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Methionine regulates copper/hydrogen peroxide oxidation products of Aβ

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Abstract: Metal-catalysed oxidation (MCO) may play a causative role in the pathogenesis of Alzheimer's disease (AD). Amyloid β peptide (Aβ), the major biomarker of AD, in the presence of copper ions reduces Cu^{2+} to Cu^{+} and catalyses the formation of H_2O_2 that subsequently induces radicals through Fenton chemistry. Aβ is also subject to attack by free radicals, where the presence of Cu^{2+} in conjunction with H_2O_2 catalyses oxygenation, primarily at the methionine sulfur atom. This work investigates MCO of Aβ, to gain further insight into the role of oxidative stress in AD. By combining a fluorescence assay with gel electrophoresis to monitor MCO reactions of Aβ (1–28) in the presence and absence of methionine it was determined that methionine can both protect some residues against MCO and promote the oxidation of Tyr(10) specifically. Electrospray ionization mass spectrometric analysis of methionine MCO products indicated the formation of methionine sulfoxide, methionine sulfone and related hydroxylated products. Similar products could be formed from the oxidation of Met(35) of Aβ and may relate to changes in properties of the peptide following MCO.

Keywords: Alzheimer's disease; amyloid β-peptide; Aβ; copper; metal catalysed oxidation; methionine oxidation; hydrogen peroxide; oxidative stress

INTRODUCTION

Metal-catalysed oxidation (MCO) gives rise to highly reactive intermediates such as hydroxyl radicals which lead to damage of bio-molecules and are implicated in aging and the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD) [1,2]. AD is characterized by the accumulation of senile plaques mainly composed of aggregated amyloid β-peptide (Aβ). Aβ is primarily a 40–42 amino acid peptide that is a proteolytic product derived from the β amyloid precursor protein (APP) (for review see [3–5]).

There are many indications that oxidative damage plays a causative role in AD [6], including: metal ions, especially iron, copper and zinc, being elevated in the brains of AD patients [7–10] and implicated in the catalytic activity that may produce free radicals; antioxidants and metal chelators have shown promise as potential treatments for AD [11–15]; and the toxicity of Aβ is eliminated by free radical scavengers *in vitro* [16,17].

Different studies have indicated that the formation of free radicals by Aβ occurs through interactions with Cu^{2+} where the peptide reduces Cu^{2+} to Cu^{+} [18–23]. This catalytic process leads to the formation of H_2O_2 with the subsequent production of radicals such as the hydroxyl radical through Fenton chemistry [18,20,24]. Additionally, Aβ itself is subject to attack by free radicals, where the presence of Cu^{2+} can catalyse oxygenation, mainly at the methionine sulfur atom Met(35) [25,17], and dityrosine formation [26,23]. The most common source of reactive oxygen species (ROS) is H_2O_2, which in the presence of redox active metal ions Fe^{2+} or Cu^{+} generates the hydroxyl radical (Fenton reaction).

Met(35) has been reported to play an important role in the redox mediated toxicity of Aβ [1,27,17]. As reviewed by Varadarajan *et al.* [1], the accumulated data support a vital role for the methionine residue of Aβ peptide in oxidative stress and neurotoxicological properties of this peptide. Evidence includes: elevation of methionine oxidation in senile plaques which recently has been shown by Raman spectroscopy [28]; formation of ROS by the shorter fragments of β-amyloid peptide that contain the methionine residue, such as Aβ(25–35) and Aβ(32–35) [29]; substitution of the methionine by norleucine prevents toxicity [30]; and replacement of a single sulfur atom in the methionine residue of the peptide with a methylene (CH_2) has been reported to inhibit the toxicity of Aβ and the associated oxidative

Abbreviations: Aβ, β-amyloid peptide; AD, Alzheimer's disease; Boc, t-butyloxycarbonyl; C_{ortho}, carbon atom at ortho position in phenol ring; ECL, enhanced chemiluminescence; EDTA, ethylenediamine tetra-acetic acid; ESI-MS, electrospray ionization-mass spectrometry; Fmoc-Cl, 9-fluorenylmethoxycarbonyl chloride; HFIP, hexafluoroisopropanol; MCO, metal catalysed oxidation; Met(O), methionine sulfoxide; PBS, phosphate buffered saline; PCET, proton-coupled electron transfer; PDA, photo diode array; ROS, reactive oxygen species; RP-HPLC, reversed phase-high performance liquid chromatography; SDS, sodium dodecyl sulfate.

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stress to cultured hippocampal neurons [31]. Therefore, it has been hypothesized that the oxidative state of the Met(35) residue sulfur in Aβ has implications for initiation of free radicals and oxidative stress [32,2], and the neurotoxicity of Aβ in AD [32,31,33],

In general, MCO of methionine (methionyl) residues results in oxidation of the sulfur to methionine sulfoxide Met(O) [34,35]. This oxidation product is known to occur in the presence of H_2O_2 even without the metal ion [36,37]. MCO is suggested to result from direct oxidation [38], with a higher rate at pH 8.5 [39], and Cu^{2+} catalysed this oxidation only at low reactant concentration [39]. The rate of oxidation appears to be higher in proteins than for the free amino acid [40]. Oxidation of the sulfur increases the hydrophilicity of the protein and can induce conformational changes [41] in a variety of proteins including Aβ as shown by us and other groups [42,43,17]. Met(35) oxidation causes partial conversion of Aβ from β-sheet to random coil and decreases Aβ aggregation [42–44]. Additionally, Met(35) oxidized Aβ is both soluble and toxic to neuronal cells [17] and may prove to play an important role in AD. Methionine also has been reported to protect other residues from MCO [45]. Hence, investigation of MCO of methionine is important, as is the identification of stable and suitable MCO methionine products that may be used as a biomarker for MCO of proteins and peptides in biological systems.

The goal was to study changes to Aβ peptides under conditions of MCO, and to investigate the role that methionine plays in regulating MCO, to gain further insights into the role of oxidative stress in AD. To this end, techniques were developed to study MCO of amino acids and the study was extended to Aβ(1–28), a shorter version of the amyloid β-peptide. It was demonstrated that: (i) MCO of amino acids and peptides can be monitored using a fluorescence plate reader, with His and Tyr able to be selectively monitored depending upon the wavelength chosen; (ii) His, Phe and Tyr are most sensitive to MCO and also most readily monitored using this technique; and (iii) Met appears to protect His of Aβ(1–28) but promotes Tyr oxidation during MCO. This report shows that in the presence of exogenous methionine, MCO of Aβ(1–28) generated fluorescent products specific for tyrosine modification and caused formation of higher oligomers of Aβ(1–28), while in the absence of methionine, MCO of Aβ(1–28) generated fluorescent products mainly due to histidine and phenylalanine modifications, illustrating the role of methionine in regulating the oxidation products of Aβ.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were purchased from BDH Chemicals (Poole, England), Sigma Chemical Co. (St Louis, USA) and Musashi (Melbourne, Australia) with the highest available quality and purity. All solvents were HPLC grade.

Aβ peptides were synthesized using manual solid-phase (Boc) amino acid chemistry as described previously [46].

Electrophoretic molecular weight markers and reagents for enhanced chemiluminescence (ECL) were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). Precast Tris-tricine gels (Novex) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA) and Trans-Blot nitrocellulose membrane were purchased from BioRad Laboratories (Hercules, CA, USA).

Instrumentation

High performance liquid chromatography (HPLC) was performed using a Waters system with dual 510 pumps and a model 996-photodiode array detector controlled using Millennium software (Milford, USA). Data were recorded on a Millennium 32 data collection package.

A fluorescence microplate reader (fMax) with incubator and SOFTmaxPRO software (Sunnyvale, USA) were used. Fluorescence plate reader measurements were carried out at 25 °C using the following filters: (λ_{ex} 355 nm/λ_{em} 460 nm, λ_{ex} 485 nm/λ_{em} 538 nm, λ_{ex} 544 nm/λ_{em} 590 nm and λ_{ex} 584 nm/λ_{em} 612 nm).

Electrospray ionization mass spectrometry (ESI-MS) was performed using a Micromass Quattro II triple quadrupole instrument (Manchester, UK) using positive ion mode. Samples were introduced to the source via a Hewlett Packard (HP1100) LC system (Palo Alto, USA) equipped with solvent degasser, binary pumps and auto-sampler. Different buffer solutions (either 0.1% formic acid or ammonium formate in MilliQ water : acetonitrile (50 : 50)) were fed at 40 µl/min to the MS probe. ESI-MS samples were dissolved in appropriate volumes of 1% formic acid. Chelex 100 was used for removal of Cu^{2+} to avoid suppression of the MS signal by the metal ion. Data were acquired in MCA mode from 90 to 1000 Da in 10 s scans, with an ion source at 80 °C and sampling cone voltages of 25 or 30, 40 or 60 V. The voltages were selected and the high voltage value (60 V) used for fragmentation of tested samples and further identification.

Methods

Amino acid-/metal catalysed oxidation. 5 mg of each amino acid was dissolved in 10 ml of PBS (pH 7.4); 4 ml of each solution was mixed with 46 µl of 20 mM $CuCl_2$ by stirring at 37 °C; the samples were sonicated for 2 min and kept in closed vials, bubbled with nitrogen gas for 3 min to ensure removal of oxygen then 40, 30, 20 and 10 µl of 3% H_2O_2 was added over 3 days. Excess amounts of Chelex 100 were added to the flasks at reaction end to ensure removal of any trace copper ions. The reaction was quenched by freezing under liquid nitrogen and freeze-dried. The dried samples and the controls were redissolved in 4 ml of MilliQ water and analysed using a fluorescence microplate reader and Fmoc amino acid analysis.

An alternative method was used to suit the conditions required for ESI-MS analysis. The size of the samples was multiplied by 10; PBS was replaced by ammonium acetate buffer with the same pH and H_2O_2/amino acid 10 : 1 molar ratio was added immediately. In addition, the incubation time was reduced to 1 day. Excess amounts of Chelex 100

Table 1 Scheme Used to Determine Metal Catalysed Oxidation of Aβ(1–28) Peptide

	Solution added (molar ratio)					
Sample number[a]	H_2O_2[b] (10)	Cu^{+}[c] (100)	Cu^{2+}[d] (100)	Methionine[e] (1)	Horseradish peroxidase[f] (10)	Aβ(1–28)[g] (1)
1						√
2	√					√
3	√	√				√
4	√	√		√		√
5	√		√			√
6	√		√	√		√
7					√	√
8	√				√	√

[a] All the samples were made up to 1 ml by addition of 10 mM ammonium acetate buffer (pH 7.4) and the control samples included the same compositions as 1–8 but $CH_3CN:H_2O$ replaced the peptide solution.
[b] 10 µl of 3.0 mM H_2O_2.
[c] 100 µl of 3.0 mM CuCl.
[d] 100 µl of 3.0 mM of $CuCl_2$.
[e] 10 µl of 300 µM methionine.
[f] 10 µl of horseradish peroxidase solution (0.1 mg in 1 ml of ammonium acetate buffer).
[g] 100 µl of 30 µM Aβ (1–28) (0.1 mg dissolved in H_2O : CH_3CN, 1 : 1 v/v).

were added to the flasks at reaction end to ensure removal of any trace copper ions. The reactions were decanted into clean tubes and frozen in liquid nitrogen and lyophilized. The dried samples were analysed using ESI-MS and fractions were collected from HPLC for Fmoc-amino acid analysis.

Aβ peptide metal catalysed oxidation. Aβ(1–28) (0.01 mg/ml or 3 µM) in ammonium acetate buffer (0.01 M, pH 7.4) was incubated with H_2O_2 (1 µg/ml or 30 µM) in CuCl (3 µg/ml or 31 µM) or $CuCl_2$ (4 µg/ml or 31 µM), with and without methionine (0.46 µg/ml or 3.1 µM) (see Table 1). These solutions were vortexed for 10 s and incubated for 1 day at 37°C. Chelex 100 was added to remove Cu ions and the samples freeze-dried. The samples and the controls were redissolved in MilliQ water or MilliQ water : acetonitrile (1 : 1) and analysed by ESI-MS, UV/Vis, fluorescence microplate reader and gel electrophoresis-polyacrylamide gel electrophoresis utilizing the WO2 antibody as described below.

All MCO reactions were done in the absence of light to avoid photochemical reactions [47].

Fmoc-amino acid analysis. Fmoc (fluorenylmethoxycarbonyl) amino acid analysis was carried out using a slight modification of Haynes's method [48,49]. 100 µl of amino acid with MCO reaction mixture or the control sample (with the same concentration of the amino acid but without MCO components) in 1.5 ml Eppendorf tube was mixed with 5 µl of borate buffer (pH 8.5, 16 mM), followed by addition of 100 µl of Fmoc reagent solution (6 mg in 1.5 MilliQ water), agitated for 90 s and followed by the addition of 60 µl cleavage reagent (680 µl of 0.1 M EDTA and 20 M NaOH + 300 µl of hydroxylamine hydrochloride in 1 ml MilliQ water + 80 µl of 2-(methylthio)ethanol) prepared fresh daily. The reaction components were mixed and allowed to stand for 3.5 min. The reaction was stopped by addition of 140 µl of the quenching reagent (2 ml glacial acetic dissolved in 8 ml acetonitrile acid). 20 µl of the reaction mixture was injected into the HPLC system.

Polyacrylamide gel electrophoresis (PAGE) and Western blotting. MCO of Aβ(1–28) was monitored by SDS–PAGE. The dried samples were dissolved in sample buffer (8 M urea, 8% sodium dodecyl sulfate (SDS), 30% glycerol, 100 mM tricine, 0.01% phenol red and 10% mercaptoethanol), and subjected to PAGE and calibrated Western blot according to previously described procedures [50]. Western blots were probed for Aβ species using the monoclonal antibody WO2 which recognizes residues 5–8 [51].

RESULTS

Metal Catalysed Oxidation of Amino Acids

As a prelude to attempting to understand MCO modifications to Aβ, initially MCO mediated changes to the individual amino acids were investigated. The object of this study was to develop an assay for monitoring the changes in peptide residues due to Cu^{2+} and H_2O_2 oxidation. Fmoc amino acids analysis was used to monitor the impact of MCO on the different amino acids. Selected amino acids were assayed using a fluorescence plate reader after MCO. Two of the readily available plate filters were found to be specific for monitoring the modification of tyrosine, phenylalanine and histidine.

Fmoc-amino acid analysis. Fmoc-amino acid analysis was used to overcome difficulties in analysis by RP-HPLC of amino acids and their products from MCO [48]. The amino acid was derivatized with the Fmoc group

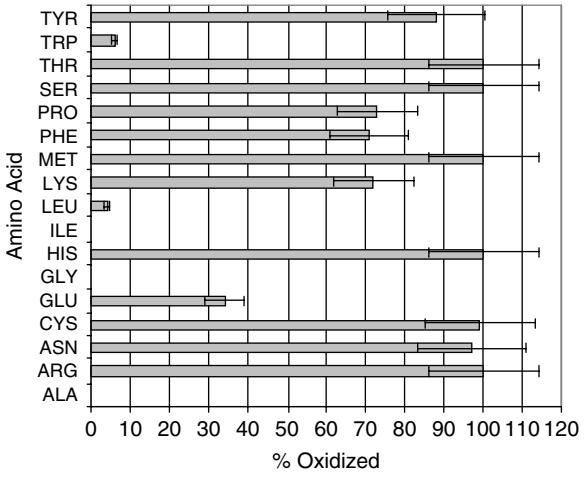

Figure 1 Percentage of amino acid oxidized by MCO after incubation for 3 days with Cu^{2+}/H_2O_2 in PBS (pH 7.4) at 37 °C. Results obtained from HPLC analysis of Fmoc amino acid.

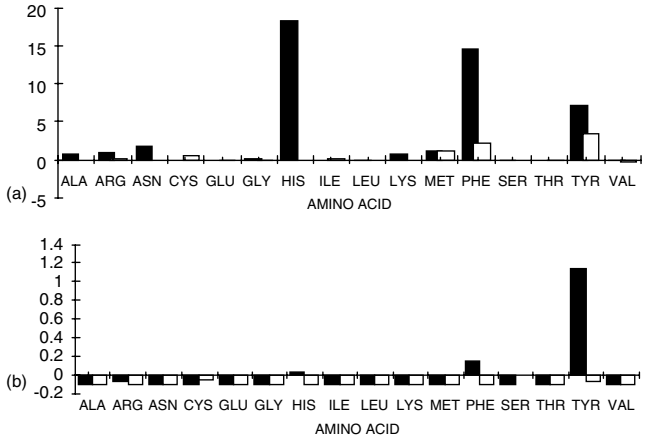

Figure 2 Change in fluorescence intensity after reaction of amino acids with Cu^{2+}/H_2O_2 as monitored by fluorescence microplate reader (□, amino acid + Cu^{2+} prior to reaction; ■, amino acid + Cu^{2+}/H_2O_2 after reaction). (a) Plate reader filter (λ_{ex} 355 nm/λ_{em} 460 nm); (b) Plate reader filter (λ_{ex} 485 nm/λ_{em} 538 nm).

to reduce polarity and prevent the amino acid from eluting with the solvent peak. Furthermore, the Fmoc group enhances the sensitivity of amino acids to UV-Vis and fluorescence detectors. This method was applied to quantify amino acid oxidation in the Cu^{2+}/H_2O_2 system. Oxidation was quantified by calculating the difference in peak area for the starting Fmoc-amino acid before and after reaction (Figure 1). The results show that aliphatic amino acids have the greatest stability against MCO, especially alanine, isoleucine, leucine and glycine. The remaining amino acids, except tryptophan, are modified under conditions of MCO.

Fluorescence microplate reader analysis of MCO of amino acids. A fluorescence microplate reader was used to determine any changes in fluorescence of amino acids following oxidation conditions as a possible assay for oxidation of Aβ residues. The four available filters with the microplate reader (λ_{ex} 355 nm/λ_{em} 460 nm, λ_{ex} 485 nm/λ_{em} 538 nm, λ_{ex} 544 nm/λ_{em} 590 nm and λ_{ex} 584 nm/λ_{ex} 661 nm) were tested; but only the first two filters showed positive results (Figure 2).

Filter (λ_{ex} 355 nm/λ_{em} 460 nm) showed an increase in fluorescence from MCO of histidine, phenylalanine and tyrosine. Both histidine and phenylalanine MCO products produced the largest increase in fluorescence (see Figure 2(a)). Consequently, this filter can be used to monitor MCO of histidine and phenylalanine in peptides and proteins. The filter (λ_{ex} 485 nm/λ_{em} 538 nm) was the most sensitive to the fluorescent products of tyrosine oxidation (Figure 2(b)). Aromatic residues are known to fluoresce, so it is not surprising that His, Phe and Tyr showed the strongest fluorescence after MCO. It should be noted that the presence of Cu^{2+} quenched the fluorescence signal of some amino acids, especially tyrosine products (such as dityrosine) since Cu^{2+} was not removed from the crude solutions; this indicated that the MCO products of some amino acids formed strong ligands for Cu^{2+}.

ESI-MS identification of MCO of methionine. ESI-MS analysis of the Cu^{2+}-MCO reaction mixtures of methionine showed fragmented forms of the amino acids (data not shown) even at low cone voltage (20 V). Methionine showed a peak with loss of a CO_2H group (45.1 mu). Other masses for fragmented methionine were detected and could be due to the catalytic activity of copper ions causing fragmentation of amino acids in the ESI [52–54]. Fmoc-amino acid analysis was used to isolate the modified forms of methionine by MCO. ESI-MS data indicated the formation of the usual oxidation products as found for H_2O_2 without Cu^{2+}, i.e. formation of Met(O), but also showed formation of methionine sulfone (dioxide) Met$(O)_2$. In addition, the hydroxylated form also appeared in the MS, as indicated by the additional proton (i.e., O + 1 mu) and/or sodium mass (i.e., O + 23 mu).

Metal catalysed oxidation of Aβ (1–28). Aβ(1–28) was used as a model for Aβ(1–40) and Aβ(1–42) because it is more soluble, aggregates less, contains all the non-aliphatic amino acids (except Met (35)) and all the residues required for metal binding [55,22]. The addition of exogenous methionine has previously been shown to induce redox reactions [22] with Aβ(1–28) and Cu^{2+} and was, therefore, added (1 : 1 molar ratio) to some samples to test its role in MCO of the peptide. Also Cu^{+} and Cu^{2+} were used to identify the importance of the metal ion oxidation state to the conversion and formation of reactive oxygen species (ROS) in MCO and Fenton reaction (conversion of Cu^{+} to Cu^{2+}).

Fluorescence microplate reader analysis of MCO of Aβ (1–28). The fluorescence microplate results

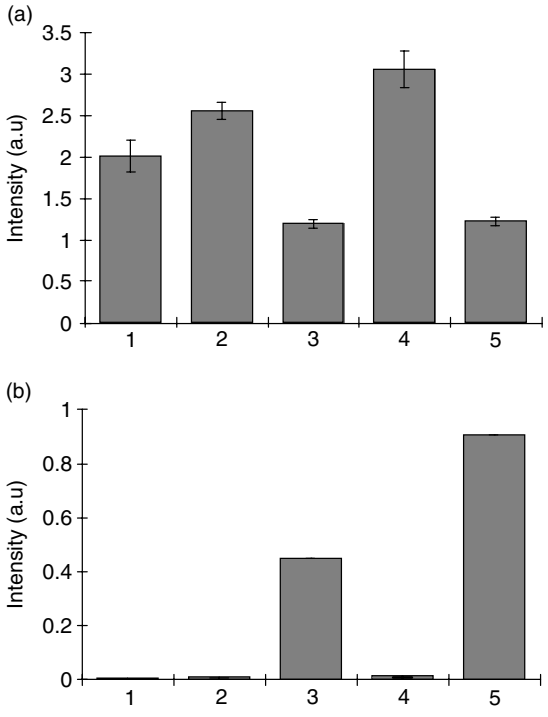

Figure 3 Fluorescence intensity of Aβ(1–28) and H_2O_2 after incubation with Cu^+ or Cu^{2+} and methionine. The samples are: 1 (H_2O_2 + Aβ(1–28)), 2 (H_2O_2 + Aβ(1–28) + Cu^+), 3 (H_2O_2 + Aβ(1–28) + Cu^+ + methionine), 4 (H_2O_2 + Aβ(1–28) + Cu^{2+}) and 5 (H_2O_2 + Aβ(1–28) + Cu^{2+} + methionine). (a) Plate reader filter (λ_{ex} 355 nm/λ_{em} 460 nm); (b) Plate reader filter (λ_{ex}485 nm, λ_{em} 538 nm).

for the peptide under different MCO reaction conditions are shown in Figure 3. Using the filter (λ_{ex} 355 nm/λ_{em} 460 nm) indicated a slight modification of Aβ(1–28) in the presence of H_2O_2 and Cu^+ or Cu^{2+} in comparison to that with H_2O_2 alone. Formation of more fluorescent products was primarily due to both histidine and phenylalanine modifications, as can be seen with the individual amino acids in Figure 2(a). Surprisingly, this modification was inhibited by the addition of methionine. However, the presence of methionine appeared to promote the modification of tyrosine (Tyr(10)) as indicated by the increase in the fluorescence intensity using the λ_{ex}485 nm/λ_{em}538 nm filter (see Figure 2(b) and Figure 3(b)). The methionine itself does not cause an increase in fluorescence as oxidized Met products do not fluoresce (Figure 2) but the addition of methionine results in an increase in oxidized tyrosine products. These results illustrate the importance of methionine in MCO, in facilitating the modification of Tyr(10) in Aβ(1–28). Unlike full-length Aβ, Aβ(1–28) does not contain a Met residue, yet MCO of Tyr(10) is enhanced by the addition of free methionine.

Gel electrophoresis-polyacrylamide gel electrophoresis. Gel electrophoresis was used to determine the aggregated state of Aβ(1–28) after MCO modification.

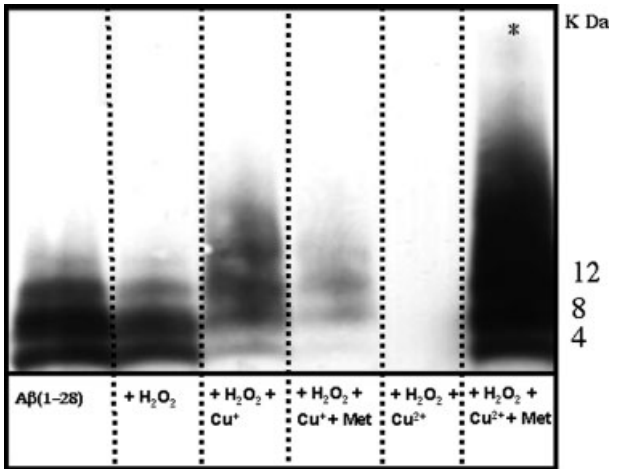

Figure 4 Western blot of Aβ(1–28) samples under different MCO conditions. The gel shows oligomerization and modification of the peptide, especially in presence of Cu^{2+} and methionine.

The results are summarized in Figure 4 and show that Aβ(1–28) in the presence of H_2O_2 and Cu ions aggregated more readily to form higher oligomers. The peptide with H_2O_2 and Cu^+ showed some aggregation but with H_2O_2 and Cu^{2+} showed little intensity on the gel, suggesting that modifications to the epitope of the WO2 antibody had occurred. The published epitope for this antibody is residues 5–8 (RHDS) [51]. In the presence of methionine, H_2O_2 and Cu^+ a similar loss of intensity was observed suggesting modifications to the antibody epitope, i.e. MCO of histidine. The addition of methionine to the H_2O_2 and Cu^{2+} reaction with Aβ(1–28) changed the profile of the reaction such that massive aggregation was observed and there was no evidence of any interference with the antibody epitope as immunoreactivity to the WO2 antibodies was still observed, i.e. MCO of tyrosine, which would not affect the RHDS residues. These differences in the effects of Cu^+ and Cu^{2+}, reflect the formation of different oxidation products of Aβ(1–28) with modification of histidines and formation of dityrosines [22], respectively.

DISCUSSION

Monitoring the modification of Aβ by MCO is difficult partly because a variety of residues can undergo oxidation and each can form multiple oxidation products (see Figure 1). In addition, binding of metal ions such as Cu^{2+} to a specific region may cause changes in the conformation of the peptide that promote aggregation [56] making analysis more difficult. Nevertheless, specific changes were observed, allowing conclusions to be made about the role of specific residues such as methionine and tyrosine in MCO of Aβ.

Under oxidative conditions the Cu^{2+} bound to the histidine residues of Aβ [22] induces modifications to a variety of different residues. NMR results showed that the imidazole ring is modified in the case of histidine, and the results of amino acid analysis and HPLC showed that phenylalanine formed Tyr, o-Tyr and m-Try following MCO (data not shown). However, the presence of methionine alters the profile of products from the MCO, possibly by reducing Cu^{2+} to Cu^{+} as shown by Curtain *et al.* [22], such that modification of tyrosine residues is promoted (see Figures 2–4). It was recently shown that when Cu^{2+} bound to Aβ(1–42) is reduced to Cu^{+} by a reductant this process is facilitated via a proton-coupled electron transfer (PCET) involving Tyr(10). As the electron passes from the reductant to the copper there is a concerted transfer of a proton to Tyr(10), resulting in the phenol oxygen being protonated and Tyr(10) being susceptible to oxidative modification [57,23]. Reactions involving PCET have become increasingly implicated in a range of biological systems including charge transport in DNA and enzymatic oxygen production [58].

Modifications to tyrosine residues include the formation of 3,4-dihydroxyphenylalanine (DOPA), dopamine, dopamine quinone, dityrosine (DT), isoDT and dihydroxyindol [57]. DT ($C_{ortho} - C_{ortho}$ linkage) and isoDT ($C_{ortho} - O$ linkage) are the most stable structures of these products [59,60]. Recent results from Atwood *et al.* [26] have shown that Aβ(1–42) in the presence of Cu/H_2O_2 forms dityrosine. These different tyrosine linkages lead to the formation of higher oligomers and facilitate peptide aggregation as shown in Figure 4. In addition, Schoeneich and Williams [61] studying the reaction of Aβ 1–16 with Cu/H_2O_2 by HPLC-MS/MS detected 2-oxo-histidine, which is generated from the oxidation of histidine residues of Aβ 1–16. Our current study sits between these two reports and is able to explain the apparent disparity in the results since Aβ(1–28) does not contain Met(35). Without methionine, histidines are modified and when methionine is added to Aβ(1–28), modified forms of tyrosine are produced, including dityrosine linkages. Our results support recent work which showed a modification in both histidine and tyrosine residue contents of Aβ peptides by MCO of $Cu^{2+} + H_2O_2$ [62]. In addition, the data support the suggestion that histidine and tyrosine residues are most vulnerable to metal mediated oxidative attack [62].

Oxidation of the sulfur atom of the methionine residue during MCO of Aβ peptides plays an important role in peptide modification as recently reported [25,2], where Met(35) is proposed to be the main source of ROS from Aβ [2]. It was previously shown [17] that oxidation of Met(35) leads to changes in Aβ structure and lipid interactions but that the peptide is still neurotoxic. Additionally Dong *et al.* have shown evidence that extensive side chain oxidation occurs in Met(35) of Aβ in the senile plaque of diseased brain [28].

The Western blot results revealed that the aggregation of Aβ(1–28) in the presence of copper ions induces modifications which prevent the antibody binding these forms (Figure 4) as seen for A$\beta(1–28) + H_2O_2 + Cu^{2+}$, which suggests that in this case residues 5–8 (RHDS) are modified. The presence of methionine inhibited these modifications, and instead promoted modification of Tyr10 (as shown by fluorescence plate reader results). The results support the hypothesis of methionine acting as a scavenger, which prevents the modification of amino acids such as histidine in Aβ but allows modification to tyrosine which is not part of the epitope of the WO2 antibody.

Methionine adducts modified by MCO were identified by ESI-MS. The modified forms of methionine were isolated as Fmoc amino acid derivatives (Figure 1(b)) and were not detected by direct RP-HPLC analysis as they are most likely to be of higher polarity than methionine. These products are summarized in Figure 5. Higher oxidized compounds of methionine may have an additional oxygen at the sulfur atom or to one of the carbon atoms, or addition of a perhydroxyl group (OOH) at the sulfur atom [63,64]. Sulfides oxidized to sulfoxides and then sulfones are known reactions for sulfur atoms in different compounds [65], but it has been shown by MS that copper ions catalysed the formation of these products in addition to the other active oxidation products. Some of these products have been identified recently in other oxidation reactions, e.g. oxohydroxylsulfur produced from the photochemical reaction of H_2O_2 with DMSO [66]. Furthermore, the hydroxyl radical formed from a Fenton-like reaction [67] is known to react with sulfoxide compounds to

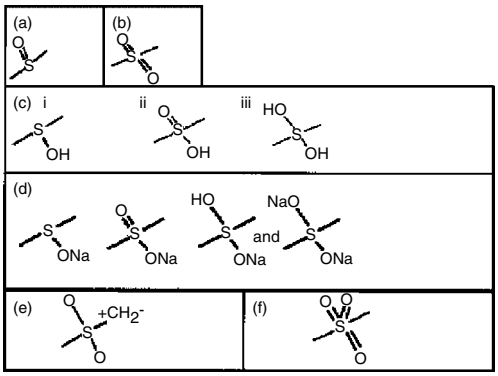

Figure 5 Different forms of sulfur in methionine residue as a result of metal catalysed oxidation (MCO). These include formation of: (a) known sulfoxides or oxo-sulfur Me(O), (b) sulfones or dioxosulfur (methionine sulfone) Met(O_2), and (c) other hydroxyl sulfur compounds, such as (i) hydroxyl sulfur, (ii) oxohydroxylsulfur, (iii) dihydroxyl and (d) their sodium salts. Low yields of higher oxidized forms could induce (e) methylide sulfoxonium ion, and (f) sulfonic acid derivative with additional oxygen atoms Met(O_3).

form oxohydroxylsulfur derivatives which decay with a 100 ns half-life to form sulfone derivatives [67], but our MS suggests that the oxohydroxylsulfur derivative of methionine is more stable. Similar products may be formed from the oxidation of Met(35) of Aβ and have implications to conformational changes in the peptide [68,69]. Further, oxidized products of MCO of Aβ may be detected by fluorescence assays and used to study structure-function relationships.

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REFERENCES

1. Varadarajan S, Yatin S, Aksenova M, Butterfield DA. Review: Alzheimer's amyloid β-peptide-associated free radical oxidative stress and neurotoxicity. *J. Struct. Biol.* 2000; **130**: 184–208.
2. Schoneich C. Redox processes of methionine relevant to β-amyloid oxidation and Alzheimer's disease [Review]. *Arch. Biochem. Biophys.* 2002; **397**: 370–376.
3. Masters CL, Beyreuther K. Science, medicine, and the future: Alzheimer's disease [Review]. *Br. Med. J.* 1998; **316**: 446–448.
4. Golde TE, Eckman CB, Younkin SG. Biochemical detection of Aβ isoforms: implications for pathogenesis, diagnosis, and treatment of Alzheimer's disease. *Biochim. Biophys. Acta* 2000; **1502**: 172–187.
5. Clippingdale AB, Wade JD, Barrow CJ. The amyloid-β peptide and its role in Alzheimer's disease. *J. Pept. Sci.* 2001; **7**: 227–249.
6. Bush AI. The metallobiology of Alzheimer's disease. *Trends Neurosci.* 2003; **26**: 207–214.
7. Campbell A, Smith MA, Sayre LM, Bondy SC, Perry G. Mechanisms by which metals promote events connected to neurodegenerative diseases. *Brain Res. Bull.* 2001; **55**: 125–132.
8. Atwood CS, Huang X, Moir RD, Tanzi RE, Bush AI. Role of free radicals and metal ions in the pathogenesis of Alzheimer's disease. *Met. Ions Biol. Syst.* 1999; **36**: 309–364.
9. Rao KSJ, Rao RV, Shanmugavelu P, Menon RB. Trace elements in Alzheimer's disease brain: a new hypothesis. *Alzheimer's Rep.* 1999; **2**: 241–246.
10. Christen Y. Oxidative stress and Alzheimer's disease(1). *Am. J. Clin. Nutr.* 2000; **71**: 621S–629S.
11. Muench G, Thome J, Foley P, Schinzel R, Riederer P. Advanced glycation endproducts in aging and Alzheimer's disease. *Brain Res. Rev.* 1997; **23**: 134–143.
12. Vatassery GT, Bauer T, Dysken M. High doses of vitamin E in the treatment of disorders of the central nervous system in the aged [Review]. *Am. J. Clin. Nutr.* 1999; **70**: 793–801.
13. Cherny RA, Barnham KJ, Lynch T, Volitakis I, Li Q-X, McLean CA, Multhaup G, Beyreuther K, Tanzi RE, Masters CL, Bush AI. Chelation and intercalation: complementary properties in a compound for the treatment of Alzheimer's disease. *J. Struct. Biol.* 2000; **130**: 209–216.
14. Cherny RA, Atwood CS, Xilinas ME, Gray DN, Jones WD, McLean CA, Barnham KJ, Volitakis I, Fraser FW, Kim Y-S, Huang XD, Goldstein LE, Moir RD, Lim J, Beyreuther K, Zheng H, Tanzi RE, Masters CL, Bush AI. Treatment with a copper-zinc chelator markedly and rapidly inhibits β-amyloid accumulation in Alzheimer's disease transgenic mice. *Neuron* 2001; **30**: 665–676.
15. Ritchie CW, Bush AI, Mackinnon A, Macfarlane S, Mastwyk M, MacGregor L, Kiers L, Cherny R, Li QX, Tammer A, Carrington D, Mavros C, Volitakis I, Xilinas M, Ames D, Davis S, Beyreuther K, Tanzi RE, Masters CL. Metal-protein attenuation with iodochlorhydroxyquin (clioquinol) targeting Aβ amyloid deposition and toxicity in Alzheimer disease: a pilot phase 2 clinical trial. *Arch. Neurol.* 2003; **60**: 1685–1691.
16. Behl C, Davis JB, Lesley R, Schubert D. Hydrogen peroxide mediates amyloid β protein toxicity. *Cell* 1994; **77**: 817–827.
17. Barnham KJ, Ciccotosto GD, Tickler AK, Ali FE, Smith DG, Williamson NA, Lam Y-H, Carrington D, Tew D, Kocak G, Volitakis I, Separovic F, Barrows CJ, Wade JD, Masters CL, Cherny RA, Curtain CC, Bush AI, Cappai R. Neurotoxic, Redox-competent Alzheimer's β-amyloid is released from lipid membrane by methionine oxidation. *J. Biol. Chem.* 2003; **278**: 42 959–42 965.
18. Huang X, Atwood CS, Hartshorn MA, Multhaup G, Goldstein LE, Scarpa RC, Cuajungco MP, Gray DN, Lim J, Moir RD, Tanzi RE, Bush AI. The Aβ peptide of Alzheimer's disease directly produces hydrogen peroxide through metal ion reduction. *Biochemistry* 1999; **38**: 7609–7616.
19. Huang X, Cuajungco MP, Atwood CS, Hartshorn MA, Tyndall JDA, Hanson GR, Stokes KC, Leopold M, Multhaup G, Goldstein LE, Scarpa RC, Saunders AJ, Lim J, Moir RD, Glabe C, Bowden EF, Masters CL, Fairlie DP, Tanzi RE, Bush AI. Cu(II) potentiation of Alzheimer Aβ neurotoxicity — correlation with cell-free hydrogen peroxide production and metal reduction. *J. Biol. Chem.* 1999; **274**: 37 111–37 116.
20. Bush AI. Metals and neuroscience. *Curr. Opin. Chem. Biol.* 2000; **4**: 184–191.
21. Huang X, Cuajungco MP, Atwood CS, Moir RD, Tanzi RE, Bush AI. Alzheimer's disease, β-amyloid protein and zinc. *J. Nutr.* 2000; **130**: 1488S–1492S.
22. Curtain C, Ali F, Volitakis I, Cherny R, Norton R, Beyreuther K, Barrow C, Masters C, Bush A, Barnham K. Alzheimer's disease amyloid-β binds copper and zinc to generate an allosterically ordered membrane-penetrating structure containing superoxide dismutase-like subunits. *J. Biol. Chem.* 2001; **276**: 20 466–20 473.
23. Barnham KJ, Haeffner F, Ciccotosto GD, Curtain CC, Tew D, Mavros C, Beyreuther K, Carrington D, Masters CL, Cherny RA, Cappai R, Bush AI. Tyrosine gated electron transfer is key to the toxicity mechanism of Alzheimer's disease β-amyloid. *FASEB J.* 2004, doi:10.1096/fj.04-1890fje.
24. Lynch T, Cherny RA, Bush AI. Oxidative processes in Alzheimer's disease: the role of Aβ-metal interactions. *Exp. Gerontol.* 2000; **35**: 445–451.
25. Nishino S, Nishida Y. Oxygenation of amyloid β-peptide (1–40) by copper(II) complex and hydrogen peroxide system. *Inorg. Chem. Commun.* 2001; **4**: 86–89.
26. Atwood CS, Perry G, Zeng H, Kato Y, Jones WD, Ling KQ, Huang X, Moir RD, Wang D, Sayre LM, Smith MA, Chen SG, Bush AI. Copper mediates dityrosine cross-linking of Alzheimer's amyloid-β. *Biochemistry* 2004; **43**: 560–568.
27. Butterfield DA, Lauderback CM. Lipid peroxidation and protein oxidation in Alzheimer's disease brain: potential causes and consequences involving amyloid β-peptide-associated free radical oxidative stress [Review]. *Free Radical Biol. Med.* 2002; **32**: 1050–1060.
28. Dong J, Atwood CS, Anderson VE, Siedlak SL, Smith MA, Perry G, Carey PR. Metal binding and oxidation of amyloid-β within isolated senile plaque cores: Raman microscopic evidence. *Biochemistry* 2002; **42**: 2768–2773.
29. Subramaniam R, Howard BJ, Hensley K, Aksenova M, Carney JM, Butterfield DA. β-amyloid (32–35) toxicity to biomolecules: implications to Alzheimer's disease. *Alzheimer's Res.* 1995; **1**: 141–144.

30. Varadarajan S, LaFontaine M, Dass C, Butterfield DA. Alzheimer's β-amyloid peptide associated free radicals: Mechanistic investigations. *Proceedings, 216th ACS National Meeting, Boston, August 23–27*, Abstracts of Papers of the American Chemical Society 216: U558 043-Toxi; Part 1, 1998, Amer. Chemical Soc., Washington DC, USA.
31. Varadarajan S, Yatin S, Butterfield D. Alzheimer's amyloid β-peptide (1–42) fibrils are not always neurotoxic. *Alzheimer's Rep.* 2000; **3**: 71–76.
32. Yatin S, Varadarajan S. *In vitro* and *in vivo* oxidative stress associated with Alzheimer's amyloid β-peptide (1–42). *Neurobiol. Aging* 1999; **20**: 325–330.
33. Kanski J, Varadarajan S, Aksenova M, Butterfield D. Role of glycine-33 and methionine-35 in Alzheimer's amyloid β-peptide 1–42 associated oxidative stress and neurotoxicity. *Biochim. Biophys. Acta* 2002; **1586**: 190–198.
34. Toennies G, Kolb JJ. Methionine studies. VI. DL-Methionine sulfone. *J. Biol. Chem.* 1941; **140**: 131–134.
35. Amici A, Levine RL, Tsai L, Stadtman ER. Conversion of amino acid residues in proteins and amino acid homopolymers to carbonyl derivatives by metal-catalyzed oxidation reactions. *J. Biol. Chem.* 1989; **264**: 3341–3346.
36. Christensen BW, Kjaer A. Absolute configuration of methionine sulfoxide. *Chem. Commun.* 1965; 225–226.
37. Marastoni M, Salvadori S, Balboni G, Borea PA, Marzola G, Tomatis R. Synthesis and activity profiles of new dermorphin-(1–4) peptide analogs. *J. Med. Chem.* 1987; **30**: 1538–1542.
38. Steffek RP, Thomas MJ. Hydrogen peroxide modification of human oxyhemoglobin. *Free Radical Res. Commun.* 1991; **12–13**: 489–497.
39. Boonvisut S, Aksnes A, Njaa LR. Oxidation of methionine. Effects of hydrogen peroxide alone and in combination with iodide and selenite. *Food Chem.* 1982; **9**: 183–194.
40. Cuq JL. Comparative study of the oxidation of methionine and tryptophan by hydrogen peroxide. *Sci. Aliments* 1992; **12**: 315–337.
41. Chao CC, Ma YS, Stadtman ER. Modification of protein surface hydrophobicity and methionine oxidation by oxidative systems. *Proc. Natl Acad. Sci. USA* 1997; **94**: 2969–2974.
42. Watson AA, Fairlie DP, Craik DJ. Solution structure of methionine-oxidized amyloid β-peptide (1–40). Does oxidation affect conformational switching? *Biochemistry* 1998; **37**: 12 700–12 706.
43. Palmblad M, Westlind-Danielsson A, Bergquist J. Oxidation of methionine 35 attenuates formation of amyloid β-peptide 1–40 oligomers. *J. Biol. Chem.* 2002; **277**: 19 506–19 510.
44. Bitan G, Tarus B, Vollers SS, Lashuel HA, Condron MM, Straub JE, Teplow DB. A molecular switch in amyloid assembly: Met35 and amyloid β-protein oligomerization. *J. Am. Chem. Soc.* 2003; **125**: 15 359–15 365.
45. Such L, O'Connor J-E, Saez GT, Gil F, Beltran JF, Moya A, Alberola A. Flow cytometric analysis of peroxidative activity in granulocytes from coronary and peripheral blood in acute myocardial ischemia and reperfusion in dogs: protective effect of methionine. *Cytometry* 1999; **37**: 140–146.
46. He W, Barrow CJ. The Aβ 3-pyroglutamyl and 11-pyroglutamyl peptides found in senile plaque have greater β-sheet forming and aggregation propensities *in vitro* than full-length Aβ. *Biochemistry* 1999; **38**: 10 871–10 877.
47. Verweij H, Dubbelman TM, Van Steveninck J. Photodynamic protein cross-linking. *Biochim. Biophys. Acta* 1981; **647**: 87–94.
48. Haynes PA, Sheumack D, Greig LG, Kibby J, Redmond JW. Applications of automated amino acid analysis using 9-fluorenylmethyl chloroformate. *J. Chromatogr.* 1991; **588**: 107–114.
49. Haynes PA, Sheumack D, Kibby J, Redmond JW. Amino acid analysis using derivatization with 9-fluorenylmethyl chloroformate and reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 1991; **540**: 177–185.
50. Cherny RA, Legg JT, McLean CA, Fairlie DP, Huang X, Atwood CS, Beyreuther K, Tanzi RE, Masters CL, Bush AI. Aqueous dissolution of Alzheimer's disease Aβ amyloid deposits by biometal depletion. *J. Biol. Chem.* 1999; **274**: 23 223–23 228.
51. Ida N, Hartmann T, Pantel J, Schroder J, Zerfass R, Forstl H, Sandbrink R, Masters CL, Beyreuther K. Analysis of heterogeneous A4 peptides in human cerebrospinal fluid and blood by a newly developed sensitive Western blot assay. *J. Biol. Chem.* 1996; **271**: 22 908–22 914.
52. Gatlin CL, Turecek F, Vaisar T. Gas-phase complexes of amino acids with Cu(II) and diimine ligands. Part I. Aliphatic and aromatic amino acids. *J. Mass Spectrom.* 1995; **30**: 1605–1616.
53. Gatlin CL, Turecek F, Vaisar T. Gas-phase complexes of amino acids with Cu(II) and diimine ligands. Part II. Amino acids with O, N and S functional groups in the side-chain. *J. Mass Spectrom.* 1995; **30**: 1617–1627.
54. Wen D, Yalcin T, Harrison AG. Fragmentation reactions of Cu^+-cationated α-amino acids. *Rapid Commun. Mass Spectrom.* 1995; **9**: 1155–1157.
55. Miura T, Suzuki K, Kohata N, Takeuchi H. Metal binding modes of Alzheimer's amyloid β-peptide in insoluble aggregates and soluble complexes. *Biochemistry* 2000; **39**: 7024–7031.
56. Suzuki K, Miura T, Takeuchi H. Inhibitory effect of copper(II) on zinc(II)-induced aggregation of amyloid β-peptide. *Biochem. Biophys. Res. Commun.* 2001; **285**: 991–996.
57. Ali FE, Barnham KJ, Barrow CJ, Separovic F. Copper catalysed oxidation of amino acids and Alzheimer's disease. *Lett. Peptide Sci.* (in press) 2004.
58. Cukier RI, Nocera DG. Proton-coupled electron transfer. *Annu. Rev. Phys. Chem.* 1998; **49**: 337–369.
59. Eickhoff H, Jung G, Rieker A. Oxidative phenol coupling — tyrosine dimers and libraries containing tyrosyl peptide dimers. *Tetrahedron* 2001; **57**: 353–364.
60. Shamovsky IL, Riopelle RJ, Ross GM. Ab initio studies on the mechanism of tyrosine coupling. *J. Phys. Chem. A* 2001; **105**: 1061–1070.
61. Schoeneich C, Williams TD. Cu(II)-Catalyzed oxidation of β-amyloid peptide targets His13 and His14 over His6: Detection of 2-oxo-histidine by HPLC-MS/MS. *Chem. Res. Toxicol.* 2002; **15**: 717–722.
62. Atwood CS, Huang X, Khatri A, Scarpa RC, Kim Y-S, Moir RD, Tanzi RE, Roher AE, Bush AI. Copper catalyzed oxidation of Alzheimer Aβ. *Cell Mol. Biol.* 2000; **46**: 777–783.
63. Jensen F, Greer A, Clennan EL. Reaction of organic sulfides with singlet oxygen. A revised mechanism. *J. Am. Chem. Soc.* 1998; **120**: 4439–4449.
64. McKee ML. A theoretical study of unimolecular reactions of dimethyl persulfoxide. *J. Am. Chem. Soc.* 1998; **120**: 3963–3969.
65. Angyan JG, Bonnelle C, Daudel R, Kucsman A, Csizmadia IG. The use of theoretical indexes for the characterization of sulfur-oxygen linkage multiplicity. *Theochemistry* 1988; **42**: 273–287.
66. Woodward JR, Lin T-S, Sakaguchi Y, Hayashi H. Detection of transient intermediates in the photochemical reaction of hydrogen peroxide with dimethyl sulfoxide by time-resolved EPR techniques. *J. Phys. Chem. A* 2000; **104**: 557–561.
67. Veltwisch D, Janata E, Asmus KD. Primary processes in the reaction of hydroxyl radicals with sulfoxides. *J. Chem. Soc. Perkin Trans. 2* 1980; 146–153.
68. Hou L, Kang I, Marchant RE, Zagorski MG. Methionine 35 oxidation reduces fibril assembly of the amyloid Aβ-(1–42) peptide of Alzheimer's disease. *J. Biol. Chem.* 2002; **277**: 40 173–40 176.
69. Hou L, Shao H, Zhang Y, Li H, Menon NK, Neuhaus EB, Brewer JM, Byeon IJ, Ray DG, Vitek MP, Iwashita T, Makula RA, Przbyla AB, Zagorski MG. Solution NMR studies of the Aβ(1–40) and Aβ(1–42) peptides establish that the Met35 oxidation state affects the mechanism of amyloid formation. *J. Am. Chem. Soc.* 2004; **126**: 1992–2005.

