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Surprising toxicity and assembly behaviour of amyloid β -protein oxidized to sulfone

Panchanan Maiti*, Roberto Piacentini†, Cristian Ripoli†, Claudio Grassi†, and Gal Bitan^{*,†,§,1}

*Department of Neurology, David Geffen School of Medicine, University of California at Los Angeles, 635 Charles E. Young Drive South, Los Angeles, CA 90025, U.S.A

†Institute of Human Physiology, Catholic University Medical School, Largo F. Vito 1, Rome 00168, Italy

‡Brain Research Institute, University of California at Los Angeles, 635 Charles E. Young Drive South, Los Angeles, CA 90025, U.S.A.

§Molecular Biology Institute, University of California at Los Angeles, 635 Charles E. Young Drive South, Los Angeles, CA 90025, U.S.A

Abstract

A β (amyloid β -peptide) is believed to cause AD (Alzheimer's disease). A β 42 (A β comprising 42 amino acids) is substantially more neurotoxic than A β 40 (A β comprising 40 amino acids), and this increased toxicity correlates with the existence of unique A β 42 oligomers. Met³⁵ oxidation to sulfoxide or sulfone eliminates the differences in early oligomerization between A β 40 and A β 42. Met³⁵ oxidation to sulfoxide has been reported to decrease A β assembly kinetics and neurotoxicity, whereas oxidation to sulfone has rarely been studied. Based on these data, we expected that oxidation of A β to sulfone would also decrease its toxicity and assembly kinetics. To test this hypothesis, we compared systematically the effect of the wild-type, sulfoxide and sulfone forms of A β 40 and A β 42 on neuronal viability, dendritic spine morphology and macroscopic Ca²⁺ currents in primary neurons, and correlated the data with assembly kinetics. Surprisingly, we found that, in contrast with A β -sulfoxide, A β -sulfone was as toxic and aggregated as fast, as wild-type A β . Thus, although A β -sulfone is similar to A β -sulfoxide in its dipole moment and oligomer size distribution, it behaves similarly to wild-type A β in its aggregation kinetics and neurotoxicity. These surprising data decouple the toxicity of oxidized A β from its initial oligomerization, and suggest that our current understanding of the effect of methionine oxidation in A β is limited.

Keywords

Alzheimer's disease; amyloid β -protein; methionine; oxidation; oligomer

¹To whom correspondence should be addressed (gbitan@mednet.ucla.edu).

AUTHOR CONTRIBUTION

Panchanan Maiti, Roberto Piacentini and Cristian Ripoli performed the research and analysed the data. Claudio Grassi and Gal Bitan designed the research and analysed the data. Panchanan Maiti and Gal Bitan wrote the manuscript.

INTRODUCTION

AD (Alzheimer's disease) is characterized by memory loss and progressive cognitive decline [1]. Extracellular amyloid plaques comprising predominantly fibrillar A β (amyloid β -protein) and intracellular neurofibrillary tangles made of hyperphosphorylated tau are neuropathological hallmarks of AD [2]. Abundant evidence suggests that assembly of A β into pre-fibrillar neurotoxic oligomers initiates the disease process in AD, and formation of amyloid plaques and neurofibrillary tangles follow neuronal injury by A β oligomers [3].

A β exists predominantly in two major forms comprising 40 (A β 40) or 42 (A β 42) amino acid residues. Genetic, physiological and biochemical evidence indicates that A β 42 plays a predominant role in the pathogenesis of AD [4], although it is ~10-fold less abundant than A β 40 [5]. In the AD brain, A β 42 is the main component of parenchymal plaques, whereas A β 40 is the main component of vascular deposits [6]. A β 42 aggregates faster and is substantially more neurotoxic than A β 40 [7].

Study of A β oligomerization *in vitro* using PICUP (photo-induced cross-linking of unmodified proteins) and ion-mobility MS has revealed that the oligomer size distributions of A β 40 and A β 42 are distinct [8,9]. A β 40 exists mainly as a mixture of monomer, dimer, trimer and tetramer units, whereas A β 42 preferentially forms pentamer and hexamer units, termed paranuclei, which self-associate into higher-order oligomers [8,9]. The distinct oligomerization patterns of A β 40 and A β 42 correlate with the difference in the toxicity of the two A β alloforms and may explain these differences. This suggests that the C-terminal region of A β is a crucial factor in A β assembly and toxicity. Supporting this hypothesis, structural studies using experimental and computational methods have identified substantial differences in the flexibility and conformational preferences of the C-terminal region of A β 40 and A β 42 [10–12].

The single methionine residue in A β , Met³⁵, is located in the middle of the hydrophobic C-terminal region of A β and is readily oxidized *in vivo* [13]. The dramatic increase in the polarity of the methionine side chain that occurs upon oxidation (Figure 1) has a profound effect on the hydrophathy of the entire region [14]. A β in which Met³⁵ is oxidized to sulfoxide (A β -sulfoxide) has been found in amyloid plaques of AD brain [13], and consequently has been the subject of many studies [15]. Despite the abundance of literature on the subject, it is not clear whether A β -sulfoxide contributes to AD aetiology or results from the highly oxidative environment around amyloid plaques where fibrillar A β may be trapped for long periods and get oxidized.

In addition to oxidation to sulfoxide, methionine can undergo a second oxidation reaction yielding methionine-sulfone (Figure 1). This reaction is less common than oxidation of methionine to methionine-sulfoxide *in vivo*, yet methionine-sulfone has been observed in the brain of patients with AD or Parkinson's disease [16] in the antioxidant protein DJ-1. Ali et al. [17] have proposed that A β -sulfone also might form *in vivo* and this has not so far been observed, simply because it has not been looked for [17].

Oxidation of Met³⁵ to sulfoxide has been reported to decrease formation of A β 40 trimers and tetramers [18], lower A β assembly kinetics [11,19] and reduce A β neurotoxicity

[15,20]. Solution-state NMR studies have shown that the lower assembly kinetics and toxicity correlated with higher conformational flexibility in the C-terminal region of A β -sulfoxide relative to WT (wild-type) A β [19]. The assembly kinetics and toxicity of A β -sulfone have not been reported previously.

A PICUP study of the effect of Met³⁵ oxidation in A β revealed striking differences between A β 40 and A β 42 [14]. The oligomer size distribution of A β 40 was unaffected by oxidation of Met³⁵. In contrast, oxidation of Met³⁵ in A β 42 to either sulfoxide or sulfone was found to abolish paranucleus formation and produce oligomer size distributions indistinguishable from those of A β 40 [14]. In agreement with these observations, molecular dynamics simulations of the WT, sulfoxide and sulfone forms of A β 42 monomers showed that the two oxidized forms adopted conformations that were distinct from that of WT A β 42, both in secondary structure and in global fold [21]. In particular, long-range interactions between the C-terminal region and the CHC (central hydrophobic cluster), which were prominent in WT A β 42 and have been observed in other modelling studies of A β 42 monomers [22] and oligomers [10,23], were absent in the simulations of the two oxidized forms [21]. Rather, the sulfoxide and sulfone groups, which are hydrogen-bond acceptors, formed hydrogen bonds with backbone amide groups in the C-terminal regions stabilizing short-range turn conformations. In modelling studies comparing A β 40 and A β 42 oligomerization [10,23] the interaction between the C-terminus and the CHC regions was substantially less prominent in A β 40 compared with A β 42, suggesting that this structure may be necessary for paranucleus formation.

Attempting to correlate the structural and functional data, we hypothesized that the A β oligomer size distributions observed using PICUP correlated with toxicity. If this hypothesis were correct, oxidation of A β 40 would be predicted to have little effect on toxicity, whereas oxidation of A β 42 to sulfoxide or sulfone would result in toxicity lower than that of WT A β 42 and similar to that of A β 40. The lower toxicity of A β 42-sulfoxide reported in the literature [15,20,24] supported this hypothesis, yet we found a dearth of information regarding the effect of oxidation of methionine to methionine-sulfoxide in A β 40 or oxidation of either A β alloforms to sulfone.

Because methionine-sulfoxide can be reduced to methionine *in vivo* by methionine-sulfoxide reductases [25], when cells are treated with A β -sulfoxide, they also are treated with unoxidized A β . The ratio of A β -sulfoxide to WT A β depends on the kinetics of methionine-sulfoxide reduction relative to peptide degradation. Additional factors determining the observed toxicity are the assembly state of each A β form in the context of the cellular environment [26] and the mechanisms by which each form causes toxicity, all of which are poorly understood. Because methionine-sulfone is not reduced *in vivo* [27], studying the effect of A β -sulfone on cultured neurons is a 'clean' system compared with A β -sulfoxide. Thus, to test our hypothesis of the effect of methionine oxidation on A β assembly and toxicity, we compared the WT, sulfoxide and sulfone forms of A β 40 and A β 42 using assays that measure assembly kinetics, cell viability and cellular function in primary cortical or hippocampal neurons. Surprisingly, we found that, contrary to our prediction, A β -sulfone behaved similarly to unoxidized A β and distinctly from A β -

sulfoxide, raising new questions regarding the mechanisms by which each A β form causes toxicity.

EXPERIMENTAL

Peptide synthesis

A β 40, [Met(O)³⁵]A β 40, [Met(O₂)³⁵]A β 40, A β 42, [Met(O)³⁵]-A β 42 and [Met(O₂)³⁵]A β 42 were synthesized by incorporating Fmoc (fluoren-9-ylmethoxycarbonyl)-Met(O) or Fmoc-Met(O₂) (EMD Biosciences) in position 35 where appropriate. They were then purified and characterized in the UCLA Biopolymers Laboratory. Quantitative amino acid analysis and MS were used to characterize the expected compositions and molecular masses respectively, for each peptide.

Preparation of peptide solutions

Purified peptides were stored as freeze-dried powders at – 20°C. Before use, peptides were treated with HFIP (1,1,1,3,3,3-hexafluoroisopropanol; TCI America) to disassemble pre-formed aggregates, and stored as dry films at – 20°C as described previously [28]. Immediately before use, films were dissolved in 60 mM NaOH at 10% of the desired volume. For biophysical measurements, the solution was then diluted to 50% of the desired volume with deionized water (18.2 M Ω produced by a Milli-Q system, Millipore) and sonicated for 1 min. Then, the solution was diluted with 20 mM sodium phosphate (pH 7.4), to 10 μ M. The pH was adjusted, if necessary, with H₃PO₄ or 1 M NaOH. Alternatively, peptides were prepared by dilution into cell culture medium, similar to the preparation for toxicity experiments. For toxicity experiments, peptides were diluted with cell culture medium after initial dissolution in 60 mM NaOH, and then sonicated for 1 min. The peptides were then diluted in culture medium to the final concentration, which was 10 μ M unless otherwise stated.

Animals

Experiments performed in UCLA were compliant with the National Research Council Guide for the Care and Use of Laboratory Animals and were approved by the UCLA Animal Research Council and the Ethics Committee. Experiments performed in the Catholic University of Rome complied with Italian Ministry of Health guidelines, with national laws (Legislative decree 116/1992) and with European Union guidelines on animal research (No. 86/609/EEC). For experiments in UCLA, pregnant [E (embryonic day) 18] Sprague–Dawley rats were purchased from Charles River Laboratory (Wilmington, MA). In the Catholic University of Rome, Sprague–Dawley rats were bred in-house.

Cell culture

For TUNEL (terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling) assay and dendritic spine morphology experiments, primary cortical or hippocampal neurons respectively, were prepared as described previously by Segal and Manor [29]. Briefly, E18 pregnant rats were killed with CO₂ and the pups were collected immediately. The brains were dissected in chilled Leibovitz's L-15 medium (A.T.C.C.) in the presence of 1 μ g/ml penicillin/streptomycin (Invitrogen). The tissue was incubated with 0.25% trypsin/EDTA

(A.T.C.C.) for 30 min and then mechanically dissociated in a small volume of Leibovitz's L-15 medium using a fire-polished Pasteur pipette. Cortical neurons were used for cell-viability assays. They were suspended in DMEM (Dulbecco modified Eagle's medium, obtained from A.T.C.C.) containing 10% heat-inactivated FBS (fetal bovine serum, A.T.C.C.) and 1 $\mu\text{g/ml}$ penicillin/streptomycin, and plated in poly-D-lysine (0.1 mg, Sigma)-coated coverslips (Fisher Scientific) at a density of 3×10^5 cells/ml. Hippocampal neurons were used for dendritic spine studies. They were suspended in neurobasal media (Gibco) containing B-27 growth factor and 20 mM glutamine and plated on poly-D-lysine (0.1 mg%, Sigma)-coated coverslips. The cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 6 days for cell viability experiments and for 21 days for dendritic spine experiments. At 24 h after plating, the medium was replaced with fresh DMEM/neurobasal medium supplemented with 5 μM cytosine β -D-arabinofuranoside (Sigma) to inhibit the proliferation of glial cells.

For measurements of Ca²⁺ currents, primary cortical neurons were obtained from E17–E19 rat embryos as described previously by Dravid and Murray [30] with minor modifications. Briefly, cortices were dissected and incubated for 10 min at 37°C in PBS containing 0.025% trypsin/0.01% EDTA (Biochrom AG). The tissue was then mechanically dissociated at room temperature (23–25°C) using a fire-polished Pasteur pipette and the cell suspension was harvested and centrifuged at 235 g for 8 min. The pellet was suspended in 88.8% minimum essential medium (Biochrom), 5% FBS, 5% horse serum, 1% glutamine (2 mM), 0.2% gentamicin (0.1 mg/ml) and glucose (25 mM). Cells were plated on to 20-mm coverslips pre-coated with poly-L-lysine (0.1 mg/ml, Sigma) at a density of 1×10^5 cells/well. At 24 h after plating, the culture medium was replaced with a medium containing 97.3% neurobasal medium (Invitrogen), 2% B-27 (Invitrogen), 0.5% glutamine (2 mM) and 0.2% gentamicin (0.1 mg/ml). Finally, after 72 h, the culture medium was replaced with a similar medium lacking glutamine and cells were grown for 4–7 more days before experiments.

TUNEL staining

Cortical neurons were treated with A β analogues for 48 h and then stained using a TUNEL assay kit (APO-BrdU apoptosis detection kit, Invitrogen) as described previously [31]. Fluorescent signals were visualized using a Nikon Eclipse E400 microscope (Nikon Instruments) at $\lambda_{\text{ex}} = 480$ nm and $\lambda_{\text{em}} = 530$ nm. Images were merged using the bundled software 'Picture Frame' (Optronics). Images were taken from multiple fields in three independent experiments and the number of TUNEL-positive cells divided by the total number of counted cells was expressed as the percentage of apoptotic death (means $\pm$ S.E.M.).

Patch-clamp recording of Ca²⁺ currents

According to standard protocols, Ba²⁺ was used as a charge carrier rather than Ca²⁺ [32]. Macroscopic Ba²⁺ currents flowing through VGCCs (voltage-gated calcium channels) were recorded by patch-clamp in whole-cell configuration [33] using an Axopatch 200B amplifier (Molecular Devices). Stimulation and data acquisition were performed using the Digidata 1200 series interface and pCLAMP 9.2 software (Molecular Devices), as described previously [20,33]. The external solution contained 125 mM NaCl, 10 mM BaCl₂, 1 mM

MgCl₂, 10 mM Hepes and 2×10^{-4} mM tetrodotoxin to block Na⁺ channels. The pH was adjusted to 7.3 with NaOH. The standard internal solution consisted of 110 mM CsCl, 10 mM tetraethylammonium chloride, 2 mM MgCl₂, 10 mM EGTA, 8 mM glucose and 10 mM Hepes. To minimize current run-down during experiments, 4.0 mM ATP magnesium salt, 0.25 mM cAMP sodium salt and 4.0 mM phos-phocreatine disodium salt were added to this solution. The pH was adjusted to 7.3 with CsOH. The cell membrane was depolarized every 6 s (pulse duration 200 ms) to voltages ranging from – 50 to +50 mV from the holding potential of –80 mV. Compensation for capacitative transients and leakage currents was achieved online with the clamp-amplifier settings and off-line by subtraction of Cd²⁺-insensitive currents (200 μ M Cd²⁺). Current density (pA/pF) was calculated by dividing current amplitude by cell membrane capacitance, which was measured with the membrane test feature of the pCLAMP 9.2 software. Cells were treated with 10 μ M of each A β analogue for 12 h before experiments. All recordings were made at 23–25°C.

Dendritic spine morphology

Primary hippocampal neurons were used to study the dendritic spine morphology as described previously [34]. Briefly, neurons were treated with 3 μ M of each A β analogue for 72 h. The cells were then fixed with 4% paraformaldehyde and individual neurons were stained with DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate, Sigma) as described previously [34] using a Model 5070 micromanipulator (Eppendorf). The DiI-labelled neurons were imaged at 100 $\times$ magnification (oil-immersion objective) using a confocal laser-scanning microscope (Leica). Images were magnified further using a 3 $\times$ zoom so the morphology of individual spines could be determined and subsequently quantified. The Z-stack images were collected at 0.3 μ m intervals to cover the full depth of the dendritic arbors (20–30 μ m) and compressed into a single JPEG image. The numbers of dendritic spines were counted per 100 μ m length of dendritic branches using ImageJ. The dendritic segments were selected randomly among secondary dendrites from apical branches. At least 50 dendritic segments were used for morphometric analysis. In total, 10–15 individual neurons were analysed for each experimental group.

CD spectroscopy

Samples were incubated at 25°C with continuous agitation at 200 rev./min using an orbital shaker. Spectra were recorded every 2 h during the first 12 h, and then at 24, 48 and 72 h, using a J-810 spectropolarimeter (Jasco) equipped with a thermostable sample cell at 25°C using 1-mm path-length cuvettes. Spectra were collected from 190 to 260 nm with a 1 s response time, 50 nm/min scan speed, 0.2 nm resolution and 2 nm bandwidth, and averaged after background subtraction. The data are representative of three independent experiments.

Electron microscopy

Samples were incubated at 25°C with continuous agitation using an orbital shaker at 200 rev./min. Aliquots (8 μ l) were applied to glow-discharged carbon-coated Formvar grids (Electron Microscopy Science) for 20 min, fixed with 5 μ l of 2.5% glutaraldehyde (Sigma) for 4 min, and stained with 5 μ l of 1% uranyl acetate (Sigma) for 3 min. The solution was wicked off and the grids were air-dried. The morphology was visualized using a CM120 transmission electron microscope (FEI-Philips).

RESULTS

Effect of WT and oxidized A β on neuronal viability

To investigate the structure–activity relationship of native and oxidized A β variants and gain insight into the mechanism of toxicity, we used TUNEL staining in rat primary cortical neurons (Figure 2). The TUNEL assay indicates DNA fragmentation as a measurement of apoptosis [35].

A β 40 was found to cause $14 \pm 1\%$ apoptosis (Figure 2). A β 40-sulfoxide caused $11.5 \pm 0.7\%$ apoptosis, whereas A β 40-sulfone had a similar effect to WT A β 40, $14.3 \pm 0.6\%$ apoptosis. Overall, the differences observed among the A β 40 analogues were not statistically significant. Under the same conditions, A β 42 caused $20.5 \pm 0.5\%$ apoptosis (Figure 2). Similar to previously described data [20], A β 42-sulfoxide was significantly less toxic, causing $13.0 \pm 0.7\%$ apoptosis, i.e. a similar level of toxicity to A β 40, as predicted. Surprisingly, however, A β 42-sulfone showed similar toxicity to WT A β 42, causing $19.7 \pm 0.8\%$ apoptosis. These results were unexpected in view of the oligomer size distribution of A β 42-sulfone, which was similar to that of A β 42-sulfoxide and distinct from that of WT A β 42 [14]. Similar trends were observed in both cortical and hippocampal neurons using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] reduction and LDH (lactate dehydrogenase) release assays (results not shown). Thus three different assays showed that despite the similar increase in dipole moment upon oxidation of Met³⁵ to sulfoxide or sulfone [14,21], and despite the fact that oxidation had a similar effect on both the sulfoxide and sulfone forms of A β 42 [14], only the sulfoxide form behaved as we predicted and showed similar toxicity to WT A β 40.

Effect of WT and oxidized A β on macroscopic Ca²⁺ currents

Next we asked whether the behaviour of the oxidized forms of A β correlated with more subtle toxic effects. Disruption of Ca²⁺ homeostasis and excitotoxicity are thought to be important mechanisms by which A β disrupts synaptic activity and eventually lead to neuronal death [36]. Disruption of intracellular Ca²⁺ homeostasis has long been implicated both in normal brain aging and in the pathogenesis of AD. Both conditions are characterized by impairment of the neurons' ability to control Ca²⁺ fluxes and recover from Ca²⁺ loads [37]. To assess the effect on Ca²⁺ currents, we measured A β -induced changes in currents flowing through VGCCs in primary cortical neurons.

In whole-cell patch-clamp recordings, we measured macroscopic Ba²⁺ currents after treatment with the different A β 40 or A β 42 analogues. Previously, treatment of IMR32 cells with 10 μ M A β 42 for 6–24 h was found to yield maximal increase in depolarization-induced Ca²⁺ influx at 12 h [20]. Therefore we measured Ba²⁺ currents in the experiments described here at 12 h post-treatment.

Both A β 40 and A β 42 caused a significant increase ($P < 0.001$) in total Ba²⁺ current densities in cortical neurons (Figures 3A and 3B), similar to the previous results obtained using IMR32 cells [20], suggesting that each of them triggered dysregulation of Ca²⁺ channels. The effect of A β 42 was stronger than that of A β 40 (+69% compared with +55% respectively), although the difference was not statistically significant. In contrast,

treatment of the cells with A β 40- or A β 42-sulfoxide had no effect on total Ba²⁺ currents. In agreement with the measurements of cell viability, the sulfone form of A β 40 and A β 42 caused an increase in Ba²⁺ current densities to the same magnitude as the corresponding WT A β form (Figures 3A and 3B). The data suggest that in contrast with the WT and sulfone forms of A β , under the experimental conditions used in the present study, the weak toxicity afflicted by A β -sulfoxide was not associated with significant changes in VGCC signals.

Previously, L-type Ca²⁺ (Ca_v1) channels were found to contribute significantly to the current increase induced by A β 42 in IMR32 cells, and Ca²⁺ influx through these channels was found to be responsible for the calcium-mediated activation of pro-apoptotic pathways underlying the stronger neurotoxicity of WT A β 42 relative to A β 42-sulfoxide [20]. To determine whether a similar correlation exists between L-current increases and A β neurotoxicity in cortical neurons, and to what extent it is activated by each of the oxidized forms of A β , we studied the A β -induced modulation of current amplitudes before and after applying 5 μ M of the selective L-channel blocker nifedipine. Analysis of the data revealed that peak Ba²⁺ currents flowing through L-channels significantly after treatment with WT A β 40 or A β 42 (+80% and +98% respectively; $P < 0.01$), or their respective sulfone forms (+51% and +80% respectively; $P < 0.01$; Figures 3C and 3D), whereas A β 40- or A β 42-sulfoxide did not produce significant changes.

A β -induced perturbation of dendritic spines

Dendritic spines are semi-autonomous compartments containing molecular machinery important for synaptic transmission and plasticity [38]. The number of dendritic spines per unit length of the dendrite in the adult brain is indicative of the number of excitatory synapses present. Examination of post-mortem brains from patients with AD or Down syndrome dementia has shown substantial loss of dendritic spines [39].

We analysed dendritic spines from apical branches of hippocampal pyramidal neurons after treatment with A β 42 or its oxidized analogues (Figure 4). In initial experiments, we found that 3 μ M A β 42 induced a robust effect on dendritic spine number and morphology in this assay, whereas 10 μ M led to overt cell death that precluded observation of the effect on dendritic spines. Therefore we used 3 μ M for comparison of WT and oxidized A β 42 analogues. Because the toxicity of A β 40 was substantially lower than that of A β 42 in cell viability assays (Figure 2, and data not shown for MTT and LDH assays), we did not use A β 40 in this assay.

After treatment with 3 μ M A β 42, the dendrites showed abundant varicosities and the number of dendritic spines per 100 μ m decreased to <30% of that in cells treated with medium (Figures 4B and 4E). Consistent with the cell viability assays described above, A β 42-sulfoxide showed little induction of varicosities and had a significantly weaker effect on dendritic spine density compared with WT A β 42, decreasing the number of dendritic spines per 100 μ m to ~70% of medium-treated cells (Figures 4C and 4E). In contrast, and consistent with the other assay results, A β 42-sulfone induced abundant varicosities and decreased the dendritic spine density to the same level as WT A β 42 (Figures 4D and 4E).

Assembly kinetics of WT and oxidized A β

Because the biological activity of A β 42-sulfone contradicted our predictions, which were based on the initial oligomer size distributions for the WT and oxidized A β 42 analogues, we asked whether the bioactivity correlated with aggregation kinetics. Early studies of the effect of methionine oxidation to sulfoxide on A β assembly have led to mixed results [40,41], whereas most of the later studies reported that assembly kinetics of A β -sulfoxide was decreased relative to that of WT A β .

We used CD spectroscopy to study the effect of Met³⁵ oxidation on β -sheet formation during A β fibrillogenesis at the same concentration as used for cell viability measurements (Figure 5). All of the peptides showed an initial CD spectrum characterized by a minimum at 196–200 nm, suggesting that the conformation was predominantly statistical coil. A β 40 showed a gradual decrease in the magnitude of this minimum over 72 h, at which point a maximum at 197–198 nm appeared (Figure 5A), suggesting an increase in β -sheet content. Observation of the β -sheet-characteristic minimum at 215–218 nm required a higher concentration of A β 40 (results not shown). For consistency with other measurements, we kept the concentration at 10 μ M in the presented experiments. A β 40-sulfoxide (Figure 5B) and A β 40-sulfone (Figure 5C) showed somewhat slower conformational conversion during the first 24 h, although the overall rate of conformational change over 72 h was similar to that of WT A β 40. In both cases, at 48–72 h the spectrum did not show the expected maximum at 197–198 nm and instead appeared nearly flat. However, in each case the solution remained free of particulate material or precipitates, suggesting that the spectrum reflected *bona fide* conformational change rather than precipitation of aggregated material.

The spectrum of A β 42 showed a conversion from a minimum at 197 nm into a maximum at 198 nm within 24 h, with concomitant appearance of a minimum at 217–218 nm, indicating conversion from statistical coil into a β -sheet-rich conformation (Figure 5D). The spectra showed an isodichroic point at 210 nm, suggesting that other conformations did not accumulate to a significant extent. In contrast, the spectrum of A β 42-sulfoxide did not change during the first 24 h of measurement (Figure 5E), consistent with previous observations of delayed aggregation and β -sheet formation of this peptide relative to WT A β 42 [42]. At later time points (48–72 h), A β 42-sulfoxide did show slow conversion into a typical β -sheet-rich conformation (results not shown). The slow conformational transition of A β 42-sulfoxide correlated with the lack of paranucleus formation by this peptide [14] and with the low toxicity described above. Also in correlation with its biological activity, A β 42-sulfone behaved similarly to WT A β 42. After a short lag-phase, the CD spectrum changed from predominantly statistical coil to β -sheet-rich, with an isodichroic point at 210 nm.

To test whether the conformational transition observed by CD spectroscopy correlated with fibril formation, we examined morphological changes in A β 40 and A β 42 in their native, sulfoxide or sulfone forms by electron microscopy (Figure 6). All of the peptides initially had non-fibrillar morphology and showed structures consistent with oligomers. The morphology of A β 40 remained non-fibrillar for 48 h. At 72 h of incubation, typical amyloid fibrils were present together with remaining non-fibrillar structures, and by 96 h, fibrils largely predominated the morphology of A β 40. A β 40-sulfoxide and A β 40-sulfone both followed similar, albeit slower, morphological changes. Both peptides were still non-fibrillar

at 72 h and formed fibrils by 96 h. Thus fibril formation was delayed by ~24 h relative to WT A β 40. The fibrils of A β 40-sulfoxide were scarce relative to those of WT A β 40 or A β 40-sulfone. These data were consistent with the kinetic trends observed by CD.

As expected, fibril formation by A β 42 analogues was faster than by A β 40 analogues. Correlating with the conformational transition observed by CD and with the toxicity data, A β 42 and A β 42-sulfone had similar kinetics and showed abundant fibrils following 24 h of incubation, whereas A β 42-sulfoxide was non-fibrillar at this time point and showed fibril formation only after 48 h.

The environment the peptides experience in cell culture experiments is different from the hypotonic buffer used in the CD and electron microscopy experiments in the present study and may affect peptide assembly. Detailed structural investigation of peptide conformation and morphology using CD and electron microscopy respectively, is not possible in the cell culture environment, which includes medium, serum and the cells themselves. However, investigating the structures in the cell culture medium (without serum or cells) may provide clues to the behaviour of the peptides in this environment. Therefore we incubated the peptides at the same concentration (10 μ M) in DMEM at 37°C and investigated their conformation and morphology periodically for several days. The results are shown in Supplementary Figures S1 and S2 (at <http://www.BiochemJ.org/bj/433/bj4330323add.htm>).

CD spectra could be obtained in medium only down to 203 nm because at lower wavelengths the dynode voltage exceeded the instrument's recommended limits. Thus the minimum and maximum molar ellipticity at 195–198 that characterize statistical coil and β -strand respectively, could not be observed. In addition, the noise level was higher than in the spectra obtained in buffer. Nevertheless, temporal changes were observed consistent with conformational transition. Under these conditions, the conformational transition happened in all cases with faster kinetics than in buffer and appeared to be complete or nearly complete by 24 h (Supplementary Figure S1). A β 40 (Supplementary Figure S1A) and A β 40-sulfone (Supplementary Figure S1C) appeared to have similar kinetics of conformational transition, whereas the kinetics of A β 40-sulfoxide was somewhat slower (Supplementary Figure S1B). A similar trend was observed for the A β 42 analogues (Supplementary Figure S1D–S1F), although the high noise levels made it difficult to draw unambiguous conclusions.

Consistent with the CD data, all of the peptides showed amorphous structures at $t = 0$ h, although not in all cases could these be distinguished from structures observed in the medium itself (Supplementary Figure S2). All of the peptides formed fibrils within 24 h of incubation in medium. A β 40 formed abundant short fibrils that were mixed with amorphous structures at 24 and 48 h. At 24 h, the fibrils of A β 40-sulfoxide and A β 40-sulfone appeared longer than those of WT A β 40 and looked ribbon-like, comprising up to seven protofilaments. Amorphous structures were observed together with the fibrils at this time point in A β 40-sulfoxide, but not A β 40-sulfone samples. Interestingly, by 48 h, the fibrils of both oxidized forms appeared shorter than at 24 h. A β 42 analogues behaved similarly and were assembled into fibrils by 24 h. At this time point, amorphous aggregates were observed together with fibrils for A β 42-sulfoxide, but not for WT A β 42 or A β 42-sulfone. The fibrillation process for the latter two peptides appeared to be complete by 24 h.

DISCUSSION

The high toxicity we found for A β 42-sulfone (Figures 2–4) contradicted our expectations, which were based on the difference between the oligomer size distributions of A β 42-sulfoxide and A β 42-sulfone, and that of WT A β 42 [14], the calculated dipole moments of methionine, methionine-sulfoxide and methionine-sulfone (Figure 1), and structures predicted in several modelling studies [10,21–23]. Despite the similarity between the behaviours of the sulfoxide and sulfone forms of A β 42, and the difference between the two oxidized forms and WT A β 42, A β 42-sulfone was as toxic as WT A β 42. Several mechanisms may explain these unexpected observations. In the present study we tested the hypothesis that the toxicity correlated with aggregation rather than with early oligomerization.

The toxicity levels of the WT and oxidized A β 42 analogues correlated with their kinetics of β -sheet and fibril formation (Figures 5 and 6). One interpretation of the data is that the toxic effect may be mediated by later-forming large oligomers or aggregates rather than by initial small oligomers. Currently, there is no consensus regarding the size and type of A β oligomers that contribute the most to toxicity, or even concerning whether certain oligomers are more important than others for AD pathology. Side-by-side comparison of small and large oligomer populations made of synthetic A β and fractionated from the same initial source has suggested that larger structures were more toxic than small ones [43]. A recent examination of individual A β 40 oligomers stabilized by PICUP has shown a correlation between increased oligomer size and toxicity [44], yet the study found substantial toxicity already for dimers, trimers and tetramers. Comparison of memory impairment in rats induced by A β oligomers of different sizes from synthetic, cell culture or transgenic animal brain sources has suggested that small oligomers were more toxic than larger ones [45].

Although high-resolution structures for A β oligomers are beginning to emerge [46], direct structure–activity comparisons among small and large oligomers are difficult. Thus whether or not the trends we observed among assembly kinetics and toxicity of the different A β analogues represent cause and effect relationship or a mere correlation is an open question. An answer to this question may be provided in future studies by electrophysiological experiments comparing the sulfoxide and sulfone forms of A β 42 because such experiments can measure toxic effects induced by A β oligomers within minutes, probably before substantial amounts of larger oligomers form [47].

Alternatively, we may explain our findings by questioning our fundamental assumptions. Based on the similarity in the dipole moments of the sulfoxide and sulfone groups (Figure 1) and the similarity between the oligomer size distributions of A β 42-sulfoxide and A β 42-sulfone, we assumed that the three-dimensional structure of the oligomers formed by the two oxidized A β 42 analogues was similar (and distinct from that of WT A β 42). We postulated that because of the large increase in dipole moment upon oxidation, the hydrophobic methionine side chain, which presumably is shielded from the aqueous solvent in A β 42 oligomers, would partition preferably into the solvent once the sulfide group is oxidized to sulfoxide or sulfone [14]. If such a structural change occurs in A β 42-sulfoxide, but not in A β 42-sulfone, it would explain why the sulfone form is as toxic as WT A β 42. This

explanation is unlikely and does not account for the similarity in the oligomer size distribution between A β 42-sulfoxide and A β 42-sulfone.

Yet another alternative explanation of the data is that the intrinsic toxicity of the WT, sulfoxide and sulfone forms of A β is similar and, in the case of A β 42, is unrelated to the oligomer size distributions observed using PICUP, but a cellular response unique to the sulfoxide form reduces its toxicity relative to the WT and sulfone forms. One such cellular response might be an increase in expression levels of MsrA (methionine-sulfoxide reductase A), which was reported recently to increase in cells treated by A β 42-sulfoxide, but not WT A β 42 [48], and may provide a protective effect to the cells. Our data suggest that such protection probably is not entirely responsible for the lower toxicity of A β -sulfoxide because disruption of Ca²⁺ current through VGCC, an important mechanism contributing to A β toxicity, was substantially perturbed by WT A β and A β -sulfone and not at all by A β -sulfoxide. These data suggest that regardless of putative induction of a protective MsrA response, the toxicity of WT A β and A β -sulfone is mediated by at least one mechanism not shared by A β -sulfoxide. Our data also suggest that a substantial portion of A β -sulfoxide remains oxidized and does not get reduced by MsrA during the assay period.

The majority of the studies addressing Met³⁵ oxidation in full-length A β have focused on A β 42, whereas information about A β 40 has been scarce. We found that A β 40 analogues behaved generally similarly to A β 42 analogues, although the differences in toxicity and assembly kinetics were smaller between the WT and oxidized A β 40 peptides. In addition, A β 40-sulfone behaved somewhat more similarly to A β 40-sulfoxide than the counterpart A β 42 analogues. These observations are in line with the lack of change in the oligomer size distribution of WT A β 40 upon oxidation to sulfoxide or sulfone [14] and suggest that, in A β 40, the relative contribution of the C-terminal region to the assembly and toxicity behaviour is lower than in A β 42, consistent with previous reports [8,49].

The present study suggests that formation of methionyl radicals and participation of methionine in Fenton chemistry [17] probably do not contribute significantly to A β -induced toxicity because A β -sulfone is less likely than A β -sulfoxide to participate in Fenton chemistry or contribute to production of ROS (reactive oxygen species), yet it is as toxic as WT A β . Supporting this view, recently, substitution of Met³⁵ by valine or norleucine in A β 40 or A β 42 was reported to yield analogues that were as toxic as the WT peptides [31], although in other experimental systems substitution of Met³⁵ by norleucine abolished A β 42 toxicity [15,20]. Interestingly, a recent study in a transgenic mouse model of AD found that substitution of Met³⁵ by leucine had no effect on learning and memory deficits even though markers of oxidative stress were significantly decreased [50].

In summary, the results of the present study suggest a possible correlation between assembly kinetics and toxicity of native and oxidized A β , but demonstrate that the relationships among primary structure, quaternary structure and toxic activity are complex. Further study will be required to understand why the sulfoxide and sulfone forms of A β 42 have similar electronic characteristics and produce similar oligomer size distributions in PICUP experiments, yet behave distinctly in terms of their toxicity and aggregation kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used

Aβ	amyloid β -protein
Aβ40	A β comprising 40 amino acids
Aβ42	A β comprising 42 amino acids
AD	Alzheimer's disease
CHC	central hydrophobic cluster
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
DMEM	Dulbecco modified Eagle's medium
E	embryonic day
FBS	fetal bovine serum
Fmoc	fluoren-9-ylmethoxycarbonyl
HFIP	1,1,1,3,3,3-hexafluoroisopropanol
LDH	lactate dehydrogenase
MsrA	methionine-sulfoxide reductase A
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2 <i>H</i> -tetrazolium bromide
PICUP	photo-induced cross-linking of unmodified proteins
TUNEL	terminal deoxynucleotidyltransferase-mediated dUTP nick-end labelling
VGCC	voltage-gated calcium channel
WT	wild-type

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Residue	Structure	Dipole Moment [14]	Dipole Moment [21]
Methionine		1.60	1.76
Methionine-sulfoxide		5.28	3.72
Methionine-sulfone		5.20	4.30

Figure 1.
Structure and calculated dipole moments (in Debye) of methionine and its oxidized forms

Figure 2. Native and oxidized Aβ-induced apoptosis

Rat primary cortical neurons were cultured for 6 days on poly-D-lysine-coated coverslips and treated with 10 μ M of each A β analogue for 48 h. The cells were then probed with the APO-BrdU kit for analysis of DNA fragmentation. (A) Representative micrographs of multiple fields. Red colour indicates normal cells. Green/yellow colour indicates TUNEL-positive cells. (B) The number of apoptotic cells was divided by the total number of cells and expressed as a percentage of apoptotic death. The results are means $\pm$ S.E.M. for three independent experiments. ** $P < 0.01$.

Figure 3. Effect of native and oxidized Aβ analogues on calcium currents

Rat primary cortical neurons were treated with 10 μ M of each Aβ40 or Aβ42 analogue for 12 h. Macroscopic Ba²⁺ currents flowing through VGCCs were recorded using patch-clamp in a whole-cell configuration. Current density (pA/pF) was calculated by dividing current amplitude by cell membrane capacitance. (**A** and **B**) Current-to-voltage relationships in neurons treated with Aβ40 and Aβ42 analogues respectively ($n = 15$ for each group). (**C** and **D**) Peak current densities of L-type currents in neurons treated with Aβ40 analogues or Aβ42 analogues respectively. ** $P < 0.01$; n.s., not significant.

Figure 4. Native and oxidized Aβ42-induced changes in dendritic spine morphology

Rat primary hippocampal neurons were grown for 21 days, treated with 3 μM of each Aβ42 analogue for 72 h, and stained with DiI. Micrographs are representative of secondary apical branches from multiple neurons. (A) Medium alone, (B) WT Aβ42, (C) Aβ42-sulfoxide and (D) Aβ42-sulfone. (E) The total number of spines in three independent experiments was counted and expressed as means ± S.E.M. ** $P < 0.01$.

Figure 5. Time-dependent conformational transition in native and oxidized A β analogues
 Time-dependent conformational changes in A β 40, A β 42 and their respective oxidized analogues were recorded using CD spectroscopy. HFIP-treated peptides were incubated in 10 mM sodium phosphate (pH 7.4). The spectra are representative of at least three independent experiments. (A) WT A β 40, (B) A β 40-sulfoxide, (C) A β 40-sulfone, (D) WT A β 42, (E) A β 42-sulfoxide and (F) A β 42-sulfone.

Figure 6. Morphology of native and oxidized A β analogues

A β analogues were incubated in sodium phosphate (pH 7.4), and aliquots were spotted on glow-discharged, carbon-coated grids, stained with 1 % uranyl acetate, and examined by electron microscopy. Each image is representative of three independent experiments.