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Specific reaction of Met 35 in amyloid beta peptide with hypochlorous acid

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Abstract
The reaction of the amyloid beta peptide (Aβ) with hypochlorous acid and hydroxyl radicals was analysed by spectrophotometry and mass spectrometry. N-acetylmethionine, Aβ25-35 and Aβ1-42 reacted rapidly with hypochlorous acid. The relative reaction rates of N-acetylmethionine and Aβ with hypochlorous acid was in the order N-acetylmethionine > Aβ25-35 >Aβ1-42. While the reaction of Aβ25-35 in the presence of a slight excess of hypochlorous acid resulted in complete conversion of Met35 to Met35 sulphoxide, Aβ1-42 required more than a 4-fold excess of hypochlorous acid for complete conversion of Met35. Identical products were obtained when Aβ25-35 and Aβ1-42 were treated with a hypochlorous acid generating system. Conversion of Met35 to Met35 sulphoxide in Aβ abolished the aggregation of Aβ25-35. Reaction of Aβ with hydroxyl radicals resulted in limited conversion of Met35 to Met35 sulphoxide. The specific reaction of Met35 in Aβ with hypochlorous acid to form Met35 sulphoxide has been analysed.

Keywords: *Amyloid-beta peptide, hypochlorous acid, Alzheimer's disease, antioxidant, oxidative stress.*

Abbreviations: *Aβ, amyloid beta peptide; AD, Alzheimer's disease; MPO, myeloperoxidase; MCD, monochlorodimedone; N-acetyl Met, N-acetylmethionine; SPs, senile plaques; ThT, Thioflavine T.*

Introduction

Accumulation of the amyloid beta peptide (Aβ) in the form of senile plaques (SPs) is a pathological hallmark of Alzheimer's disease (AD) [1]. Although the progression of AD is linked to inflammatory and oxidative processes [1,2], the biochemical mechanism underlying AD is uncertain. Several markers of oxidative stress such as hydroxynonenal (HNE), Cl-tyrosine, nitrotyrosine and 8-OH deoxyguanosine are increased in SPs during disease progression [3–6]; oxidative stress is an early event in AD [3]. SPs consist of Aβ1-42 and Aβ1-41 [1] and contain high concentrations of iron, copper and zinc [7]. Transition metal-induced oxidative stress such as hydroxyl radical generation is well documented [3,8–10]. On the other hand, it is widely believed that Aβ is responsible for the neurodegeneration found in AD [11–13]. Aβ binds an equimolar amount of copper with high affinity; the complex can produce H_2O_2, which leads to oxidative damage in cells [14,15]. Although the Cu(II)-Aβ complex is more toxic to neurons in culture than Aβ alone, the complex is less toxic than free copper [16]. If the

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Cu(II)-Aβ complex is incubated in the presence of H_2O_2 or ascorbate, the copper binding site is oxidatively modified [17,18]. In contrast, several lines of evidence have shown that Aβ serves as an antioxidant and protects neuronal cells from oxidative stress [19–24]. Recent findings show that myeloperoxidase (MPO) and microglia are co-localized with SPs [6]. In addition to phagocytosis, microglia secrete proteases that degrade Aβ [25]. In addition, microglia are activated by Aβ *in vivo* and generate H_2O_2 [26]. The interaction of Aβ with microglia is reported to stimulate the inflammatory process and may contribute to neuronal damage [27]. Met35 in Aβ is sensitive to oxidation and the extensive conversion of Met35 to Met sulphoxide has been observed in SP cores [28]. In this study, we focused on the reaction of Aβ with MPO-derived oxidants including hypervalent MPO and hypochlorous acid. We demonstrated the selective reaction of Met 35 in either Aβ25-35 or Aβ1-42 with hypochlorous acid, forming Met35 sulphoxide.

Materials and methods

Materials

Aβ25-35 and Aβ1-42 were purchased from the Peptide Institute Inc. (Osaka, Japan). Myeloperoxidase (MPO) from human leucocytes, N-acetylmethionine (N-acetyl Met) and monochlorodimedone (MCD) were purchased from Sigma-Aldrich Japan (Tokyo). Sodium hypochlorite solution (NaOCl) and luminol were obtained from Wako Pure Chemical Industry (Osaka, Japan). Thioflavin T (ThT) was obtained from Nacalai Tesque (Kyoto, Japan). Reactions were carried out at 25°C in 25 mM potassium phosphate buffer (pH 7.4) unless otherwise stated. Spectrophotometric and chemiluminescence of luminol were analysed using a Shimadzu MPS-2000 spectrophotometer and a luminometer (Aloka BLR-301, Japan), respectively.

Aggregation of Aβ

ThT shows strong fluorescence when it is incorporated in amyloid fibrils [29,30]. Sample solutions containing 100 μM Aβ25-35 or Aβ1-42 were incubated at 37°C for 48 or 100 h in 10 mM phosphate buffer (pH 7.4). The composition of the fluorescence measurement solution was 50 mM Gly-NaOH (pH 9.0), 5 μM ThT and 1 μM Aβ. The fluorescence spectra (excitation at 450 nm) was obtained with a Shimadzu spectrofluorometer RF-5300PC. Each spectrum is the mean of three experiments.

Reactions of Aβ with HOCl

The concentration of HOCl was determined by the reaction of HOCl with MCD. The concentration of HOCl in the mixtures was calculated from the molar absorption coefficient of MCD at 290 nm [31]. A HOCl solution was added last to reaction mixtures containing MCD and Aβ with continuous stirring. Based on the rate constant for the reaction of HOCl with MCD [32,33], the rate constants for the reaction of HOCl with N-acetyl Met, Aβ25-35 and Aβ1-42 were estimated from competition kinetics [31,34].

Analysis of reaction products of Aβ with HOCl or hydroxyl radicals

The reaction mixtures containing 100 μM Aβ25-35 or Aβ1-42 were treated with various concentrations of HOCl for 24 or 48 h or with hydroxyl radicals (H_2O_2 and $FeCl_2$) [24]. Aliquots of the reaction mixture were analysed by MALDI-TOF spectrometry with a Bruker Daltonics Reflex III. To confirm the formation of Met sulphoxide in Aβ25-35, HOCl-treated Aβ25-35 was sequenced by post-source decay analysis [35].

Results

We analysed the effect of HOCl on the aggregation of Aβ25-35 (Figure 1). Aggregation was assessed by the change in the fluorescence of ThT after incubation with Aβ25-35 for 48 h. Addition of an equimolar amount of HOCl to a solution containing Aβ25-35 resulted in the reduction of fluorescence by 80% (Figure 1B). Although there are several candidates for the reaction site of Aβ25-35 with HOCl, a specific reaction with the Met35 residue was suspected [32,33]. Relative reaction rates for the reaction of

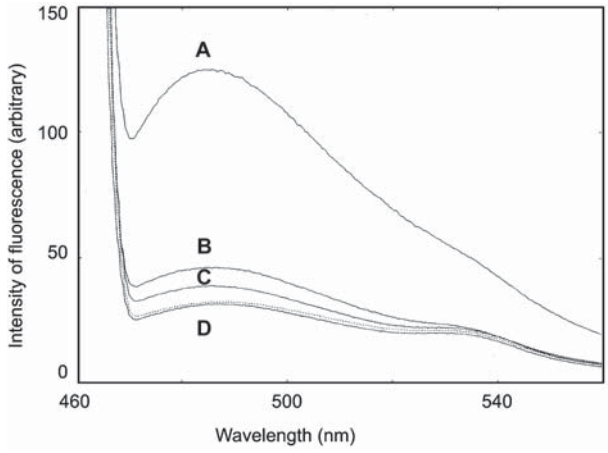

Figure 1. Effect of HOCl upon the aggregation of Aβ25-35. Reaction mixtures contained 100 μM Aβ25-35 in 25 mM phosphate buffer were incubated for 48 h in the (A) absence or presence of (B) 100, (C) 200 or (D) 300 μM HOCl. Aggregation of Aβ25-35 was detected by the increase in fluorescence of ThT. When Aβ25-35 was treated with HOCl-generating system (100 μM H_2O_2, 10 mM KCl and 0.2 units MPO at pH 6.0), the reproducibility of the results was poor. Measurement was as described in Materials and methods.

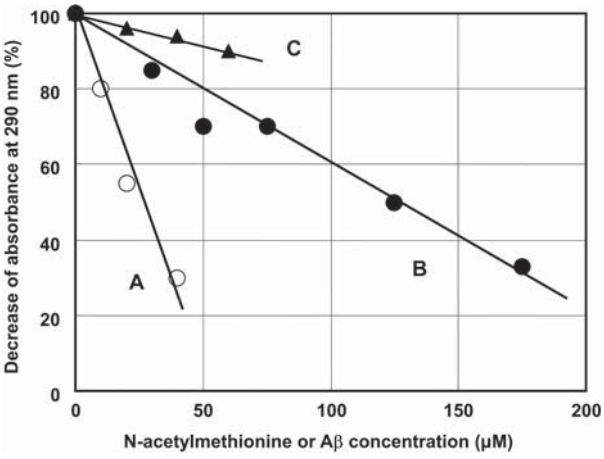

Figure 2. Effect of Aβ concentrations upon the reaction of MCD with HOCl. Reaction mixtures contained 50 μM MCD and 45 μM HOCl. Reactions were carried out in the absence or presence of varied concentrations of (A) N-acetylmethionine, (B) Aβ25-35 or (C) Aβ1-42 and initiated by the addition of HOCl.

N-acetyl Met, Aβ25-35 and Aβ1-42 with HOCl were estimated based on the reaction of MCD with HOCl [32]. Aβ25-35 reacted rapidly with HOCl, although the reaction rate was 5-times slower than that of N-acetyl Met with HOCl (Figure 2). The reaction rates were in the order of N-acetyl Met > Aβ25-35 > Aβ1-42 (Table I).

Chemiluminescence (CL) of luminol was used for the detection of HOCl generated by myeloperoxidase system ($MPO/H_2O_2/Cl^-$). We previously elucidated the one- and two-electron oxidation mechanism of luminol by peroxidase systems leading to chemiluminescence [36]. CL of luminol by the MPO/H_2O_2 system (Figure 3A) was increased 15-fold in the presence of 10 mM Cl^- (Figure 3B). When CL of luminol by the $MPO/H_2O_2/Cl^-$ system was observed in the presence of the same concentration of Aβ25-35 or Aβ1-42, Aβ25-35 (Figure 3C) was more inhibitory than Aβ1-42 (Figure 3D). This result is compatible with the estimated rate constants (Table I).

Reaction of HOCl with biomolecules can result in the modification of amino acid side chains [32,33,37]. The results, to date, investigating the oxidation of Met35 indicate a role in the aggregation of Aβ and damage to cells; oxidation of Met35 in Aβ retarded aggregation and reduced damage to cells [38–41]. Figure 4A shows the mass spectrum of Aβ25-35,

Table I. Rate constant for the reaction of Aβ with hypochlorous acid.

Aβ	Rate constant ($M^{-1}s^{-1}$)
N-Acetyl Met	7×10^6
Aβ25-35	1.4×10^6
Aβ1-42	1×10^6
Aβ1-12	2×10^5

Experimental conditions and calculation procedure for the rate constant are described in the Materials and methods section.

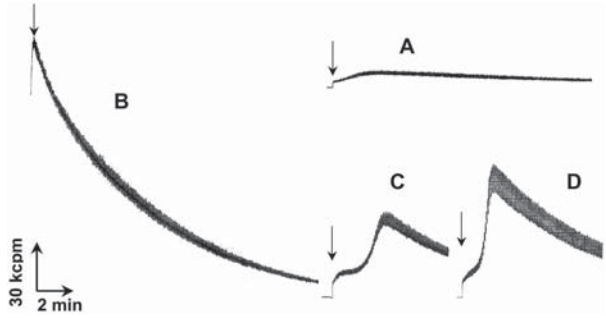

Figure 3. Inhibition of Aβ25-35 and Aβ1-42 upon the chemiluminescence of luminol by MPO system ($MPO/H_2O_2/Cl^-$). Reaction mixtures contained 100 μM H_2O_2, 10 mM KCl, 0.02 units MPO and 100 μM luminol. Reactions were carried out in the (B) absence or presence of (C) 100 μM Aβ25-35 or (D) Aβ1-42, and initiated by adding H_2O_2. Chemiluminescence of luminol by MPO was shown in the absence of KCl and Aβ (A).

which indicates a major peak assigned as Aβ25-35 ($m/z=1060$) and a minor peak ($m/z=1076$). Addition of one molar equivalent of HOCl to Aβ25-35 changes the spectrum (Figure 4B). The resulting spectrum indicates addition of one oxygen atom addition to Aβ25-35 along with a trace amount of fragmentation. The extent of conversion to the new peak mirrored the loss of ThT fluorescence. When Aβ25-35 was treated with an HOCl generating system ($MPO/H_2O_2/Cl^-$), an identical peak was observed (Figure 4C). Figure 5 displays the values of b- and y-ions associated with untreated Aβ25-35 (Figure 5A) and HOCl-treated Aβ25-35 (Figure 5B). A 16-Da increase was assigned to Met35 after HOCl-treatment. The data indicate a one-oxygen atom addition to Met35 in Aβ25-35. Full length Aβ was treated with either an equimolar (Figure 6B) or 2.0-fold (Figure 6C) molar excess of HOCl. A substantial new peak ($m/z=4527$)

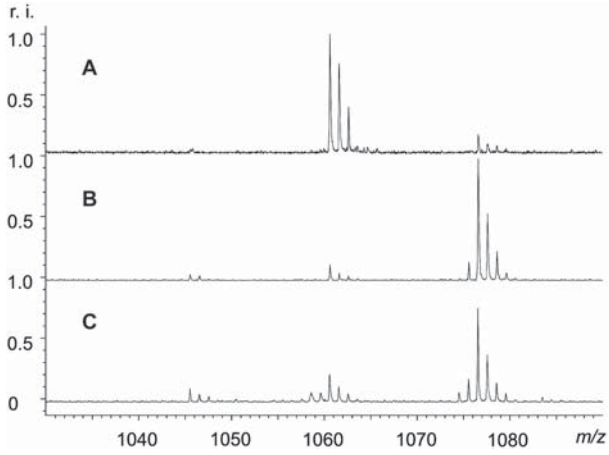

Figure 4. Mass spectra of Aβ25-35 in the (A) absence or presence of (B) HOCl or (C) MPO system ($MPO/H_2O_2/Cl^-$). Reaction mixtures containing 100 μM Aβ25-35 were incubated for 24 h in the (A) absence or presence of (B) 100 μM HOCl and (C) MPO system (100 μM H_2O_2, 10 mM KCl and 0.2 units MPO). Aliquots of the reaction mixture were analysed by MALDI-TOF spectrometry.

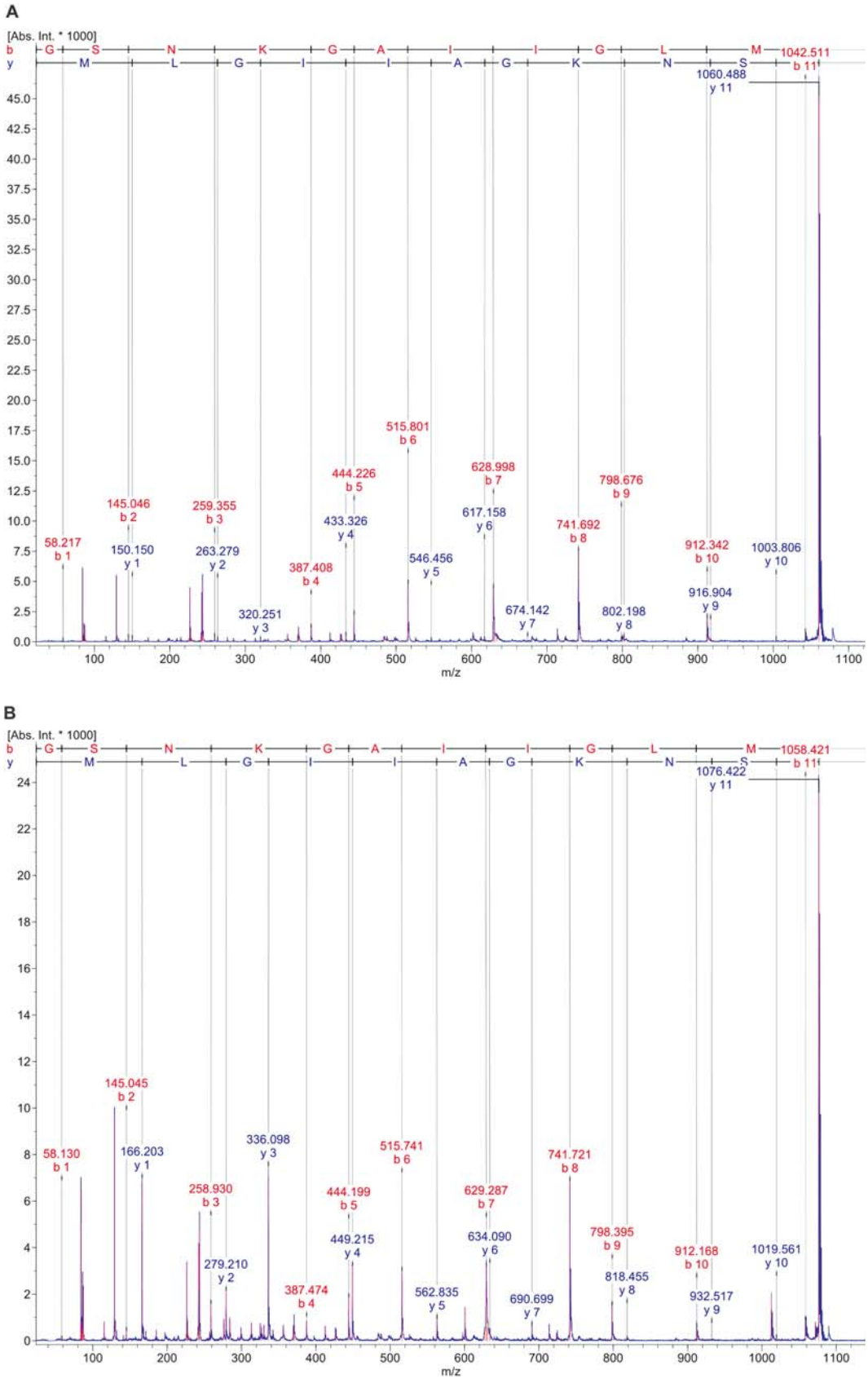

Figure 5. Spectral analysis of (A) Aβ25-35 and (B) Aβ25-35 treated with HOCl. Reaction conditions are as described in Figure 4.

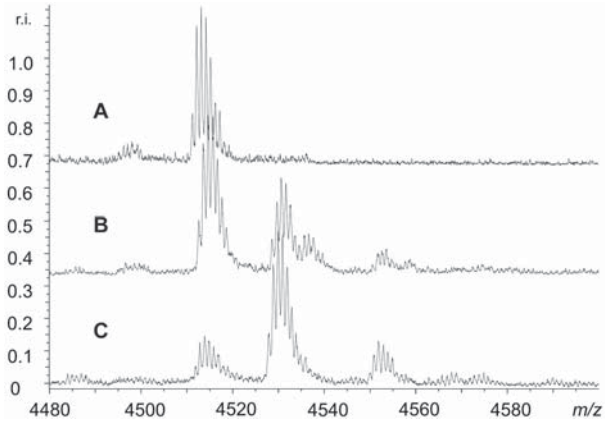

Figure 6. Mass spectra of Aβ1-42 after reaction with HOCl. The reaction mixtures containing 100 μM Aβ1-42 were incubated for 48 h in the (A) absence or presence of (B)100 or (C) 200 μM HOCl. Minor peaks at m/z =4533 and 4549 were sodium adduct of Aβ1-42 and of a new peak, respectively (B, C).

appeared, although more than 50% of the Aβ1-42 was unchanged (Figure 6B). A complete loss and fragmentation of Aβ1-42 (m/z=4511) were observed by the addition of 4-fold excess HOCl. Formation of chlorotyrosine is a marker of oxidative stress produced by neutrophils [6]. It is important to note that no chlorinated Aβ1-42 was observed under these conditions.

A high concentration of iron is present in SPs, thus iron-catalysed oxidation of Aβ and iron-catalysed Haber-Weiss reaction (hydroxyl radical generation) would occur in SPs. Aβ25-35, Aβ1-40 and Aβ1-42 were incubated in the presence of ferric iron, yet no detectable Met sulphoxide was observed (Figure 7A). The results indicate that iron-catalysed oxidation of Met35 in Aβ does not readily proceed. To confirm the reaction of Met35 in Aβ with hydroxyl radicals, Aβ25-35 and Aβ1-42 were treated with hydroxyl radicals (H_2O_2 and $FeCl_2$) (Figure 7B). The resulting spectrum shows that more than 90% of the Aβ1-42 was

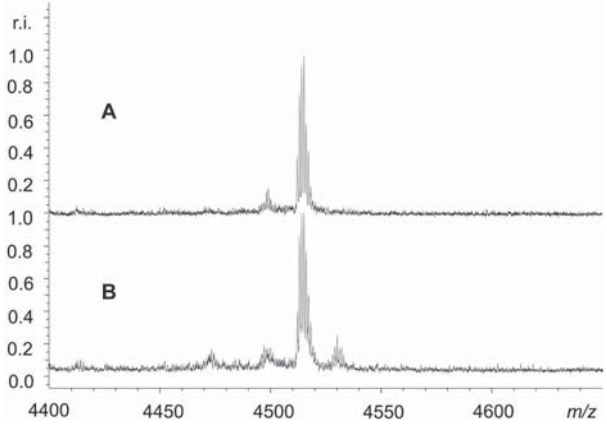

Figure 7. Mass spectra of Aβ1-42 after reaction with hydroxyl radicals. The reaction mixtures containing 100 μM Aβ1-42 were incubated for 48 h in the presence of (A) 200μM Fe^{3+} or (B) 200 μM Fe^{2+} +200 μM H_2O_2.

unchanged, only a slight conversion producing an oxidized peak was observed. Hydroxyl radicals react with all biomolecules including amino acids so rapidly that no selective reaction of amino acid side chain in Aβ1-42 should be expected.

Discussion

Met35 in Aβ1-40 and Aβ1-42 plays a role in the cellular toxicity of Aβ [41]; Aβ shows cell toxicity during the course of aggregation [1,38–42]. Aβ, present as a monomer, polymerizes to form fibrils through the transient generation of oligomers. These oligomers of Aβ interact with the membranes of cells and mitochondria, which leads to dysfunction of membrane permeability and of mitochondrial respiratory control [40,41]. The C-terminus of Aβ1-42 is hydrophobic and is responsible for aggregation; the peptide fragment Aβ25-35 is used as a model of aggregation, since aggregation of Aβ25-35 is more reproducible than Aβ1-42 [40]. Oxidation of Met35 to a sulphoxide results in reduced cell toxicity [41]. After reaction, oxidized Aβ25-35 exhibits reduced aggregation. In the present study, oxidation of Met35 through the reaction with HOCl reduces Aβ aggregation.

Reaction of HOCl with biomolecules has been reported in detail [32,33,43,44]. HOCl is an electrophilic oxidant and reacts rapidly with Met and Cys side chains in proteins. The rate constant for the reaction of N-acetyl Met and Aβ with HOCl are in the order N-acetyl Met > Aβ25-35>Aβ1-42 (Table I). Met35 in Aβ25-35 reacts more rapidly with HOCl than Met35 in Aβ1-42. Figure 5B indicates that one oxygen atom is added to Met35 in Aβ25-35 after the incubation of Aβ25-35 with equimolar HOCl. No further oxygen addition was observed after addition of 2–5-fold greater excess of HOCl. It has been reported that a free sulphydryl group consumes 3 mole equivalents of HOCl and reaction of Met with HOCl produces Met sulphoxide [44]. One-electron oxidation of Aβ1-40 with azide radicals has been reported [45]. Met35 in Aβ1-40 is a target of azide radicals and the reaction leads to a formation of Met sulphoxide, while no oxidation is observed for the reaction of the reverse peptide, Aβ40-1, with azide radicals. Reaction of a primary amine with HOCl forms chloramine, which then decomposes [37]. Chlorine transfer from chloramine to Met or Cys has been reported [44]. When Aβ1-12 was treated with one molar equivalent of HOCl, a 34-Da increase and a 44-Da loss of Aβ1-12 were observed (data not shown). The results suggest the chlorination of Asp1 and Tyr10. A 44-Da loss of Aβ1-12 was explained by the chlorination of terminal amine (Asp1), which was followed by a decarboxylation of the residue. Contrary to this, the current results (Figures 4 and 6) indicate that reaction of Aβ25-35 and Aβ1-42 with

Table II. Effect of HOCl or hydroxyl radicals upon the formation of methionine sulphoxide.

	Oxidant	
Aβ	HOCl	Hydroxyl radicals[b]
Aβ25-35	100[a]	11
Aβ1-40	45	< 10
Aβ1-42	34	< 10

Experimental conditions are described in the Materials and methods section. The results are representative of three experiments.
[a]Reaction of HOCl with Aβ25-35 as control (Figure 4B).
[b]Hydroxyl radicals were produced by the reaction of Fe^{2+} with H_2O_2.

HOCl produces no chlorinated compounds and both contain Met sulphoxide through the reaction with HOCl. These results demonstrate the preferential reactivity of Met35 with HOCl relative to terminal amine and Tyr10 in the full length Aβ.

Myeloperoxidase (MPO) and microglia are co-localized with SPs [6]. Reactive oxygen species are believed to be generated by activated microglia. Microglia activated by Aβ, *in vitro*, produce reactive oxygen species, which is ascribed to the action of NADPH oxidase [26]. HOCl is generated by the action of MPO: MPO reacts with H_2O_2 to form Compound I, which subsequently oxidizes Cl^- to form HOCl [31]. It has been shown that SP cores from AD patients contain sulphoxide compounds. Raman spectra analyses indicate that extensive Met oxidation is found in the intact plaques [28]. MPO and inactive MPO are correlated with the progression and continuation of inflammation in neurodegenerative disease [25]. Chloride ions are present in large quantities in the brain [45]. We have treated Aβ25-35 and Aβ1-42 with either the MPO/H_2O_2 or the MPO/H_2O_2/Cl^- system. A marked increase in Met sulphoxide was observed when Aβ species were treated with the HOCl generating system, whereas no formation of Met sulphoxide was found when either peptide was treated with hypervalent MPO (MPO/H_2O_2). MPO catalyses the oxidation of phenolic compounds such as tyrosine [46], while our results indicate no reactivity of MPO with the tyrosine residue in Aβ1-40 and Aβ1-42.

Transition metal-induced oxidative stress is reduced when the reactions are conducted in the presence of Aβ [16,23,24,42]. SPs consist of Aβ1-42 and Aβ1-40 and contain high concentrations of transition metals [1,7]. Aβ1-42 binds one mole of copper with high affinity and site-specific oxidation occurs in the presence of H_2O_2 [14]. The ligands of Cu(II) are postulated to be 3N1O (three nitrogens and one oxygen) and the reaction yields oxo-His [17,47]. Figure 7A indicates that iron-catalysed oxidation of Met35 in Aβ is difficult to proceed. When Aβ1-42 was treated with hydroxyl radicals ($FeCl_2 + H_2O_2$), a trace amount of Met sulphoxide in Aβ1-42 was observed (Figure 7B). No significant increase of Met sulphoxide formation was observed with an increasing concentration of reactants ($FeCl_2 + H_2O_2$). The result cannot rule out the possibility that Aβ inhibits the reaction of Fe^{2+} with H_2O_2. The present study suggests that Met35 in Aβ1-40 and Aβ1-42 selectively reacts with HOCl, rather than hydroxyl radicals, leading to extensive Met sulphoxide formation (Table II).

Taken together, our results suggest that SPs are modified by astrocytes, presumably via myeloperoxidase, since transition metals poorly oxidize Met35 in Aβ, the major constituent of plaques. Myeloperoxidase has been localized to plaques [48]. This ties in well with the observation that SPs modified by astrocytes are resistant to removal by microglia [49], explaining the persistence of plaques in the AD brain.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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