (10 μM) and/or $CuCl_2$ (10 μM) and/or ascorbate (30 μM) in various combinations, all in 10 mM phosphate buffer, pH 7.4. Mean values are shown in both absorbance at 450 nm and hydrogen peroxide concentration on the *y*-axes. Samples were incubated for 60 min at 37 °C prior to reagent addition. Plot shows the average ODAD assay result for samples, based on 18 or 24 replicates, with standard error bars plotted. Dashed line represents background noise level observed in the assay (m = monomeric, f = fibrillar).

(no copper) causes a slight reduction in absorbance from baseline levels due to interactions with the assay reagents. Monomeric Aβ40, in the absence of Cu^{2+}, gives a slight H_2O_2 signal (1.5 $\pm$ 0.6 μM); however, this is clearly not caused by trace amounts of Cu^{2+} because addition of 1 mol equiv of Cu^{2+} (10 μM) to Aβ produces the same amount of H_2O_2 (1.0 $\pm$ 0.4 μM). The addition of a reducing agent, ascorbate, to Aβ40 (in the absence of $CuCl_2$) does not generate H_2O_2 either. Similarly, fibrillar Aβ40, and monomeric and fibrillar Aβ42, also showed no significant difference in absorbance at 450 nm with or without the presence of Cu^{2+} ions. This indicates that the small absorbance at 450 nm observed here is not the result of Fenton's cycling of Cu^{2+} to Cu^{+}. The very weak absorbance values are simply an artifact of turbidity in this optically based assay. Aβ28 plus Cu^{2+}, which we later show does not generate Cu^{+} ions and therefore cannot perform Fenton's cycling, also produces a slight absorbance at 450 nm comparable to Aβ40 and Aβ42. A cutoff at which turbidity in the assay can affect apparent absorbance is shown in Figure 1 set at 0.04 absorbance unit.

These experiments indicate that Cu-Aβ40 and Cu-Aβ42 in their monomeric or fibrillar form do not generate H_2O_2. The only samples to produce H_2O_2 (15−20 μM) are those that contain Cu^{2+} with physiological levels of ascorbate, irrespective of the presence or absence of Aβ.

Further studies using the ODAD and peroxidase assay focused on testing the additional effect of a physiological reducing agent, ascorbate. As before, samples were monitored at 450 nm after 60 min incubation at 37 °C but this time contained a mixture of ascorbate (30 μM) with/without $CuCl_2$ (10 μM) with/without various Aβ peptide fragments (10 μM). Note, extracellular levels of ascorbate are typically between 30 and 100 μM (*15*). The nonpeptide sample of ascorbate and $CuCl_2$ showed production of ~19.5 μM H_2O_2 after 60 min at 37 °C (see Figure 1). When we directly compare this to samples that additionally contain Aβ peptides, it is evident that the amount of detectable H_2O_2 is slightly reduced in the presence of Aβ rather than increased. The exact degree of H_2O_2 depletion varies with peptide fragment, with detected H_2O_2 ranging between 78% and 99% compared to that of $CuCl_2$ with ascorbate on its own. It is evident from Figure 1 that the monomeric forms of full-length Aβ (Aβ40 and Aβ42) show a greater capacity to decrease detectable H_2O_2 compared to the fibrillar forms, 78−83% H_2O_2 for monomeric Aβ compared to the Cu^{2+} and ascorbate sample, while fibrillar forms show 94−99% H_2O_2. The shorter more soluble Aβ28 fragment also demonstrates a capacity to deplete H_2O_2 generated (see Figure 1) comparable to full-length Aβ.

These data indicate that in the presence of a physiological reductant (ascorbate) the Aβ peptides will not increase the amount of H_2O_2 generated, compared to the ascorbate and $CuCl_2$ sample alone. This was a surprise as there have been reports that the Cu−Aβ complex generates H_2O_2 in the presence of an additional reductant (*33*). The latter studies were performed in the presence of EDTA, so for this reason we repeated these types of experiments with some additional controls. We see from Figure 1 that the presence of EDTA (50 μM) dramatically decreases the amount of H_2O_2 generated by the ascorbate (30 μM) and $CuCl_2$ (10 μM) mixture to baseline levels. It has been suggested that the addition of Aβ to this mixture of ascorbate, $CuCl_2$, and EDTA increased H_2O_2 production (*33*); however, we found no such difference between ascorbate, $CuCl_2$, and EDTA with or without the presence of Aβ (10 μM). Indeed, both samples were below the cutoff for the baseline levels as shown in Figure 1.

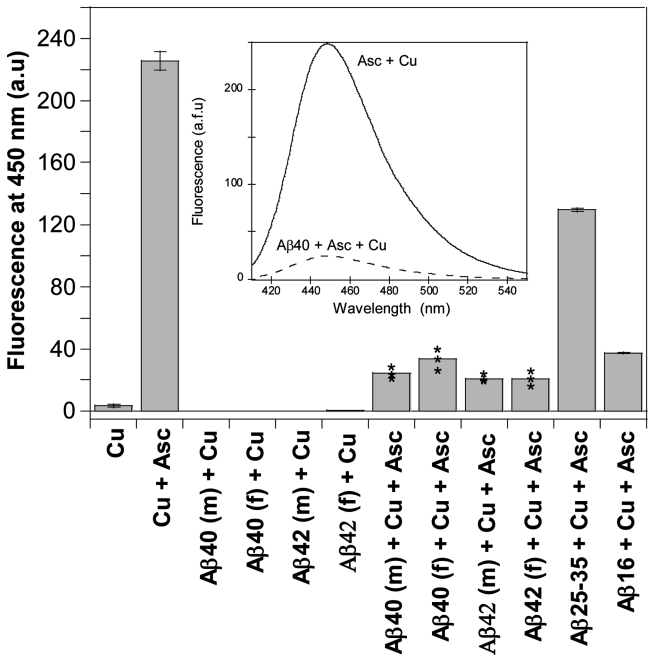

FIGURE 2: Hydroxyl radical production by Cu^{2+} and ascorbate in the presence of monomeric and fibrillar Aβ, monitored using 3-CCA assay. Aβ16/28/25−35/40/42 (10 μM) and/or $CuCl_2$ (10 μM) and/or ascorbate (30 μM) in various combinations, with 1 mM fluorophore 3-CCA. All are in 40 mM phosphate buffer, pH 7.4. Plot shows the average 3-CCA result for samples, based on three or more replicates of each sample condition. Samples were incubated for 60 min at 37 °C (m = monomeric, f = fibrillar).

The latter studies were performed at a 1:1 ratio of peptide to Cu^{2+}. Further studies testing above a stoichiometric ratio of peptide to Cu^{2+} (i.e., 1:2), with Cu^{2+} loaded as $Cu(Gly)_2$, showed similar behavior. In the presence of Aβ the amount of H_2O_2 generated was depleted compared to the ascorbate and $Cu(Gly)_2$ control (see Supporting Information Figure S3).

Influence of Aβ on Hydroxyl Radical Production by $CuCl_2$ and Ascorbate. Next, the redox properties of the Cu−Aβ complex were studied by monitoring hydroxyl radical production. Hydroxyl radicals form an important part of redox chemistry; they are highly reactive and therefore highly toxic to cells. The quantitative detection of hydroxyl radicals was carried out using the 3-CCA fluorescence assay, monitoring the product 7-OHCCA fluorescing at 450 nm. Reaction mixtures were incubated for 1 h at 37 °C. Sample conditions were $CuCl_2$ (10 μM) and Aβ peptide fragments (10 μM), with/without ascorbate (30 μM) at pH 7.4 under aerobic conditions.

As expected, the copper and ascorbate mixture produced appreciable amounts of hydroxyl radicals, as seen by the intense fluorescence signal (Figure 2). In contrast, incubation of $CuCl_2$ with Aβ40 or Aβ42, both monomeric and fibrillar, did not generate any fluorescence signal. It is clear that $CuCl_2$ with Aβ does not generate any hydroxyl radicals, in agreement with the previous H_2O_2 assay, and that reactive oxygen species are not generated in the absence of ascorbate.

We then went on to study the effect of ascorbate on the Cu−Aβ system. Incubation of $CuCl_2$ and Aβ with ascorbate for 1 h generates appreciable fluorescence signal, although the intensity is ∼ 10−20% that of $CuCl_2$ and ascorbate on its own. In particular, both monomeric and fibrillar full-length Aβ (Aβ40 and Aβ42) show a marked reduction in fluorescence, with ∼90% depletion in the amount of hydroxyl radicals detected compared to the $CuCl_2$ and ascorbate without Aβ.

Fragments of Aβ (Aβ16 and Aβ28) were also studied, and the depletion (∼85%) of hydroxyl radicals detected with these fragments is comparable with the full-length peptide (Aβ40 and Aβ42) results. However, Aβ(25−35), that contains Met^{35} but not the histidines of the Cu^{2+} binding site, is much less effective at reducing hydroxyl radicals detected and only shows ∼40% reduction relative to the $CuCl_2$ and ascorbate sample.

It is clear from the detection of hydroxyl radicals that Aβ does not silence redox cycling of the Cu^{2+} ions in the presence of a physiological reductant (ascorbate). Interestingly, from the ODAD assay it is clear the amount of H_2O_2 generated by $CuCl_2$ and ascorbate is only slightly reduced in the presence of Aβ by between 1% and 22%. In contrast, the 3-CCA assay, which monitors hydroxyl radicals, suggests that the appreciable amounts of hydroxyl radicals generated by $CuCl_2$ and ascorbate are substantially decreased by the presence of Aβ by ∼80−90%.

Rather than Aβ acting to redox silence Cu^{2+} ions, it appears to quench the hydroxyl radicals generated through Fenton−Haber Weiss reactions involving $CuCl_2$ and ascorbate. This quenching results in appreciable oxidation of Aβ itself (described later) as observed by ^{1}H NMR and mass spectrometry.

As with $CuCl_2$-containing samples, experiments using $Cu(Gly)_2$ as the source of Cu^{2+} ions also showed a marked reduction (∼80%) in detectable hydroxyl radicals in the presence of Aβ (i.e., Aβ40 plus $Cu(Gly)_2$ and ascorbate samples) compared to the $Cu(Gly)_2$ and ascorbate control (see Supporting Information Figure S4).

Cu^{2+} Reduction to Cu^+ by Aβ. Key reactions associated with oxidative stress are the redox cycling of $Cu^{2+/+}$ ions; this process is a major source of reactive oxygen species *in vivo* via the Fenton−Haber Weiss reactions (*15*). To examine any inherent ability of the Aβ peptide to reduce Cu^{2+} to Cu^+, both fragments and full-length Aβ in monomeric and fibrillar form were studied using the BC assay. The $Cu^+(BC)_2$ complex produces a characteristic purple color with an absorption maximum at 483 nm, allowing any Cu^+ production to be quantitatively detected. In Figure 3 the reduction of Cu^{2+} by three different Aβ fragments is shown. The absorbance band at 483 nm was monitored after 60 s (Figure 3a) and 60 min (Figure 3b) incubations at 37 °C under aerobic conditions. Control samples in the presence of BC (360 μM) and $CuCl_2$ (10 μM), in which no Aβ fragments were present, are also shown for both incubation times. We see from Figure 3 that BC alone is able to reduce very small amounts of Cu^{2+} itself, as has previously been noted (*56*). Contributions to Cu^{2+} reduction from the BC alone were shown to be ∼1.0 μM after 60 s and ∼2.2 μM after 60 min. The effect of this background reduction of Cu^{2+} by BC alone is highlighted in the data, shown in Figure 3.

From the 60 s sample incubations, it is evident that the shorter, more soluble fragment Aβ28 actually shows less Cu^+ production than the nonpeptide sample, detecting ∼0.8 μM Cu^+ compared to the ∼1.0 μM by BC alone (see Figure 3a). A similar result was observed for other shorter fragments tested, including Aβ16 and Aβ(25−35) (data not shown) with these fragments showing Cu^{2+} reduction less than or equivalent to that by BC alone. In contrast, both the full-

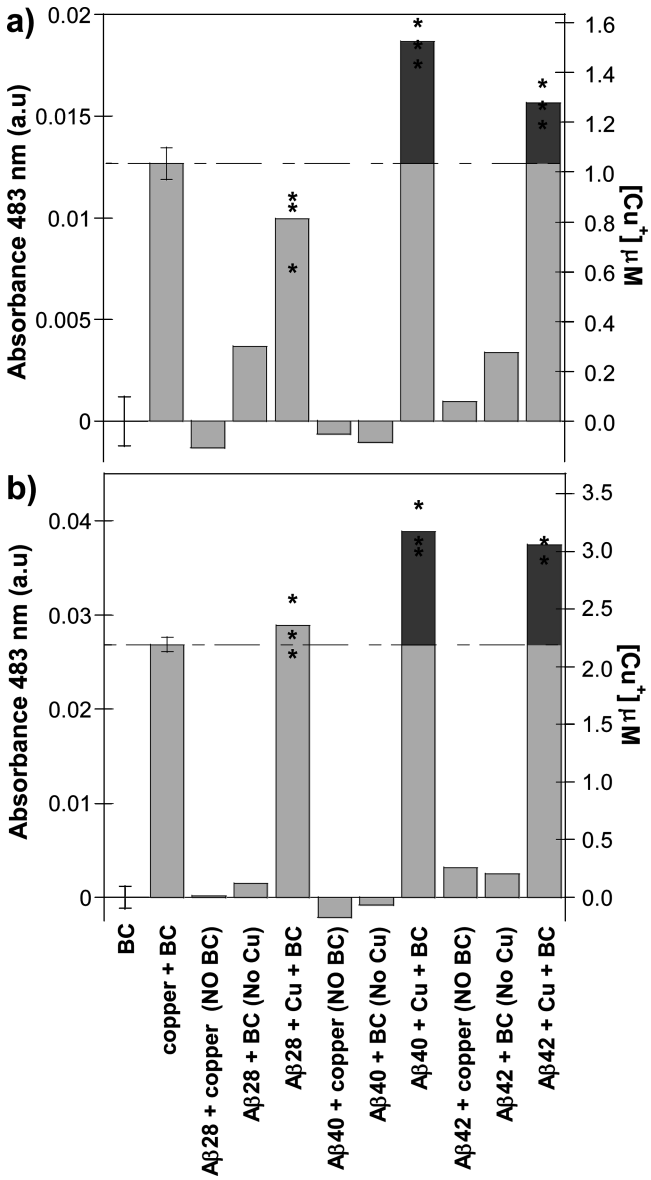

FIGURE 3: Cu^{2+} reduction by Aβ, monitored using the BC assay. Incubations contained Aβ28/40/42 (10 μM) and/or $CuCl_2$ (10 μM) and/or BC (360 μM). Dashed line represents Cu^+ reduction level by BC alone. Results are shown in both absorbance at 483 nm and Cu^+ concentration on the *y*-axes. Plot shows the average BC assay results, based on three replicates, at two different incubation periods: (a) 60 s incubation at 37 °C and (b) 60 min incubation at 37 °C.

length fragments, Aβ40 and Aβ42, produced slightly greater amounts of Cu^{2+} reduction to Cu^+ than BC alone after the 60 s incubation at 37 °C. Levels of Cu^+ ions detected in the presence of full-length peptides are ~1.5 and ~1.3 μM (Aβ40 and Aβ42, respectively) compared to the ~1.0 μM reduced by BC alone. This suggests that under these conditions Aβ possesses some intrinsic ability to convert Cu^{2+} to Cu^+ by donation of an electron. With longer incubation times (60 min at 37 °C) (see Figure 3b), the Cu^{2+} reduction in the presence of Aβ40 and Aβ42 is more apparent. Specifically, the data in Figure 3b show that full-length Aβ reduces ~3.2 and ~3.1 μM (Aβ40 and Aβ42, respectively) compared to the ~2.2 μM reduced by BC alone after the longer incubation time.

Cu^{2+} reduction by fibrillar Aβ40 and Aβ42 was studied to compare with the reducing capacity demonstrated by the monomeric forms of the peptide. Fibrillar full-length Aβ40 and Aβ42 also showed some Cu^{2+} reduction capabilities beyond the intrinsic ability of BC, which was comparable to monomeric Aβ. However, the extent of increase was variable and compromised by turbidity in the assay for these fibrillar samples.

Samples of Aβ were incubated at 37 °C with Cu^{2+} for 1 h with or without the presence of BC. Samples incubated for 1 h in the absence of BC, after which the BC was allowed to react for just 60 s, did not show any additional Cu^{2+} reduction beyond that seen in the 60 s incubations. It appears that BC drives the reduction of Cu^{2+} to Cu^+ more readily in the presence of Aβ.

It is clear then that fragment size of the Aβ peptide has an influence on its Cu^{2+} reduction capabilities. The full-length fragments (Aβ40 and Aβ42) have the ability to promote the reduction of Cu^{2+} in the presence of BC, whereas the shorter fragments Aβ16 and Aβ28, which lack the hydrophobic C-terminus and the highly oxidizable Met^{35}, do not promote reduction of Cu^{2+} ions.

We were also interested in the effect of ascorbate on Cu−Aβ samples. Using the BC assay we saw complete and rapid reduction of all the Cu^{2+} present irrespective of the presence or absence of Aβ. It is clear the Aβ does not redox silence Cu^{2+} ions.

Oxidative Modification of Aβ by a Cu^{2+}/Ascorbate System. In addition to monitoring the effect of Aβ on ROS production by a copper and ascorbate, we also examined the effects of this ROS generation on Aβ itself. Full-length Aβ42 was studied using ^{1}H NMR spectroscopy and monitored over a period of 60 h in the presence of $CuCl_2$ and ascorbate (or H_2O_2) as shown in Figure 4. In the absence of ascorbate, the paramagnetic effect of small amounts of Cu^{2+}, 0.05 mol equiv, on the Aβ42 histidine resonances and other signals is minimal, with only slight broadening observed (see Supporting Information Figure S5). Incubation of Cu−Aβ in the absence of ascorbate showed no oxidation of the peptide. Furthermore, upon the addition of ascorbate the effect on the line width or intensity of most of the Aβ resonances was not marked. Even after 60 h incubation the total loss of Aβ signals was ~35%. This is attributed to gradual precipitation/fibrillization of some Aβ. However, the histidine and methionine resonances were specifically perturbed.

Figure 4a shows ^{1}H NMR spectra of Aβ42 in the aromatic region, comparing Aβ42 in the absence of $CuCl_2$ (gray top spectra) and Aβ42 incubated with $CuCl_2$ (0.05 mol equiv) and ascorbate (10 mol equiv) at various incubation times. The ϵCH and δCH resonances of all three histidines (His^6, His^{13}, and His^{14}) gradually lose their intensity; after 60 h the histidine resonances are considerably diminished. There is a shift to low field due to a drop in pH of 0.1 unit. With greater amounts of ascorbate (20 mol equiv) almost complete loss of histidine resonances in the ^{1}H NMR spectra occurs within ~1 h of the addition of ascorbate to the Cu−Aβ complex. In addition to loss of the histidine signals, two new resonances at 6.25 and 6.29 ppm are observed with $CuCl_2$ and ascorbate incubation, and an enlargement of this signal is shown as an insert in Figure 4a. This new resonance can be assigned to the δH (C4H) of the 2-oxohistidine species (*57*). The 2-oxohistidine species results from oxidation of the histidine imidazole side chain to 2-oxoimidazole, with CϵH being converted to a carbonyl group, CϵO (*58*). The new signals shown in Figure 4a are most likely a combination

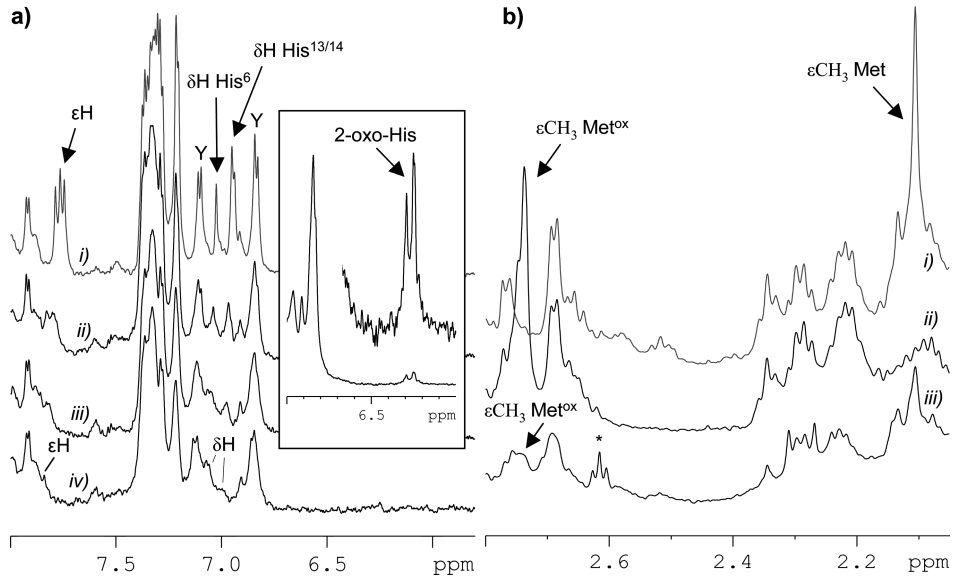

FIGURE 4: 1D proton NMR spectra of Aβ42 in the presence of $CuCl_2$ and ascorbate. (a) Aromatic region: (i) Apo Aβ42 (100 μM) in 10 mM phosphate buffer (pH 7.4) (top gray spectra), (ii−iv) a sample of Aβ42 with 5 mM (0.05 mol equiv) $CuCl_2$ and 1 mM (10 mol equiv) ascorbate in 10 mM phosphate buffer, pH 7.4 at 37 °C. Incubation times shown are (ii) ∼20 h, (iii) ∼40 h, and (iv) 60 h post addition of $CuCl_2$ and ascorbate. Insert shows the summed spectra over the complete 60 h incubation at 37 °C in the region 7.0−6.0 ppm and a zoomed view showing the 2-oxo-His signal. Y indicates Tyr^{10} ring protons. (b) Aliphatic region: (i) Apo Aβ42 (100 μM) in 10 mM phosphate buffer (pH 7.4) at 37 °C (top gray spectra), (ii) Aβ42 plus H_2O_2 (2.5 mM) after 60 h incubation at 37 °C, and (iii) Aβ42 plus 5 mM (0.05 mol equiv) $CuCl_2$ and 1 mM (10 mol equiv) ascorbate after 60 h at 37 °C. * indicates ascorbate oxidation product.

of the three histidines (His^6, His^{13}, and His^{14}) in their oxidized form. Although the loss of signal for the unoxidized histidine is almost complete, the intensity of this 2-oxo-His signal is considerably less than that of other resonances for Aβ, ∼10% the intensity of, for example, the Tyr^{10} 2,6 ring protons. It is notable that the Tyr ring protons are largely unperturbed, suggesting that under these conditions dityrosine cross-linking is limited, although higher Cu−Aβ ratios have been shown to mediate dityrosine cross-linking (*59*).

The second marked change in the ^1H NMR of Aβ42 is shown in part of the aliphatic region (Figure 4b). In the apo (top) spectra, the characteristic singlet from ϵCH_3 of Met^{35} in Aβ is evident at 2.10 ppm. Addition of H_2O_2 (2.5 mM) causes loss of this signal and the appearance of a new singlet peak in the ^1H spectra at 2.74 ppm with similar intensity. This new resonance (and shoulder) can be assigned to the *R*- and *S*-forms of ϵCH_3 in methionine sulfoxide (Met^{ox}) ($C\alpha H-C\beta H_2-C\gamma H_2SOC_\epsilon H_3$) (*60*), in which an oxygen has been added to the sulfur atom in the methionine side chain. We note that addition of H_2O_2 alone has no effect on the histidine resonances. Furthermore, it is notable that incubations of Aβ with Cu^{2+} in the absence of ascorbate shows no marked oxidation of His or Met residues.

Incubation with $CuCl_2$ (0.05 mol equiv) and ascorbate (10 mol equiv) causes a gradual decrease in intensity of the Met^{35} ϵCH_3 signal over ∼60 h. The appearance of a new ϵCH_3 signal for the Met^{ox} species is slight, and after 60 h a small peak, ∼10% the intensity of the apo unoxidized Met^{35}, is detected. Although the unoxidized Met^{35} signal is almost completely lost, this is not accompanied by the appearance of a Met^{ox} signal of equal intensity. In contrast, studies with the Aβ(25−35) fragment under similar conditions of $CuCl_2$ and ascorbate showed a significant peak for the Met^{ox} (see Supporting Information Figure S6).

We looked for the appearance of a signal that may correspond to methionine sulfone ($C\alpha H-C\beta H_2-C\gamma H_2SO_2C_\epsilon H_3$), a product rarely formed in biological systems resulting from further oxidation of methionine sulfoxide (*61*), but none were apparent. 2-Oxo-His can also be further oxidized to asparagine/aspartic acid, but these products are also not observed. The weak intensities of the 2-oxo-His and Met^{ox} suggest possible oxidation of histidine and methionine to other unidentified species. Aβ can form oligomeric species whose line width may make them difficult to detect by NMR. However, the intensities and line widths of the rest of the Aβ spectra remain largely unaffected; even after 60 h incubation, two-thirds of the oxidized Aβ is detected in the ^1H NMR spectra.

DISCUSSION

Cu−Aβ Does Not Possess a Prooxidant Activity in a Cell-Free System. Although oxidative injury is an established characteristic of AD (*4*), the exact role that the Aβ peptide plays is disputed. Some claim that the neurotoxicity of Aβ is due to generation of ROS in the presence of the Aβ−Cu complex (*30, 32, 33*). While others point to an antioxidant role for Aβ (*35−37, 40*), until now however, studies directly measuring ROS in a cell-free system in the presence of Aβ and Cu^{2+} ions have typically suggested that Aβ has prooxidant activity (*33, 44, 45, 49*). It is now clear from our ODAD and peroxidase and 3-CCA ROS detection assays that Aβ40 and Aβ42 in either their fibrillar or monomeric state do not generate either H_2O_2 or hydroxyl radicals in the presence of Cu^{2+} ions alone. Even in the presence of an additional reducing agent, ascorbate, the peptide has no ability to enhance reactive oxygen species production above and beyond that of control samples. Indeed, levels of detectable hydrogen peroxide are the same or less than controls, and hydroxyl radicals were appreciably diminished by the presence of Aβ peptide, in the $CuCl_2$ and ascorbate samples.

Our findings are in complete contrast to *in vitro* studies published by Opazo et al. in which they state that Aβ42 with Cu^{2+} generates hydrogen peroxide catalytically by recruitment of a biological reducing agent (e.g., ascorbate) as a substrate (*33*). Variation between the methodology used by Opazo et al. and that described here includes the presence of EDTA to bind free Cu^{2+} during incubations. For this reason, additional sample incubations containing EDTA were performed using the hydrogen peroxide assay; however, we still did not observe any catalytic production of hydrogen peroxide by Aβ plus Cu^{2+} and ascorbate. The amount of H_2O_2 described to be generated by Aβ and Cu^{2+} was small in the Opazo et al. study, but no control for Cu^{2+} with ascorbate and EDTA was reported. In our study the level of H_2O_2 recorded for the control of Cu^{2+} and ascorbate with EDTA was comparable to Aβ42 with Cu^{2+}, ascorbate, and EDTA; both were very low and within the noise level of the study. The Opazo et al. study used $Cu(Gly)_2$ as the source of Cu^{2+} ions, and it has been suggested that more hydroxyl radicals are generated by $Cu(Gly)_2$ and ascorbate with the addition of Aβ (*62*); however, we have found no such difference.

Aβ Acts as a Sacrificial Antioxidant by Quenching Hydroxyl Radicals. The antioxidant-like activity of the Aβ peptide can be directly correlated to observed metal catalyzed oxidation of Aβ. The loss of ^{1}H NMR signals for unoxidized histidine and methionine is almost complete. 2-Oxo-His and Met^{ox} ^{1}H NMR resonances are also observed, although they only account for about ~10% of the total Aβ detected. Methionine and histidine residues are particularly prone to metal-catalyzed oxidation (*63*), and 2-oxo-His is considered a useful biological marker under conditions of oxidative stress (*64*). Pronounced oxidation of Aβ has been observed in plaques with the Met^{35} residue oxidized to Met sulfoxide (*5, 6*). Other studies by Schoneich et al., using HPLC-MS/MS, have shown that His^{13} and His^{14} in the Aβ sequence are targeted first for oxidation and converted to 2-oxo-His (*65*).

These results suggest that the antioxidant activity of Aβ is mediated by a type of free radical scavenging, rather than through metal chelation into a redox-inactive state. We have shown that the hydroxyl radicals generated by Fenton−Haber Weiss reactions of $CuCl_2$ and ascorbate are largely (80−90%) quenched by Aβ. The highly reactive hydroxyl radicals are very short-lived, and since copper is bound to Aβ, the radical species are produced in close proximity to the peptide, readily oxidizing Aβ.

Similar behavior is observed with the Cu^{2+} transport protein serum albumin, and this is believed to be the mechanism by which albumin acts as a principle antioxidant within blood plasma, accounting for 70% of the free radical trapping activity of serum (*66*). In addition, similar behavior is observed for Cu^{2+} bound to the prion protein and suggests that the prion protein functions as an antioxidant at the synaptic cleft during release of Cu^{2+} ions (*52*).

It has been proposed that proteins with surface-exposed methionine residues can act as antioxidants. In conjunction with the repair enzyme methionine sulfoxide reductase (Msr) the oxidation/reduction of methionines can function as a sink for ROS (*67*). Importantly, studies of Msr activity in the brains of AD patients revealed that the enzyme's activity was reduced, compared to control subjects (*68*). The methionine residue within Aβ regulates oxidation products; specifically, histidine residues in the peptide were only oxidized in the absence of the methionine (*69*).

Cu^+ Generation. Our measurements of Cu^{2+} reduction capability by assorted Aβ peptides shows variation depending on the length of the Aβ fragment used. In particular, the smaller, more soluble copper binding fragment (Aβ28) showed no ability to reduce Cu^{2+} to Cu^+. Additionally, no Cu^{2+} reduction was observed when the fragments Aβ(25−35) and/or Aβ16 were coincubated with $CuCl_2$. These results are consistent with previous reports (*44, 45, 70*). An early study suggested Cu^+ generation by Aβ(25−35) (*47*); however, this observation has never been reproduced.

In contrast, we show that both monomeric and fibrillar full-length Aβ peptides (Aβ40 and Aβ42) possess some intrinsic Cu^{2+} reduction capability, in agreement with Huang et al. (*44, 45*). Although our Cu^+ assay results did not show a distinction in reduction ability between the two full-length peptides or the fibrillar state, Cu^{2+} reduction by Aβ40/42 may involve the Met^{35} residue and hydrophobic tail not present in Aβ28.

Although full-length Aβ (monomeric or fibrillar) is capable of reducing small amounts of Cu^{2+}, this is not reflected by production of hydrogen peroxide or hydroxyl radicals. The lack of H_2O_2 and hydroxyl radicals indicates that the Cu^{2+} reduction is not significant in terms of ROS generation. The reducing capacity of Aβ seems to require the presence of the Cu^+ chelator BC to drive the reaction by chelating the Cu^+ generated. Furthermore, our ^{1}H NMR studies with Cu−Aβ42 suggest that no reactive oxygen species are generated in the absence of ascorbate.

In Support of an Antioxidant Role for Aβ. The studies shown here indicate that both the monomeric and fibrillar Aβ peptide act as an antioxidant by reaction with (quenching sacrificially) reactive oxygen species to prevent their harmful effects elsewhere, and this is supported by a range of studies. There is evidence that Aβ can act as an antioxidant for lipoproteins in both CSF and plasma (*71*). Other studies have also demonstrated Aβ to possess antioxidant function, with Zou et al. reporting on the ability of monomeric Aβ40 and Aβ42 to inhibit neuronal death (*37*). These monomeric species were shown to mimic the role of antioxidant scavengers, such as catalase and vitamin E, under the same conditions, preventing neuronal death caused by transition metal-induced oxidative damage. This neuroprotective activity of Aβ was shown to be lost on formation of oligomeric and aggregated forms of the peptide. Kontush et al. suggested that only at low concentrations (0.1−1.0 nM) did Aβ inhibit autoxidation of CSF lipoproteins and plasma low density lipoprotein (LDL) (*71*). Similarly, an earlier study by Chan et al. has reported an antiapoptotic action only at low Aβ concentrations (*40*).

A number of studies suggest Aβ is upregulated as a response to oxidative stress (*72*). It has been shown that β- and γ-secretases (BACE1 and presenilins) that cleave Aβ from its precursor protein (APP) are upregulated as a response to oxidative stress (*73*−*76*). Aβ levels are therefore possibly increased as an antioxidant defense mechanism. Studies on human neuroblastoma cells showed increased levels of Aβ, through enhancement of the amyloidogenic pathway of APP, in response to the addition of hydrogen peroxide (*77*). Increased production of Aβ has also been identified in response to injuries such as head trauma (*78*−*80*).

Aβ28 has been shown to have neurotrophic activity, by enhancing neuronal survival (*81*). An inverse correlation between amyloid deposits and 8-OHG (8-hydroxyguanosine) levels, a marker for oxidative damage, also suggests that Aβ may be produced in a compensatory response to increased oxidative stress, with oxidative damage actually reducing with senile plaque formation (*82, 83*). The neuroprotective nature of Aβ is highlighted with a negative correlation between amyloid deposits and observed oxidative damage, neuronal loss, and cognitive impairment (*84*−*86*). Similar inverse correlation between Aβ deposits and oxidative damage was detected in patients with Down's syndrome. Interestingly, Aβ deposition follows rather than precedes increased 8-OHG levels, and strikingly 8-OHG levels decline to control levels after plaques form (*87*).

ROS-Induced Toxicity Mediated by Cu−*Aβ Is Not Ruled Out.* It is important to note that a so-called prooxidant role for Aβ is not ruled out by our observations. ROS-mediated cellular toxicity of Aβ has been demonstrated by a number of groups (*14*). However, we have clearly shown that Aβ does not generate any more ROS than free Cu^{2+} ions or weakly chelated $Cu(Gly)_2$ irrespective of the concentration of Cu^{2+} ions or the monomeric/fibrillar state of Aβ. However, this does not preclude an oligomeric diffusible form of Aβ binding Cu^{2+} ion and then trafficking and concentrating the Cu^{2+} ions in a redox-active state to the neuronal cell surface.

Thus the conflicting observations that Aβ can promote lipid peroxidation (*31*) while others suggest Aβ acts as a neuronal antioxidant (*35*−*37*) may well reflect the form of Aβ. This is supported by the concentration dependence of these studies, where they only exhibit antioxidant properties at low (monomeric) levels of Aβ (*40, 71*). The hydrophobic nature of Aβ peptide means it has a strong affinity for the cell membrane, but it is likely that monomeric Aβ will interact with the cell membrane differently from oligomeric or fibrillar forms of Aβ. Monomeric Aβ may protect neurons by binding potentially toxic Cu^{2+} ions released at the synapse and so quench the hydroxyl radicals, while oligomeric Aβ could concentrate the redox-active form of copper at the membrane and so cause lipid peroxidation and eventually cell death.

The prooxidant properties of Cu−Aβ are clearly not due to the ability of Aβ to catalytically generate H_2O_2 and/or hydroxyl radicals. Rather it is Aβ's ability to chelate Cu^{2+} in a redox-active form and then concentrate Cu^{2+} ions at the membrane surface.

CONCLUSION

The amyloid cascade hypothesis indicates that in cases of familial AD it is the upregulation of Aβ42 due to mutations in APP or the presenilins that causes the cascade of events leading to dementia (*11*). We suggest that, for sporadic AD, Aβ is also upregulated as a response to oxidative stress. Interestingly, oxidative stress increases with age and is linked to head trauma and the inflammatory response (*78, 79*), both of which are risk factors associated with sporadic AD. Furthermore, Cu^{2+} levels (a major source of ROS) in the brain increase with age, and Cu^{2+} homeostasis is compromised in AD patients (*16, 88*).

Our studies, in a cell-free system, show no evidence that Aβ acts as a prooxidant in a Cu^{2+}/ascorbate system; to the contrary, we observe the opposite with Aβ exhibiting antioxidant-like properties. Thus rather than causing the oxidative stress observed in the early stages of sporadic AD (*7, 8*), the upregulation of Aβ (*73*) could be a protective response to it; initially, Aβ actually reduces oxidative stress (*37*). However, in the long term, the upregulation of Aβ then causes the neurotoxic effects of Aβ oligomers and the accumulation of amyloid plaques. Aβ oligomers could act to concentrate Cu^{2+} ions at the membrane surface and allow ROS generation (*31*), leading to neuronal cell death, which causes further marked oxidative stress and the further upregulation of Aβ as shown in Figure 5.

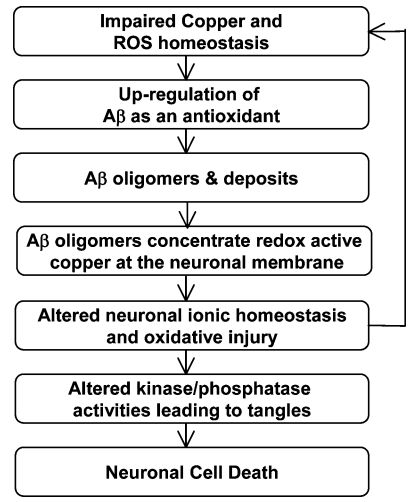

FIGURE 5: Modified amyloid cascade hypothesis. Sporadic AD is caused by upregulation of Aβ as a response to impaired copper and ROS homeostasis, both of which are linked with aging. Failure to clear increased levels of Aβ causes oligomerization and diffuse plaque formation. Diffusible oligomers could concentrate Cu^{2+} in a toxic redox-active state at the membrane. This in turn causes further oxidative stress and upregulation of Aβ. Further Aβ accumulation causes altered kinase and phosphatase activities leading to neurofibrillar tangles of tau protein and dementia.

Thus the age of onset of sporadic AD may reflect an individual's ability to maintain ROS homeostasis within the brain. Antioxidant therapeutics may therefore be the most effective treatment of sporadic AD (which accounts for 85% of AD cases) and could delay or prevent the onset of sporadic AD. Therapeutics that attempt to solublize plaques may actually release the more neurotoxic oligomeric form of Aβ and so may be counterproductive. Thus far, antioxidant therapies such as vitamin E have shown only limited efficacy (*89*).

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SUPPORTING INFORMATION AVAILABLE

Six figures showing (1) confirmation of fibril formation using a variety of methods, (2) ODAD and peroxidase, hydrogen peroxide detection assay calibration, (3) hydrogen peroxide production with higher Cu^{2+} concentrations, using

$CuGly_2$, (4) hydroxyl radical detection using Cu^{2+} ions complexed with glycine, (5) 1D proton NMR spectra of Aβ42 in the presence of $CuCl_2$, and (6) 1D proton NMR spectra of Aβ25−35 in the presence of $CuCl_2$ and ascorbate. This material is available free of charge via the Internet at http://pubs.acs.org.

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