Fractionation and characterization of oligomeric, protofibrillar and fibrillar forms of β -amyloid peptide

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The β -amyloid ($A\beta$) peptide, a major component of senile plaques in Alzheimer's disease brain, has been shown previously to undergo a process of polymerization to produce neurotoxic forms of amyloid. Recent literature has attempted to define precisely the form of $A\beta$ responsible for its neurodegenerative properties. In the present study we describe a novel density-gradient centrifugation method for the isolation and characterization of structurally distinct polymerized forms of $A\beta$ peptide. Fractions containing protofibrils, fibrils, sheet structures and low molecular mass oligomers were prepared. The fractionated forms of $A\beta$ were characterized structurally by transmission electron microscopy. The effects on cell viability of these fractions was determined in the B12 neuronal cell line and hippocampal

neurons. Marked effects on cell viability in the cells were found to correspond to the presence of protofibrillar and fibrillar structures, but not to monomeric peptide or sheet-like structures of polymerized $A\beta$. Biological activity correlated with a positive reaction in an immunoassay that specifically detects protofibrillar and fibrillar $A\beta$; those fractions that were immunoassay negative had no effect on cell viability. These data suggest that the effect of $A\beta$ on cell viability is not confined to a single conformational form but that both fibrillar and protofibrillar species have the potential to be active in this assay.

Key words: Alzheimer's disease, density-gradient centrifugation, protofibrils

INTRODUCTION

The β -amyloid ($A\beta$) peptide is a 39–43-amino-acid peptide derived from the proteolytic cleavage of the amyloid precursor protein [1]. It has been postulated that $A\beta$, a major component of senile plaques, is responsible for the neuronal degeneration observed in the vicinity of senile plaques in Alzheimer's disease brain and is thought to be responsible for the disease pathology [2,3]. *In vitro* studies in primary neurons and in cell lines show neurotoxic effects of $A\beta$ [4–6]. In its monomeric form, however, $A\beta$ is not generally thought to be inherently toxic to neurons or neuron-like cell lines. Although it is well-established that biological activity is dependent upon the aggregation state of the peptide [7,8], the identity of the active species is unclear. Fibrillar synthetic peptide deposits, resembling those observed *in vivo* [9,10], are formed during *in vitro* aggregation or 'aging' of $A\beta$ 1–40 in solution, and the presence of long straight fibrils appears to correlate with the neurotoxic properties of the peptide preparation [8,11]. Other data suggest that smaller peptide oligomers may be the toxic form [12,13]. Analysis of human brain extracts has demonstrated the presence of water soluble, non-filamentous forms of $A\beta$ [14] and dimeric and trimeric species [12]. Furthermore, the formation of small $A\beta$ oligomers has also been demonstrated in cell culture systems [15,16], suggesting that living cells have the capacity to secrete polymerized forms of the peptide.

In vitro fibril formation is dependent upon a seeded polymerization mechanism [17], where nucleation is concentration related and may possibly involve micelle formation [18]. Recent

reports, employing quasi-elastic light scattering [18], size-exclusion chromatography [19] and atomic force microscopy [20], have begun to unravel the pathways leading from $A\beta$ monomers to $A\beta$ oligomers and mature fibrils.

The neurotoxic properties of $A\beta$ have previously been described morphologically [21], by attempting to measure cell death by lactate dehydrogenase release [22] and by inhibiting 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction or exocytosis [6]. The reduction of MTT does not necessarily measure cell death, but is an index of the cell's metabolic integrity. Such compromising of cellular events appears to be an early indicator of impending cell loss, and demonstrates effects of $A\beta$ at concentrations well below those necessary to produce cell death [23]. Hence effects on MTT reduction, observed for $A\beta$, suggest an induction of mechanisms leading to cell death.

In the present study we use density-gradient centrifugation to separate different fibrillar forms of $A\beta$ formed during *in vitro* fibrillization or 'aging'. The different species have been characterized biophysically using photon correlation spectroscopy (PCS), an aggregate-specific immunoassay, electrophoresis and transmission electron microscopy, and biologically, based on the ability of cells to reduce the formazan dye MTT.

MATERIALS AND METHODS

Materials

$A\beta$ 1–40 (lot no. ME0741) was obtained from California Peptide Research (Napa, CA, U.S.A.). MTT, PBS and 1,1,1,3,3,3-

Abbreviations used: $A\beta$, β -amyloid; CV, coefficient of variation; DMEM, Dulbecco's modified Eagle's Medium; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCS, photon correlation spectroscopy; R_H , hydrodynamic radius; RP, reverse-phase.

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hexafluoro-2-propanol (HFIP) were all purchased from Sigma (Gillingham, Dorset, U.K.). Optiprep, Maxidens and the Nycomed Gradient Unloader were obtained from Robbins Scientific (Solihull, West Midlands, U.K.). Optiprep was supplied as a solution containing 60% (w/v) iodixanol. Optiprep was diluted using PBS and percentages of Optiprep refer to the final iodixanol content in the diluted solutions. B27, a serum free supplement, was purchased from Gibco BRL (Paisley, Renfrewshire, Scotland, U.K.). Trifluoroacetic acid (SPS grade), acetonitrile 190 (Far UV grade) and water (HPLC grade) were obtained from Romil (Waterbeach, Cambridge, U.K.). A Zorbax Stablebond SB-C₁₈ 300 narrowbore column (5 µm particle size, 300 Å pore diameter, 2.1 mm × 150 mm) was purchased from Anachem (Luton, Bedfordshire, U.K.). Micro sample vials (100 µl; low volume injection) were purchased from Alltech Associates (Carnforth, Lancashire, U.K.). Antibodies used in this study were the 2F12 monoclonal raised against Aβ peptide Asp¹–Lys¹⁶ [24] and the monoclonal antibody WO2 (kindly supplied by Professor Konrad Beyreuther, University of Heidelberg, Heidelberg, Germany) which has been shown to specifically recognize residues 5–9 of the Aβ peptide [25].

Preparation of Aβ peptide solutions for density-gradient centrifugation

Prior to incubation Aβ 1–40 was dissolved in sterile filtered (50 nm pore size) 0.1% acetic acid at 40 mg/ml. This solution was left for 15 min to facilitate dissolution, following which the solution was diluted in sterile filtered (50 nm pore size) PBS (pH 7.4) to 1 mg/ml and incubated at 35 °C for 30 min, 18 h or 18 days prior to fractionation. Aβ peptide was also solubilized at 1 mg/ml in the α-helix promoting solvent HFIP, and left for 30 min at 20 °C. Following evaporation of the HFIP under a stream of nitrogen gas, the peptide was resolubilized at 40 mg/ml in 0.1% acetic acid and diluted to 1 mg/ml in PBS immediately prior to use.

Density-gradient centrifugation

Density-gradient centrifugation was performed on a gradient of Optiprep, prepared by layering 0.65 ml of 50% Optiprep at the bottom of a Polyallomer Quick Seal centrifuge tube followed by 0.65 ml of 40%, 0.65 ml of 30%, 1.95 ml of 20%, 0.65 ml of 10% and 0.3 ml of 5% to give a final discontinuous gradient of 4.85 ml total volume. A 0.3 ml aliquot of incubated Aβ peptide (300 µg) was layered on to the top of the Optiprep gradient. The tubes were spun at 350 000 *g* for 3 h at 4 °C (Beckman Optima XL-100K ultracentrifuge, NVT 100 rotor). Following centrifugation, 14 fractions (0.375 ml each) were harvested by upward displacement using a Nycomed Gradient Unloader and Maxidens as the displacement solution. Fractions from the gradient were collected by upward displacement such that fraction 1 was the least dense fraction from the top of the gradient and fraction 14 was the most dense fraction from the bottom of the gradient.

PCS

PCS (also known as quasi-elastic or dynamic light scattering) measurements were performed at 23 °C using an ALV DLS/SLS-5000 goniometer system, and an ALV-5000/FAST multiple tau digital correlator (ALV Laser Vertriebsgesellschaft) with a 20 mW helium neon laser (Uniphase, Witney, Oxon., U.K.). Aβ-peptide-containing fractions were analysed immediately after harvesting from the gradient. Samples were prepared by transferring 300 µl into clean, dust-free, 1 cm cuvettes and placed into

the goniometer for light scattering analysis. The scattering angle was 90°. The intensity and the autocorrelation function of the scattered light from the samples were automatically measured for periods of 15 min. The size distribution of the fibrils in solution was determined using ALV-NonLin proprietary data analysis software [26]. A refractive index of 1.4 and viscosity of 2.23 cP (1 P = 10⁻¹ Pa·s) for a 20% solution of Optiprep were used for calculating size distributions. For HFIP, a refractive index of 1.277 and viscosity of 1.619 cP, respectively, were used. The mass fractions of individual peaks, corresponding to fibril populations, were expressed as the percentage of the total mass of all the peaks.

Electrophoretic analysis

Samples of fractionated Aβ peptide were electrophoresed using Novex precast 4–20% acrylamide gels [27]. Aβ peptide was electroblotted on to nitrocellulose membranes at 350 mA for 45 min [28] and was detected using antibody WO2 (see the Materials section) followed by an anti-mouse horse radish peroxidase antibody and enhanced chemiluminescence ('ECL[®]') detection. For the immunodetection of high molecular mass forms of Aβ peptide the SDS concentration of the sample buffer was reduced from a final concentration of 1% (w/v) to 0.1% (w/v).

Immunoassay

The polymerization of Aβ peptide was also determined by a specific immunoassay. The capture antibody comprised the 2F12 monoclonal antibody (plates coated at 2.7 µg/ml in PBS), raised to the 1–16 fragment of Aβ peptide. Detection was with a biotinylated dextran conjugate Fab fragment of 2F12 (0.8 µg/ml). Specificity for polymerized peptide was achieved by the use of the biotinylated 2F12 as the detection antibody, with only aggregated peptide that was bound to the plated 2F12 antibody providing exposed epitopes for binding of the biotinylated antibody. Quantification of immunoreactivity involved binding of streptavidin–europium to the antibody–peptide complex and detection by delayed enhanced lanthanide fluorescence immunoassay ('DELFI[®]': Wallace, Milton Keynes, U.K.). In a typical experiment 50 ng of non-aggregated Aβ per well gave a signal of approx. 20 000 light units, whereas the same quantity of fibrillized Aβ (incubated for 18 h at 37 °C) generated a signal of approx. 800 000 light units. Fractions from the Optiprep fractionation were diluted 100-fold in Tris-buffered saline, before quantification in the immunoassay, to avoid saturation of the detection system by samples containing large amounts of polymeric material.

Biological activity in B12 cells

The profile of fractionated Aβ peptide was also assessed in the B12 clonal central nervous system cell line (European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wilts, U.K.). Cells were plated in 190 µl of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum at 10 000 cells per well in Nunc 96-well tissue culture plates. After incubation of the cells for 24 h, fractionated Aβ aliquots (10 µl) from the density-gradient were added to the cells immediately after the collection of the fractions from the gradient. Following a further 30 min incubation period, the medium was aspirated from the cell monolayer. Cells were then washed once with DMEM and incubated with 100 µl of fresh medium for 24 h at 37 °C. Subsequently, MTT was added to a final concentration of 1 mg/ml and the cells were incubated for a final 4 h at 37 °C.

Figure 1 The fractionation of fibrillized and freshly prepared A β 1–40

Aliquots (300 μ g) of the following A β samples were applied to the top of a 5–50% Optiprep gradient: (a) HFIP-treated freshly prepared A β ; (b) A β incubated at 35 °C for 30 min (prepared as described in the Materials and methods section); (c) A β incubated at 35 °C for 18 h; (d) A β incubated at 35 °C for 18 days. The gradient was centrifuged at 350 000 g for 3 h, fractions were collected across the gradient and analysed by SDS/PAGE to reveal the distribution of A β and the presence of oligomers. Aliquots of each fraction were also assayed in the B12 cell toxicity assay and the distribution of A β across the fractions was determined by RP-HPLC. Data represent the means $\pm$ S.E.M. ($n = 3$).

MTT and cell medium were replaced by 100 μ l of DMSO to solubilize the cells, before the reduction of MTT, to a formazan product, was assessed by measuring the absorption at 490 nm using a Spectramax plate reader.

Biological activity in rat primary hippocampal cell cultures

Hippocampi were removed from embryonic day 17–18 Sprague–Dawley rat brains into a Ca²⁺- and Mg²⁺-free Hanks balanced salt solution and then into a 0.1 % trypsin solution for 30 min at 37 °C. The trypsin was removed by washing the tissue twice in Neurobasal[™] growth medium, containing 2 mM L-glutamine, 2 % (v/v) B27 and 10 % (v/v) fetal calf serum. The tissue was dissociated in this medium by trituration through a flame-constricted pasteur pipette (15–20 strokes) and then passed through a 70- μ m cell strainer (Falcon, Cowley, Oxon., U.K.). Cells were washed into serum free medium by centrifuging at 100 g for 5 min and then resuspending the cell pellet into Neurobasal[™] growth medium, containing 2 mM L-glutamine and 2 % (v/v) B27. For the analysis of biological activity of A β fractions, cells were plated in Neurobasal[™] growth medium, containing 2 mM L-glutamine and 2 % (v/v) B27, at 40 000 cells per well in 96-well tissue culture plates coated with 10 μ g/ml poly(L-lysine). The MTT assay was carried out exactly as described for B12 cells.

Preparation of samples for transmission electron microscopy

A β -peptide-containing fractions were analysed immediately after harvesting from the density-gradient. Samples were prepared for transmission electron microscopy in the following manner. Aliquots of sample (5 μ l) were pipetted on to the surface of Formvar/carbon-coated electron microscope grids and adsorbed for 2 min at ambient temperature. Excess sample was then removed with filter paper, while simultaneously rinsing with 20 μ l of sterile deionized water containing a 1:1000 dilution of 100 nm diameter NIST certified latex Nanospheres[™] (Duke Scientific Corp., Palo Alto, CA, U.S.A.) for size calibration. After rinsing, 5 μ l of 1 % (w/v) uranyl acetate 'negative-contrast' stain was added for 15–20 s. Grids were then blotted dry and examined in a Hitachi H7100 transmission microscope fitted with a Gatan MSC791 ccd camera (Gatan Inc., Pleasanton, CA, U.S.A.) and Gatan Digital Micrograph Version 2.5 software for measurement analysis.

HPLC analysis

A Hewlett–Packard HP1090 DR5 binary pumping system was used with Chemstation Software (Rev.A.02.02) to control chromatography. The analytical column was maintained at 60 °C in a column oven. A β peptide was eluted with a linear gradient of solvent A [0.1 % (v/v) trifluoroacetic acid] and solvent B [acetonitrile/water (4:1, v/v) containing 0.09 % (v/v) tri-

fluoroacetic acid] at a flow rate of 0.2 ml/min. Column eluate was monitored via a Diode Array detector at 214 and 206 nm. Automatic integration of peaks was via Chemstation software. The gradient employed was 5–95 % (v/v) solvent B over 36 min then held at 95 % solvent B for 5 min and then reduced to 5 % solvent B over the next 10 min. Samples (25 μ l) were loaded automatically via an autosampler.

RESULTS

Density-gradient centrifugation of A β 1–40

In order to test the ability of Optiprep gradients to fractionate the different oligomeric forms of A β , samples of A β were fractionated on 5–50 % Optiprep gradients. The distribution of A β across the gradient following centrifugation was analysed both by SDS/PAGE and reverse-phase (RP)-HPLC. While HPLC analysis gave quantitative data on the distribution of A β across the gradient, analysis by SDS/PAGE revealed the existence of oligomeric and high molecular mass forms of the A β peptide. To ensure that the RP-HPLC assay was detecting polymerized and freshly prepared A β peptide equally, aliquots of the two preparations were compared. The mean retention times

of freshly prepared A β ($n = 4$) and polymerized A β ($n = 4$) were 21.71 min [coefficient of variation (CV) = 0.057 %] and 21.85 min (CV = 0.142 %), respectively. The same analysis revealed that the mean area under the peak for freshly solubilized and polymerized A β was 11 236 (CV = 1.096 %) and 11 167 (CV = 1.018 %), respectively.

A typical distribution of HFIP-treated A β across the gradient is shown in Figure 1(a). The RP-HPLC data for HFIP-treated A β peptide revealed that 89 % of the total peptide was found in a pool corresponding to the first five fractions from the top of the gradient, with concentrations of A β as high as 20–30 μ M in the first three fractions.

Aliquots of each fraction were incubated with B12 cells to determine activity in an MTT assay; no significant levels of activity were detected (Figure 1a). The distribution of A β across the gradient was quite different when A β that had not been exposed to HFIP was incubated (37 °C for 30 min) prior to fractionation. Two distinct pools of A β were detected (Figure 1b). The major pool (fractions 1–5) comprised 76 % of the total peptide, with concentrations at the top of the gradient exceeding 20 μ M, but exhibited no activity in the MTT assay. The second pool, however (fractions 10–13), although containing only 4–7 μ M A β , produced marked effects on MTT reduction (Figure

Figure 2 Morphology of the A β polymers fractionated by Optiprep density-gradient centrifugation

Representative images of the A β polymers observed in fractions 1–4 of 30 min- and 18 h-incubated samples (A), in fractions 10–12 of 18 h-incubated samples (B) and (C), and in fraction 10 of 18-day-incubated samples (D).

1b). Extending the $A\beta$ incubation time prior to fractionation led to a significant further shift in the $A\beta$ away from the top of the gradient and toward fractions 9–12. Thus incubation for 18 h led to only 20 % of the total $A\beta$ being detected in the pool comprising fractions 1–5, while 71 % was detected in the second pool (fractions 9–12), with concentrations reaching 50 μ M in fraction 10 (Figure 1c). MTT assay on the fractions incubated for 18 h also revealed a peak of activity in the second pool (Figure 1c). Extending the incubation period to 18 days resulted in all of the $A\beta$ appearing in the second pool with none detectable in the pool at the top of the gradient (Figure 1d). The majority of $A\beta$ (87 %) was found in a single fraction (fraction 10) which was also where the peak of B12 cell activity was detected.

In addition to the experiments performed on the B12 cell line, fractions from the centrifugation of 18 h-incubated peptide were applied to rat primary hippocampal cell cultures. The results from these experiments were exactly as observed for B12 cells, with biological activity, in the form of inhibition of MTT reduction, being confined to fractions 10–12 (results not shown) and absent from fractions toward the top of the gradient, which contained significantly higher concentrations of $A\beta$ peptide.

Morphological analysis of fractionated $A\beta$ 1–40

A morphological analysis of polymerized forms of $A\beta$ peptide fractionated by density-gradient centrifugation was performed. Aliquots of each fraction were prepared for analysis by transmission electron microscopy, immediately following fractionation. Three major structural forms of polymerized $A\beta$ peptide were detected. The slowly sedimenting material in the upper pool of 30 min- and 18 h-incubated $A\beta$ peptide was characterized by the presence of large sheet-like structures of $A\beta$ peptide (Figure 2A). These were the largest structures observed, often up to 10 μ m in width and clearly separated from other fibrillar forms. In samples incubated for 18 h analysis of the second pool (fractions 9–11) revealed an abundance of rod-like structures of 30–350 nm in length and 3–4 nm in diameter, with many being < 100 nm in length (Figures 2B and 2C). They were found to have a uniform diameter, were unbranched and appeared to differ only in length. These structures correspond with 'protofibrils' (as described previously [20]). Analysis of fractions 9–11 of 18-day-incubated samples revealed the presence of longer branched fibrillar structures. These were up to 5 μ m in length and 6–12 nm in diameter (Figure 2D) and correspond with the description of 'mature fibrils' [8,11]. These mature fibrils had a tendency to self-associate and were generally found in masses of aggregated fibrils rather than in isolation.

Detection of oligomeric and high molecular mass $A\beta$ 1–40 by SDS/PAGE

Comparison of fractions from 18-day-incubated $A\beta$ peptide by SDS/PAGE revealed a migration pattern different from the equivalent fractions from an 18 h-incubated sample or HFIP-treated $A\beta$. Fractions from 18-day-incubated $A\beta$ samples demonstrated a series of bands in addition to the 4 kDa band. Figure 3(A) shows the high molecular mass (> 250 kDa) polymerized $A\beta$ peptide band detected in an 18-day-incubated sample. In addition, significant amounts of streaking were observed down to the 4 kDa marker. In the low molecular mass range, four oligomeric forms of $A\beta$ were clearly visible, ranging between 4 kDa and 17 kDa. Analysis of the equivalent fractions from 18 h-incubated protofibril-containing $A\beta$ peptide samples showed no such high molecular mass polymeric $A\beta$ peptide (Figure 3B). The high molecular mass material is, therefore, a product of the longer term incubation.

Figure 3 Migration of $A\beta$ polymers on SDS/polyacrylamide gels

Aliquots of fractions from 18 day (A) and 18 h (B) incubations were analysed by SDS/PAGE on Novex precast 4–20% acrylamide gels to reveal oligomeric and polymeric $A\beta$. Detection of $A\beta$ was with antibody W02.

PCS of fractionated $A\beta$ 1–40

PCS was used to measure the average diffusion coefficients of the isolated $A\beta$ forms that produced effects on MTT reduction. Actual particle sizes were expressed as the equivalent radius of a sphere using the Stoke–Einstein relation [29].

Electron microscopic analysis revealed that the highest abundance of protofibrils was found in fraction 10 of 18 h-incubated $A\beta$ peptide. In order to gain information on the size distribution and purity of the protofibrils in this toxic pool of peptide we analysed aliquots of fraction 10 by PCS. The data (Figure 4) supported the observations made by electron microscope analysis (rod-like structures, 30–250 nm in length) revealing a major peak

Figure 4 Analysis of $A\beta$ oligomers and polymers by PCS

Aliquots of fraction 10 from an 18 h incubation were subjected to light scattering analyses as described in the Materials and methods section. A typical example is shown.

Figure 5 Immunoassay and toxicity assay of fractionated fibrillized A β 1–40

Aliquots of fractions from an 18 h incubation were assessed by either (a) MTT assay in B12 cells or (b) immunoassay, both as described in the Materials and methods section. Data represent the means $\pm$ S.E.M. ($n = 3$).

(88 % of total peak masses) of hydrodynamic radius (R_H) ranging from 22–35 nm (mean $\pm$ S.D. 26.81 ± 0.1 nm) and a minor peak (12 %) ranging from 93–367 nm (167 ± 0.36 nm).

Due to the low concentrations of A β in fractions 9–12 from the 30 min-incubated A β , analysis of these fractions could not be undertaken. Analysis of HFIP-dissolved peptide showed that greater than 99 % of material was present as monomer-size particulates with an R_H of 0.74 ± 0.06 nm.

Detection of protofibrils and fibrils by the 2F12/2F12 immunoassay

Immunoassay of the fractions from the gradient using the 2F12 capture–biotinylated 2F12 detection system revealed A β peptide only in those fractions that were found to contain protofibrils (fractions 10–12). The peak of 2F12 immunoreactivity was also found to coincide with the peak of neuronal cell toxicity (Figure 5). A similar result was obtained following the fractionation of 18 day-fibrillized A β peptide (results not shown). The 2F12 immunoassay signal was detected in fibril-containing fractions but not in the first 9 fractions, where sheet-like structures of A β peptide were found.

DISCUSSION

The senile plaque found in affected brain regions of Alzheimer's disease patients is composed of fibrils of A β peptides of varying lengths [17,30,31]. Initial studies which provided evidence *in vitro* that A β had to be converted to a fibrillar (or 'aged') form appeared to confirm fibrillar A β as the neurotoxic moiety [32]. Nevertheless, other workers have suggested that small oligomeric non-fibrillar forms of A β may be responsible, at least in part, for the neurodegenerative properties of the peptide [12], and while senile plaques and amyloid *per se* are often defined by their Congoophilic nature [1,33], Congo Red-positive polymerized A β may not be neurotoxic [34].

We therefore fractionated our polymerized A β preparations and used the known kinetics of polymerization as a further

means of characterizing the peptide forms present. To date, the most commonly used method for the fractionation of polymerized A β peptide has been size-exclusion chromatography [19]. However, we have used the density-gradient centrifugation medium Optiprep, which is more commonly utilized for cellular and sub-cellular fractionation. This has a number of advantages for the study of the bioactivity of fractionated polymers. Firstly, fractionation can be performed rapidly and with high sample recovery since there is no solid-phase for peptide to bind to, a particular problem with the A β peptide. Secondly, density-gradient centrifugation does not lead to the significant sample dilution that is inherent in size-exclusion chromatography. Finally, fractions collected from the gradient can be used directly in bioassays since Optiprep is non-toxic and non-ionic.

On applying A β freshly dissolved in the α -helix-promoting solvent HFIP to an Optiprep gradient, the form that predominated was concentrated in a pool at the top of the gradient and lacked both identifiable electron microscope structure (results not shown) and biological activity in the MTT assay. Light scattering showed that this preparation contained only small (< 1 nm), probably monomeric peptide forms. Incubating A β for up to 18 h produced a marked shift in the fractionation of the peptide, with the appearance of immunoassay- and MTT-positive forms in a second pool of peptide, centred around fraction 10. Although peptide was still detected in the pool at the top of the gradient, and at least some of this peptide was in what appeared to be a polymerized sheet-like form, this species did not have any effect on MTT reduction and was not detected by the immunoassay. Thus assembly into polymeric structure does not necessarily confer bioactivity in the MTT assay. It is quite possible that this pool also contained soluble non-toxic forms, similar to the low molecular mass species described elsewhere [35].

Fraction 10 material from the 18 h-incubated peptide, however, revealed the presence of small rod-like structures with an R_H similar to that referred to for protofibrils [20]. The existence of such intermediates in the process appears to be a common phenomenon in the fibrillization of many proteins [36]. The presence of these protofibril forms of A β is associated with an ability of the peptide to inhibit the reduction of MTT. We are not able to say whether the biological activity generated by fraction 10 is attributable to either or both of the peaks of protofibrils, as characterized by PCS. Longer incubation periods resulted in forms which were almost totally confined to the faster sedimenting pool. Gel electrophoresis of the 18 day major pool showed some laddering, not observed at 18 h, which might be evidence of the mature fibrils, that were detected by electron microscopy.

Cell death assays generally involve the incubation of cells with A β for many days, during which time the peptide may undergo further conformational changes. We therefore chose to define the biological activity of polymeric A β in terms of MTT reduction to capitalize on the relatively short exposure time necessary to detect an effect. It was noticeable that the maximum MTT inhibition after 30 min incubation was in fractions containing less than $10 \mu\text{M}$ peptide, as determined by HPLC analysis, and based on the molecular mass of monomeric A β . After 18 h incubation, however, although the maximum MTT effect was similar, the concentration of A β necessary to cause this inhibition was around $50 \mu\text{M}$, with fractions containing less than $10 \mu\text{M}$ having negligible effects in the MTT assay. However, taking into consideration the size of the dominant species, as determined by PCS, at these time points, the data support the view that the differing forms share similar degrees of activity [35] when expressed in terms of moles of multimeric form. It would appear

that the presence of small amounts of fibrillar material after 18 days of incubation has no noticeable effect on the MTT response which was very similar to that observed at 18 h. Hence the underlying conclusion, in agreement with very recent reports [24,37], that small protofibrillar forms of $A\beta$ are neurotoxic.

The fibrillization-dependent immunoassay has previously been used to demonstrate the ability of compounds to inhibit the formation of biologically active forms of $A\beta$ [24,38,39]. While the assay was clearly shown to differentiate between monomeric and multimeric $A\beta$ [24], no further attempt was made to characterize the immunopositive form or forms. The present data demonstrate that the immunopositive $A\beta$ is that which is biologically active. Furthermore, fractions from the top of the gradient, containing large amounts of non- β -amyloid, were found to be both biologically inactive and negative in the immunoassay. As yet, however, conclusive evidence is lacking to suggest that the activity observed at 18 h is associated with a total absence of mature fibrils or that the 18 day activity is produced by a preparation completely lacking protofibrils. A clear delineation of the boundaries between non-toxic and toxic forms of $A\beta$ is clearly of considerable significance in the search for potential inhibitors of the formation of toxic peptide species, since inhibition of fibril formation *per se* could enhance toxicity by causing the accumulation of a neurotoxic protofibrillar form.

SDS/PAGE analysis of fractions from the gradient revealed significantly different migrations through the gel for protofibrillar and mature fibril-containing samples. Protofibrillar-containing samples yielded a single 4 kDa band identical to that observed for freshly solubilized peptide. Mature fibril samples, however, revealed a series of oligomeric bands and a high molecular mass band (> 250 kDa). The presence of an immunoreactive signal streaking down the gel suggests the continual disaggregation of the fibrils during electrophoresis. It is apparent, however, that the fibrillar structures are considerably more resistant to disaggregation than are protofibrils.

Molecular modelling techniques coupled with solid-state Monte Carlo simulations enabled predictions of the possible quaternary and tertiary structures of $A\beta$ peptide within protofibrils to be calculated [40]. The computer simulations employed a molecular mechanics type forcefield, an Ewald electrostatic summation and periodic boundary conditions to represent the solid-state scenario. The final predicted conformation enabled an estimate to be made for the solid volume of an individual $A\beta$ 1–40 molecule when it is packed into a solid protofibril. The calculated molecular volume is approx. 6040 Å³ per $A\beta$ 1–40 molecule in the solid structure. This is considerably larger than the 4269 Å³ calculated for an *in vacuo* $A\beta$ 1–40 molecule. The protofibril diameter (measured from electron micrographs during the course of this study) was found to be 3–4 nm. Assuming a cylindrical protofibrillar structure it is possible to derive that there are 1.2–2.1 $A\beta$ 1–40 monomers per nm of protofibril length. Therefore a 50 nm protofibril would be expected to contain between 60 and 105 monomers.

Although senile plaques comprise $A\beta$ peptides of varying lengths [41], attention has focused on the 42 amino acid form, $A\beta$ 1–42, as being particularly important in Alzheimer's disease. The highly aggregatory $A\beta$ 1–42 is the predominant species in plaques [41], and four genes known to either cause or be major risk factors for Alzheimer's disease (amyloid precursor protein, presenilin-1, presenilin-2, apolipoprotein E) have been shown to increase the production of $A\beta$ 1–42 [42]. The rate of polymerization of $A\beta$ 1–42, however, is particularly rapid and makes the capture of intermediates during the fibrillization process difficult unless conditions such as temperature and pH are manipulated, generally away from what is considered physio-

logical. Nevertheless, both $A\beta$ 1–40 and $A\beta$ 1–42 polymerize to form protofibrils [19] and toxic species [21] and both low molecular mass species [13] and fibrillar forms [11] of $A\beta$ 1–42 have been found to be neurotoxic. Further fractionation studies will be required to determine conclusively whether $A\beta$ 1–42 polymerizes through identical, toxic, protofibrillar intermediates to those observed for the shorter $A\beta$ 1–40 form.

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