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An improved method of preparing the amyloid β-protein for fibrillogenesis and neurotoxicity experiments

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KEY WORDS: Alzheimer's disease, amyloid β-protein, amyloidogenesis, fibrillogenesis, neurotoxicity

ABBREVIATIONS: AAA = amino acid analysis; Aβ = amyloid β-protein; AD = Alzheimer's disease; AFM = atomic force microscopy; CD = circular dichroism spectroscopy; ATR-FTIR = Attentuated total reflectance-Fourier transform infrared spectroscopy; HPLC = high performance liquid chromatography; LDH = lactate dehydrogenase; LMWAβ = low molecular weight Aβ (monomer or dimer); MWCO = molecular weight cut-off; RT = room temperature; TFA = trifluoroacetic acid

Abstract

Synthetic amyloid β-protein (Aβ) is used widely to study fibril formation and the physiologic effects of low molecular weight and fibrillar forms of the peptide on cells in culture or in experimental animals. Not infrequently, conflicting results have arisen in these studies, in part due to variation in the starting conformation and assembly state of Aβ. To avoid these problems, we sought a simple, reliable means of preparing Aβ for experimental use. We found that solvation of synthetic peptide with sodium hydroxide (Aβ·NaOH), followed by lyophilization, produced stocks with superior solubility and fibrillogenesis characteristics. Solubilization of the pretreated material with neutral buffers resulted in a pH transition from ~10.5 to neutral, avoiding the isoelectric point of Aβ (pI≈5.5), at which Aβ precipitation and aggregation propensity are maximal. Relative to trifluoroacetate (Aβ·TFA) or hydrochloric acid (Aβ·HCl) salts of Aβ, yields of "low molecular weight Aβ" (monomers and/or dimers) were improved significantly by NaOH pretreatment. Time-dependent changes in circular dichroism spectra and Congo red dye-binding showed that Aβ·NaOH formed fibrils more readily than did the other Aβ preparations and that these fibrils were equally neurotoxic. NaOH pretreatment thus offers advantages for the preparation of Aβ for biophysical and physiologic studies.

Introduction

Alzheimer's disease (AD) is an archetype of a class of diseases, the amyloidoses, in which otherwise normal, soluble proteins or peptides accumulate in the form of amyloid deposits[1]. Amyloid deposition causes cell and organ dysfunction, leading in many cases to death. The major protein component of amyloid deposits in Alzheimer's disease is the amyloid β-protein (Aβ)[2]. An increasing body of genetic, physiologic, and biochemical data support the hypothesis that deposition of Aβ is a seminal event in AD[3]. Aβ deposition involves changes in both peptide conformation and assembly state. Studies of senile plaque amyloid have shown that Aβ forms fibrils with cross-β pleated sheet organization[4-6]. Fibrils can also be formed *in vitro* using synthetic Aβ. These fibrils are morphologi-

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cally, tinctorially, immunologically and spectroscopically indistinguishable from *ex vivo* fibrils. For these reasons, *in vitro* systems have been used extensively to study Aβ fibrillogenesis and the biological activity of Aβ assemblies. Importantly, synthetic Aβ fibrils are neurotoxic, an activity recapitulating *in vitro* one of the key pathophysiologic effects of Aβ thought to occur *in vivo* [7-9]. In addition, recent studies have shown that non-fibrillar and protofibrillar Aβ assemblies are also toxic[10-12].

In the past, interpretation of results of structure-activity studies has often been complicated by irreproducible behavior of the Aβ peptide. For example, significant differences in fibrillogenesis kinetics and neurotoxic activity have been observed using synthetic Aβ from different manufacturers, and in fact, from different peptide lots from the same manufacturer[13-15]. Lack of reproducibility can result from the presence of preexisting aggregates, or seeds, in the lyophilized peptide stocks. In addition, effective dissolution of Aβ in physiological buffers has sometimes been problematic. To overcome these problems, strong acids or organic solvents have been employed to solubilize Aβ. These include trifluoroacetic acid (TFA)[16], hexafluoroisopropanol[17], dimethylsulfoxide[18], and aqueous acetonitrile with TFA[7]. However, these non-physiologic solvents can cause conformational changes which facilitate or inhibit fibrillogenesis, creating additional variability[19]. In addition, organic solvents or acids present in peptide stocks may be directly cytotoxic.

One obvious and important explanation for experimental variation is the presence of TFA in Aβ peptide stocks. TFA comes from the cleavage and deprotection of chemically synthesized Aβ, and from subsequent HPLC purification procedures. Upon solvation of lyophilized Aβ in physiologic buffers, the pH of the Aβ microenvironment changes from acidic (pH ~2) to neutral as residual TFA is neutralized. In the process, the solution pH passes through the isoelectric point (pI) of Aβ (5.5), where Aβ precipitation and aggregation propensity are maximal[17,20]. These pH-associated effects result directly from the ionic and conformational properties of the Aβ molecule. In turn, these properties affect Aβ fibrillogenesis behavior. In particular, significant pH-dependent morphologic and kinetic differences in fibril formation have been demonstrated[17]. These differences, coupled with variations in peptide purity, concentration, solvent composition, and experimental procedure among different laboratories, can produce serious interpretative errors and conflicting results. Other acidic ion-pairing agents used for HPLC purification, e.g., hydrochloric acid[21], would not be expected to mitigate these effects because they would also cause a pH transition through the pI of Aβ. An alternative strategy would be to prepare Aβ stocks under strongly alkaline conditions, where Aβ is freely soluble and would not encounter its pI upon dissolution into neutral buffers. We report here that sodium hydroxide pretreatment of Aβ produces peptide solutions with higher yields of LMW Aβ and with lower levels of preexistent aggregates than does either no pretreatment or pretreatment with HCl. NaOH-treated Aβ reproducibly forms fibrils with conformational and tinctorial properties typical of amyloid fibrils and which are toxic to cultured neurons. Pretreating Aβ peptide stocks with NaOH may thus facilitate detailed mechanistic studies of the formation and biological activities of Aβ assemblies.

Materials and methods

Reagents and chemicals

Chemicals were obtained from Sigma (Saint Louis, MO). Water was deionized and filtered using a Milli-Q system (Millipore Corp., Bedford, MA). Tissue culture components were obtained from GibcoBRL (Grand Island, NY) and Hyclone (Logan, UT).

Peptide synthesis and pretreatment

Aβ(1-40) (NH_2-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVV-CO_2H) and Aβ(1-42) (NH_2-DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAIIGLMVGGVVIA-CO_2H) were synthesized as described[22]. Each peptide was purified by reverse phase HPLC using a gradient of acetonitrile in 0.1% (v/v) TFA. The identity and purity of the final product, Aβ·TFA, a TFA salt of Aβ, was confirmed by amino acid analysis (AAA), matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy and reverse phase HPLC. Aβ·NaOH was produced by dissolution of 1 mg of lyophilized Aβ·TFA in 1 ml of 2 mM NaOH such that the final solution pH was ~10.5. This generally required 20 µl of 0.1 M NaOH, but varied somewhat among different peptide lots. After pH adjustment, the solution was sonicated for 1 min at room temperature (RT; ~22°C) in an ultrasonic water bath (Branson Model 1200, Danbury, CT), then lyophilized. The HCl form of Aβ, Aβ·HCl, was produced by dissolving lyophilized Aβ·TFA at a concentration of 1 mg/ml in 2 mM HCl solution and then sonicating and lyophilizing as above. To control variability in peptide preparation, Aβ·TFA was pretreated with water, sonicated, and lyophilized in an analogous manner. Although the predominant counterions in the NaOH- and HCl-treated preparations are sodium and chlorine, respectively, small amounts of residual TFA may be present. For filtration experiments only, Aβ(1-40) was also obtained from Bachem (Lot T-20513, Torrance, CA) and Quality Controlled Biochemicals (Lot 0313621C, Hopkinton, MA). All experiments, with the exception of those presented in Table 4, were done using Aβ(1-40).

Therefore, unless otherwise specified, the abbreviation Aβ refers to Aβ(1-40).

Attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR)

ATR-FTIR was used to examine the secondary structure of Aβ in lyophilizates examined as dry powders. A Nicolet 800sx IR machine with a liquid nitrogen cooled MCT detector was used. Briefly, 1 mg of solid peptide was applied to the surface of the internal reflection element. Spectra were recorded at 4 cm^{-1} resolution. One thousand interferograms were averaged to obtain each absorption spectrum. The data were Fourier-transformed with medium Norton-Bear apodization, using a single beam setting for the background. For the water vapor and sample data, the medium Norton-Bear apodization was applied in absorbance mode using the Fourier-transformed background spectrum, followed by subtracting the contribution of water vapor peaks until the 1700 to 1800 cm^{-1} region was featureless. The amide I region between 1600 and 1700 cm^{-1} was analyzed first using second derivative spectra to determine band locations. These band positions were used to fit the raw spectrum to a combination of Gaussian-Lorentzian peaks. The band positions were allowed to vary by ± 3 cm^{-1} from the values determined by the second derivative spectrum. The heights of individual peaks were allowed to vary, and the widths were relaxed gradually starting from 15 cm^{-1} until 25 cm^{-1} during the fit. The area under each peak was then used to compute the percentage of individual secondary structure component contributing to the amide I region spectrum.

Filtration experiments

Samples were prepared by dissolving 300 μg of each peptide in 400 μl of water, vortexing briefly, and then sonicating for 1 min at RT in a Branson ultrasonic water bath. Aliquots were removed for AAA and then the remaining solution was filtered through 8 mm diameter 0.2 μm nylon micro-spin Whatman filters (Clifton, NJ) at 5,000 × *g* for 10 min at RT. Aliquots of each filtrate were removed for AAA and the remaining solution filtered through 6 mm diameter Microcon 10 centrifugal filter devices (Millipore, Bedford, MA) at 14,000 × *g* for 30 min at RT. AAA was then performed on the starting solution and the two filtrates. Initial Aβ concentrations ranged from 105-123 μM. The yield of Aβ in each filtrate was calculated relative to the initial Aβ concentration (i.e., prior to any filtration). Each individual filtration experiment (i.e., solvation, 0.2 μm filtration, 10 kDa filtration) was done a minimum of three times and each resulting aliquot provided three independent samples for AAA. The molecular weight exclusion limits of both filtration devices were unaffected by NaOH, HCl, or TFA at the concentrations used in these experiments (data not shown).

Circular dichroism spectroscopy (CD)

Samples were prepared by dissolving 200 μg of each peptide in 250 μl of water, vortexing briefly, and then filtering through 8 mm diameter 0.2 μm micro-spin Whatman filters at 5,000 × *g* for 10 min at RT. Phosphate buffer (10 mM sodium phosphate, pH 7.4, containing 200 mM sodium fluoride) was filtered through 8mm diameter 0.2 μm nylon filters (Gelman Sciences, Ann Arbor, MI), after which 250 μl were added to the peptide filtrate. The sample was vortexed and then immediately transferred into a 1 mm cuvette (Hellma, Forest Hills, NY) and analyzed using an Aviv Model 62A DS spectropolarimeter (Aviv Associates, Lakewood, NJ). No differences in fibrillogenesis behavior are observed between Aβ samples prepared in phosphate-buffered saline or phosphate-buffered sodium fluoride (data not shown), however the latter solvent is superior for CD spectroscopy. Spectra were recorded at RT from ~195-240 nm at 1 nm resolution with a scan rate of 0.25 nm/s. Raw data were manipulated by smoothing and subtraction of buffer spectra, according to the manufacturer's instructions. Deconvolution of the resulting spectra was achieved using the LINCOMB program[23] and the basis set of Brahms and Brahms[24]. The relative amounts of random coil, α-helix, β-sheet, and β-turn in each sample were determined from the normalized contribution of each secondary structure element function to the observed spectrum following curve fitting. Each experiment was repeated three times and the data obtained in each were similar.

Congo red binding assay

Peptides were dissolved in 5 mM sodium phosphate, pH 7.4, containing 100 mM sodium fluoride, as described above. Following filtration, concentrations were 61 μM (Aβ·HCl), 63 μM (Aβ·NaOH), and 65 μM (Aβ·TFA). After vortexing, each sample was incubated at 37°C without agitation. Aliquots were removed after 2 h, 1 d, 2 d, and 3 d, and Congo red binding assayed essentially according to Klunk *et al.*[25], but using a microtiter plate reader (ThermoMax, Molecular Devices, Sunnyvale, CA). Briefly, 225 μl of 20 μM Congo red in 20 mM potassium phosphate, pH 7.4, containing 0.15 M sodium chloride, was added to 25 μl of sample, mixed, and incubated for 30 min at RT. The absorbance of the resulting solutions was then determined at 480 and 540 nm. The amount of Congo red bound (Cb) was calculated using the formula Cb (μM)= $(A_{540}/25{,}295) - (A_{480}/46{,}306)$. The Cb values shown were obtained after subtraction of Cb values for buffer alone. All experiments were repeated three times and each sample was analyzed in triplicate.

Atomic force microscopy (AFM)

Experiments were performed as described[26]. Briefly, 3-5 μl of evenly suspended material were placed on the sur-

face of freshly cleaved mica (Ted Pella Inc., Reading, CA). After 30 s, the surface was rinsed gently, twice, with 50 µl of MilliQ water to remove salt and loosely bound peptide. Excess water was then removed with a gentle stream of compressed tetrafluoroethane. The sample was immediately imaged in the dry state under ambient conditions using a Nanoscope IIIa Multimode scanning probe workstation (Digital Instruments, Santa Barbara, CA).

Neurotoxicity assay

Mixed primary rat neuronal cultures, containing both glia and neurons, were prepared as described[27], with slight modifications. Briefly, cells were isolated from the neocortex of E15-17 Sprague Dawley rat embryos and plated onto poly-L-lysine coated 96-well microtiter plates (Falcon, Franklin Lakes, NJ). The medium was Dulbecco's minimal essential medium (DMEM), containing 5% (v/v) bovine calf serum, 10% (v/v) Ham's F-12, HEPES (20 mM), L-glutamine (2 mM), and penicillin-streptomycin (500 units/ml and 500 µg/ml, respectively). Cell density was 2×10^4 cells/well. Cultures were used 2-4 weeks after plating. Each of the three types of Aβ peptide was preincubated at a concentration of 0.5 mM in 20 mM HEPES, pH 7.4, for 0, 1, 2 or 3 d, then diluted ten-fold in 50 µl of culture medium before addition to the neuronal cells. The aggregation state of the peptide preparations prior to addition to cells was determined by a combination of CD spectroscopy, Congo red binding, and electron microscopy (data not shown). Initially, CD analysis revealed β-sheet structure (~50%), however all samples were Congo red negative and contained few, if any, electron microscopically visible fibrillar structures. The significant β-sheet content suggested that the conformational transitions which eventually lead to fibril formation had already started. This was not unexpected considering the high initial Aβ concentration (0.5 mM). In fact, after 1 d of incubation, turbidity was apparent visually and was accompanied by increased β-sheet content, Congo red binding, and the presence of fibrils. After 2 and 3 d, strong Congo red binding was observed in all samples, but formation of large suprafibrillar aggregates made spectroscopic measurements difficult. Neurons were incubated with each Aβ preparation for 1, 2, 3, or 4 d, after which aliquots of conditioned medium were removed for assay of lactate dehydrogenase (LDH) activity, a measure of cell death[12,27]. Each aliquot was assayed in duplicate and then the resulting activities were averaged. Cells treated with buffer alone were used as controls. All data were normalized to the LDH activity determined in the buffer-treated cells at day 1, yielding a "relative mean LDH activity" value. Although LDH can be released by both neurons and glia, visual inspection of the cultures revealed clear neuronal damage and loss, whereas glial cells remained morphologically normal (data not shown). All experiments were repeated a minimum of three times.

Table 1. Secondary structure of Aβ determined by ATR - FTIR

Structure	Aβ·NaOH	Aβ·TFA	Aβ·HCl
β-sheet	46	59	50
3_{10}-helix/turn/loop	23	20	19
turn	21	8	11
α-helix			4
disordered coil	10	13	16

Lyophilized peptide stocks were studied using ATR-FTIR. Spectra were deconvoluted using Fourier self-deconvolution and second derivative resolution enhancement (see Methods). The percentage of each secondary structure element is listed.

Results

Structure of Aβ peptides in the solid state

To our knowledge, the structure of Aβ in lyophilized peptide stocks has not heretofore been determined. If preexistent aggregates or fibrillogenic Aβ conformers are present in these preparations, simply solvating the material in water or buffer could produce "prenucleated" solutions, complicating studies of fibril nucleation and elongation and contributing to experimental irreproducibility. We explored this issue by using attenuated total reflectance-Fourier transform infrared spectroscopy (ATR-FTIR) to examine the secondary structure of Aβ peptides lyophilized after pretreatment with NaOH (Aβ·NaOH), HCl (Aβ·HCl), or water (Aβ·TFA) (Figure 1). Fourier self-deconvolution and second derivative resolution enhancement were used to deconvolute the raw data set and to provide numerical estimates of the levels of each secondary structure element from curve-fitting of spectral components in the amide I region (Table 1). The secondary structure assignments were based on extensive empirical and experimental studies[28]. We note, however, that the validity of amide I absorption band position and secondary structure correlations depend on a number of factors, including the sample, data acquisition method, deconvolution algorithm, and interpretative assumptions[29]. Uncertainty in assignments may occur, especially in cases where the bands fall outside typical regions. Here, we have assigned 1680-1674 cm^{-1} to turn, 1632-1629 cm^{-1} to β-structure, and 1651-1655 cm^{-1} to α-helix. Less easily assigned bands occur at 1644 cm^{-1} (disordered), 1661 cm^{-1} (3_{10}-helix, turn, or loop), and 1691 cm^{-1} (β-structure). The 1691-1695 cm^{-1} bands could be either turn or beta structure and have been assigned as beta on the basis that if assigned as turns

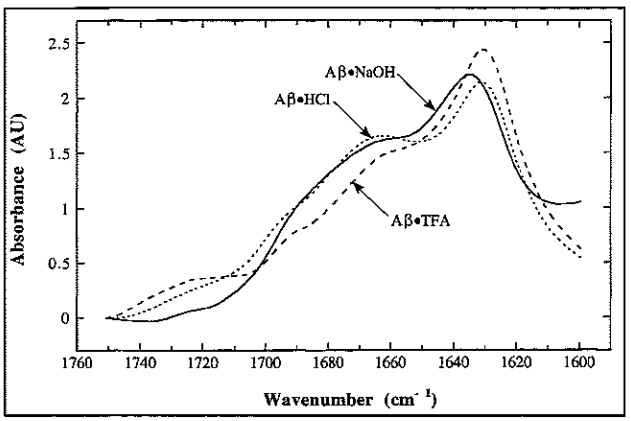

FIGURE 1: ATR-FTIR spectra of lyophilized Aβ peptides. For each peptide, Aβ·NaOH, Aβ·HCl, and Aβ·TFA, 1 mg of solid was applied to the surface of the internal reflection element and then peptide IR spectra acquired. Absorbance (AU) is plotted versus wavenumber (cm^{-1}).

the proportion of turn in the structure would be unreasonably high.

In the context of aggregation and fibril formation, the key secondary structural feature is β-structure, and with the exception of the highest frequency component, the assignments are quite clear in ascertaining the overall distribution between β-structure and helix/turn/disordered structure. Comparison of the three peptides indicates that each has a similar overall structure, but that subtle differences exist among them. Each is dominated by β-structure, with Aβ·TFA exhibiting the highest level of this component and Aβ·NaOH the lowest. Conversely, the Aβ·NaOH sample displayed the highest turn percentage, approximately twice that observed in the other samples. The significant amounts of β-structure found in the lyophilized peptides suggested that solvation of these preparations could produce solutions containing peptide aggregates. Depending on their size and structure, these aggregates could affect both the bulk solubility characteristics and the fibrillogenesis behavior of Aβ peptides in the resulting solutions.

Effects of sample pretreatment on peptide aggregation state

We employed a two-step filtration paradigm to determine the sizes and amounts of the Aβ assemblies present in solution immediately following solvation. To do so, water was added to the lyophilized peptides, which were then vortexed and sonicated briefly. Each sample was then filtered sequentially through 0.2 μm porosity and 10 kDa molecular weight cut off (MWCO) filters. Before and after each filtration procedure, aliquots from each sample were removed for AAA. Starting peptide concentrations ranged from 105-123 μM. The 0.2 μm filter retains fibrils, fibril aggregates, and other structures whose dimension parallel to the membrane surface exceed 200 nm. Protofibrils and smaller Aβ oligomers pass through. The sizes of particles retained by the 10 kDa MWCO membrane can be estimated using simple and reasonable assumptions about particle density and shape. For proteins of dry density 0.2 g/ml, globular assemblies may be modeled as spheres. As such, assemblies of 10 kDa molecular mass are 5.4 nm in diameter. Perfect cylinders and prolate ellipsoids with aspect ratios of 2 will have long axes ~7.5-8.6 nm long. These relatively compact models of Aβ geometry are consistent with NMR-derived three-dimensional models of Aβ monomer structure in water[30]. Molecules retained by the 10 kDa filter thus range in size from ~5-200 nm. Aβ molecules passing through the 10 kDa membrane are thus primarily monomeric or dimeric. We refer to these molecules as low molecular weight Aβ (LMW Aβ)[11].

Initially, we studied NaOH-, HCl-, and TFA-treated forms of Aβ(1-40) synthesized in our laboratory. No significant peptide loss (i.e., retention on the filter) was observed following filtration of Aβ·NaOH through a 0.2 μm filter, whereas losses of 10% and 17% were seen for Aβ·TFA and Aβ·HCl, respectively (Table 2). The trend toward lower recovery in these latter samples was reproducible (n=3), but was not significant statistically. NaOH pretreatment resulted

Table 2. Filtration of Aβ peptides (% yield)

Peptide Source	Pretreatment	0.2 μm	10 kDa**
Teplow Lab	NaOH	98 ± 6	68 ± 5
	TFA	90 ± 7	41 ± 4***
	HCl	83 ± 8	38 ± 6***
Bachem	NaOH	96 ± 3	60 ± 5
	TFA	74 ± 4***	37 ± 4**
	HCl	80 ± 3***	42 ± 5**
QCB	NaOH	92 ± 4	62 ± 6
	TFA	84 ± 2*	38 ± 3**
	HCl	85 ± 6	39 ± 4**

Aβ(1-40) from three different sources, pretreated in three different ways, was subjected to sequential filtration through 0.2 μm porosity and 10 kDa MWCO filters. Aβ concentrations were determined by triplicate AAA and each filtration series was performed a minimum of three times. Similar results were obtained in each case. Data from a representative experiment are presented. Initial Aβ concentrations ranged from 105-123 μM. The percent recovery of Aβ after each filtration step was calculated for each pretreatment group relative to the initial Aβ concentration. Data are presented as mean percent yield ± S.D. Statistical significance of the differences in yield between the Aβ·NaOH and Aβ·TFA samples and between the Aβ·NaOH and Aβ·HCl samples was determined using an unpaired t-test as implemented in StatView v5.0.1 (SAS Institute, San Francisco, CA). Symbols are: ***, $p<0.003$; **, $p<0.02$; *, $p<0.05$.

in improved yields following filtration of the 0.2 μm filtrates through the 10 kDa porosity membranes. For Aβ·NaOH, an average of 68% of the total starting amount was recovered after this second filtration, whereas only 41% and 38% of the Aβ·TFA and Aβ·HCl samples, respectively, were recovered. The increases in yield of LMW Aβ (66-79%) obtained by NaOH pretreatment were highly significant ($p < 0.003$).

To determine whether the improvement in yield was, in fact, due to NaOH pretreatment and not to idiotypic properties of the peptide lot, we repeated the filtration series on Aβ(1-40) samples prepared using starting peptide obtained from two commercial sources, Bachem and QCB. The same trends were observed for each of these additional peptides. For the Bachem peptide, yields of LMW Aβ were increased 43-62% by NaOH pretreatment (n=3). The QCB peptide displayed a 59-63% increase in yield (n=3). For both the Bachem and QCB peptides, the increases in yield for the Aβ·NaOH samples (versus Aβ·HCl or Aβ·TFA samples) were significant ($p<0.02$). These data show that solvating NaOH-pretreated Aβ(1-40) with water produces solutions containing few large (>0.2 μm) aggregates and significantly fewer numbers of Aβ oligomers (5-200 nm in size) than are produced using HCl-pretreated or standard TFA-containing synthetic peptides.

Effects of sample pretreatment on temporal changes in Aβ conformation

To determine whether peptide pretreatment affected the conformational changes associated with fibril formation, temporal changes in the secondary structure of Aβ were examined by circular dichroism spectroscopy (Figure 2). To do so, Aβ·NaOH, Aβ·HCl, and Aβ·TFA were dissolved in water, filtered through 0.2 μm filters, mixed with filtered phosphate buffer (pH 7.4), then allowed to fibrillize at RT. Immediately following dissolution, the spectra of the three peptides showed one minimum at 198 nm, characteristic of a predominantly random coil (RC) structure. Deconvolution of the CD spectra showed, in fact, ~70% RC structure in each of the three peptides, with the remainder being primarily β-strand (Table 3). After 2 d, the Aβ·TFA peptide displayed an increase in α-helix content and a decrease in RC content. The Aβ·HCl peptide showed a small increase in β-structure with a concomitant decrease in RC. In contrast, after 2 d, the Aβ·NaOH peptide displayed an ~2-fold increase in β-structure and an ~50% decrease in RC. In addition, 20% α-helix was observed, compared to 4% seen initially. Six hours later, the spectrum of Aβ·NaOH showed one minimum at 218 nm and a β-sheet level of 65%. It took 6 d for the Aβ·HCl and Aβ·TFA peptides to approach this level of β-sheet structure (Figures 2A and B; Table 3). Interestingly, as in the Aβ·NaOH case, a transitory increase in the level of α-helix was detected in both the Aβ·TFA and Aβ·HCl samples. Each peptide thus appeared to undergo similar transitions in secondary structure, but with NaOH pretreatment significantly accelerating the process.

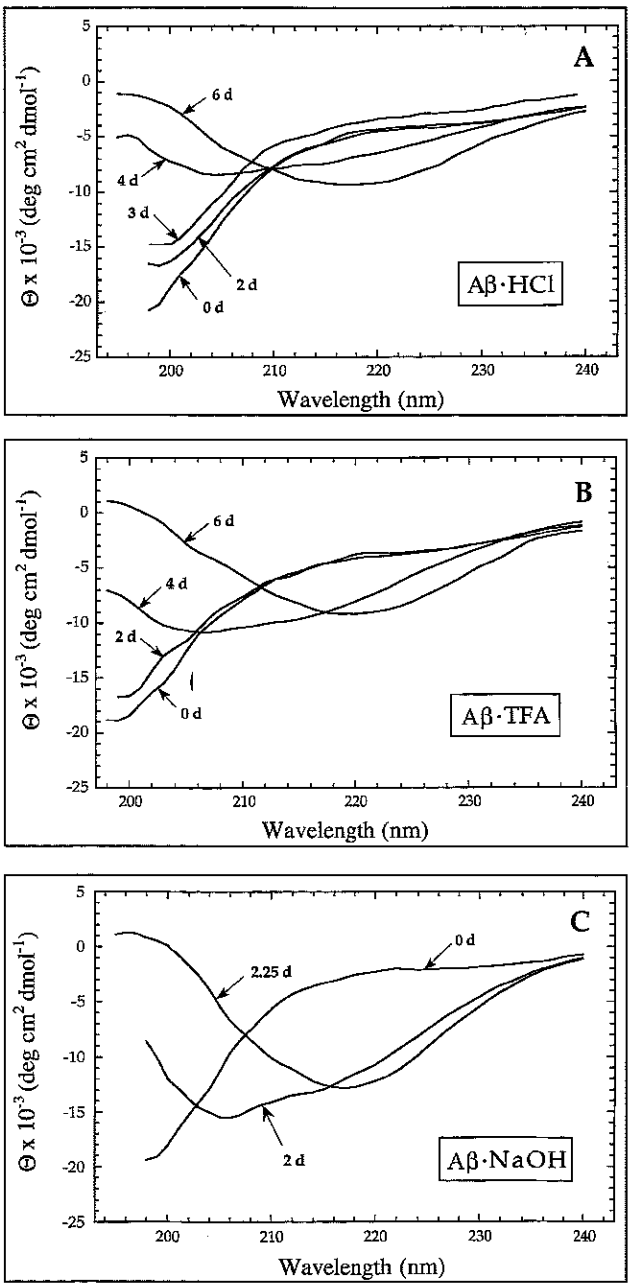

FIGURE 2: Temporal changes in the CD spectra of Aβ peptides during fibrillogenesis. CD spectroscopy was performed periodically after dissolution of Aβ·HCl (A), Aβ·TFA (B), or Aβ·NaOH (C), in phosphate buffer. Starting peptide concentration, determined by AAA, was 65 μM. Molar ellipticity (θ) is plotted versus wavelength for each of the three peptide samples.

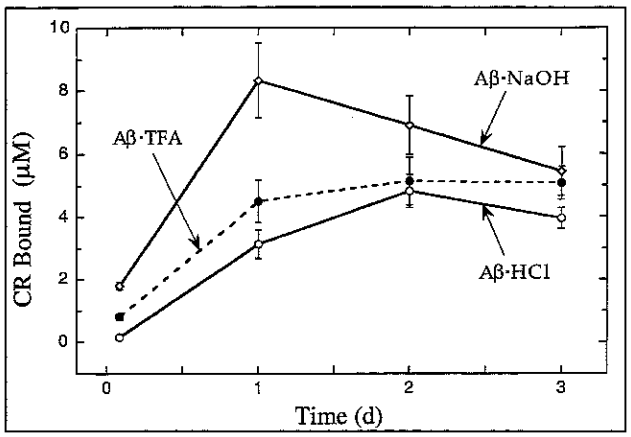

FIGURE 3: Temporal changes in Congo red binding during fibrillogenesis. Aβ·NaOH, Aβ·TFA, and Aβ·HCl peptides were dissolved in phosphate buffer, incubated at 37°C for 2h, 1 d, 2 d, and 3 d, and then assayed for Congo red binding. All samples were assayed in triplicate. Data are expressed as mean Congo red (CR) bound in units of μM ± SD.

Effects of sample pretreatment on temporal changes in Congo red binding

The previously observed differences in rates of conformational change among the NaOH-, HCl-, and TFA-treated Aβ peptides suggested that rates of amyloid fibril formation and aggregation could differ depending on sample preparation method. To examine this question, Congo red binding was used as a measure of fibril formation. Following incubation at 37°C for 2 h, 1 d, 2 d, or 3 d, samples were assayed (Figure 3). Initial (2 h) levels of Congo red binding were significantly greater ($p<0.0005$) in the Aβ·NaOH sample than in the Aβ·HCl and Aβ·TFA samples. The total amount of Congo red bound increased over time. For Aβ·NaOH, maximal binding occurred at ~1 d and was significantly higher ($p<0.01$) than the maximal levels bound by the other peptides. Maximal binding occurred later, at ~2 d, for the Aβ·HCl and Aβ·TFA samples. The increased rate and maximal levels of Congo red binding by the Aβ·NaOH sample relative to the other samples suggested that NaOH pretreatment facilitated amyloid fibril formation, as opposed to formation of disordered aggregates. In all cases, binding levels decreased after reaching their maxima. This is a common observation in fibrillogenesis experiments and likely reflects sequestration of binding sites due to fibril-fibril interaction and aggregate precipitation.

Morphology of Aβ polymers

In order to correlate the temporal changes in the secondary structure of the three different peptide preparations with their morphologies, aliquots of the samples used for CD studies were examined using atomic force microscopy. Initially, a dense layer of small, irregularly-shaped particles was observed in samples from each of the three peptides (Figure 4). The heights of these particles varied between 3 and 12 nm with the 3.5-4.5 nm size particles being the most abundant. In addition, a few short fibrils were seen which probably preexisted in the solution. After 2 d, numerous protofibrils 3.5-4.5 nm in height and 20-120 nm in length, along with globular particles 4-10 nm high, were observed in the Aβ·TFA and Aβ·HCl samples. A number of short fibrils, 6 nm in height, were also seen in the Aβ·HCl sample. In contrast, in the Aβ·NaOH sample, protofibrils were observed after 1 d (data not shown), but by 2 d many mature fibrils were visible. These fibrils ranged in height from 3.5-5.7 nm and were generally in excess of 1 μm in length, dimensions characteristic of "type 2" fibrils described previously[31]. These fibrils displayed a variety of periodic structures, some of which appeared helical and some of which displayed rod-like segments ~100 to 200 nm long with slightly tilted axes (similar to sausage links). Shorter fibrils were also seen in which axial periodicities were apparent. Whether these periodicities were due to axially-stacked globular subunits or to twisted pairs of filaments was not clear. After 6 d, fibrils with lengths exceeding 1 mm were formed by all peptides. Aβ·TFA and Aβ·HCl formed fibrils 9-10 nm in height which displayed a helical periodicity of ~40 nm ("type 1" fibrils[31]). Although type 2 fibrils were

Table 3. Conformational change in Aβ during fibrillogenesis

Pretreatment	Time (d)	α-helix	β-structure	Coil
NaOH	0	4	28	68
	2	20	54	26
	2.25	10	65	25
TFA	0	6	24	70
	2	12	25	63
	3	17	27	56
	4	21	28	51
	6	9	55	36
HCl	0	3	30	67
	2	3	35	62
	3	5	38	57
	4	18	45	37
	6	8	62	30

Aβ·NaOH, Aβ·TFA, and Aβ·HCl were incubated in phosphate buffer at RT to induce fibril formation. CD spectra were acquired periodically and were deconvoluted using the algorithm of Perzcel et al.[23] and the Brahms reference spectra library[24]. The percentage of each secondary structure element is listed. "β-structure" includes both β-sheet and β-turn. Each experiment was repeated three times, yielding similar data in each case. Results from one such experiment are presented.

occasionally observed in these samples, type 1 fibrils predominated. The opposite was true for the Aβ·NaOH samples, where type 2 fibrils predominated and type 1 fibrils were observed occasionally.

Neurotoxicity

To determine the biological activities of NaOH-, HCl-, and TFA-treated Aβ peptides, toxicity assays were performed using cultured rat primary cortical neurons. Each peptide was incubated for 0, 1, 2, and 3 d at 37°C in 20 mM HEPES buffer, then diluted ten-fold in tissue culture medium to yield a final Aβ concentration of 50 μM and added to the rat cortical cultures. Each day, for four consecutive days, cell death was assessed by measuring the release of LDH into the culture medium. A total of three experiments were performed. Results from a representative experiment are shown in Figure 5. Here, each peptide caused a reproducible, time-dependent increase in LDH release. Maximal release generally was observed after 4 d of treatment. Absolute levels of LDH release varied depending on the peptide preincubation period. In all experiments, maximal levels of LDH release were produced using Aβ peptides that either were not preincubated or were preincubated for only 24 h. In most experiments, relative to cells treated only with medium, significant increases in LDH release were observed in cells treated with peptide for 2 d or more. At these time points, for the Aβ·NaOH peptides, 24 h preincubation consistently produced the greatest LDH release. For the Aβ·HCl and Aβ·TFA preparations, the greatest effects were observed using freshly dissolved peptide or peptide preincubated for 24 h.

To confirm that the similarity in toxic behavior among the three preparations was meaningful and not due to some type of "saturation" of the assay at 50 μM Aβ concentration, cells were also treated with peptides at concentrations of 15, 30, and 45 μM. Assay of LDH release each day for three consecutive days showed that each peptide caused a time-dependent and concentration-dependent increase in LDH release (data not shown). No significant differences were observed among the three different Aβ preparations.

Table 4. Filtration of Aβ(1-42) peptides (% Yield)

Pretreatment	0.2 μm	10 kDa
NaOH	94 ± 4	31 ± 3
TFA	10 ± 1*	03 ± 1*
HCl	56 ± 3*	08 ± 2*

Aβ(1-42), pretreated in three different ways, was subjected to sequential filtration through 0.2 μm porosity and 10 kDa MWCO filters. Initial Aβ concentrations ranged from 89-97 μM. The percent recovery of Aβ after each filtration step was calculated for each pretreatment group relative to the initial Aβ concentration. Statistical significance of the differences in yield between the Aβ·NaOH and Aβ·TFA samples and between the Aβ·NaOH and Aβ·HCl samples were highly significant (*, $p<0.0001$), as determined using an unpaired t-test implemented in StatView v5.0.1 (SAS Institute, San Francisco, CA).

Discussion

Stochastic factors have often complicated experimental studies of Aβ fibril formation and of the biological activity of fibrillar and non-fibrillar Aβ assemblies[14]. An important source of variability is the Aβ peptide itself, and in particular, the method used to prepare the peptide stock prior to study. The goal of the work presented here was to develop a simple procedure for preparation of Aβ lyophilizates, which upon dissolution at physiologic pH, would produce non-aggregated starting solutions of Aβ in high yield. Importantly, these solutions would reproducibly give rise to amyloid fibrils with the conformational, tinctorial, morphologic, and neurotoxic properties ascribed to fibrils found *in vivo*. We found that dissolution of TFA salts of synthetic Aβ in dilute NaOH, to yield a pH of ~10.5, followed by lyophilization, produced peptide stocks with these desired characteristics. With respect to yields of LMW Aβ, NaOH-pretreated Aβ was a superior starting material relative to the TFA salt of the peptide and to Aβ pretreated with HCl.

The success of the method for preparing peptide stocks of Aβ(1-40) suggested that it might also be of value in the preparation of Aβ(1-42), an important and experimentally troublesome peptide. For this reason, Aβ(1-42) was substituted for Aβ(1-40) in a new series of filtration and CD experiments. Filtration data showed that NaOH-pretreatment of Aβ(1-42) produced a highly significant increase in yield relative to peptides that were untreated or were treated with HCl ($p<0.0001$, Table 4). CD analysis showed that immediately following dissolution, Aβ(1-42)·TFA and Aβ(1-42)·HCl were composed predominantly of β-sheet (71% and 69%, respectively), and that following only 2h or 5h of incubation at room temperature, respectively, both peptides were extensively aggregated (data not shown). In fact, the Aβ(1-42)·TFA sample was already turbid upon dissolution in buffer. In contrast, Aβ(1-42)·NaOH was predominantly disordered immediately after dissolution (64% RC) and did not display high levels (68%) of β-sheet structure until 18h of incubation. NaOH pretreatment is thus of significant utility for both Aβ(1-40) and Aβ(1-42).

In validating the above strategy for Aβ preparation, we studied both pre- and post-solvation characteristics of each of the three different peptide preparations. ATR-FTIR spec-

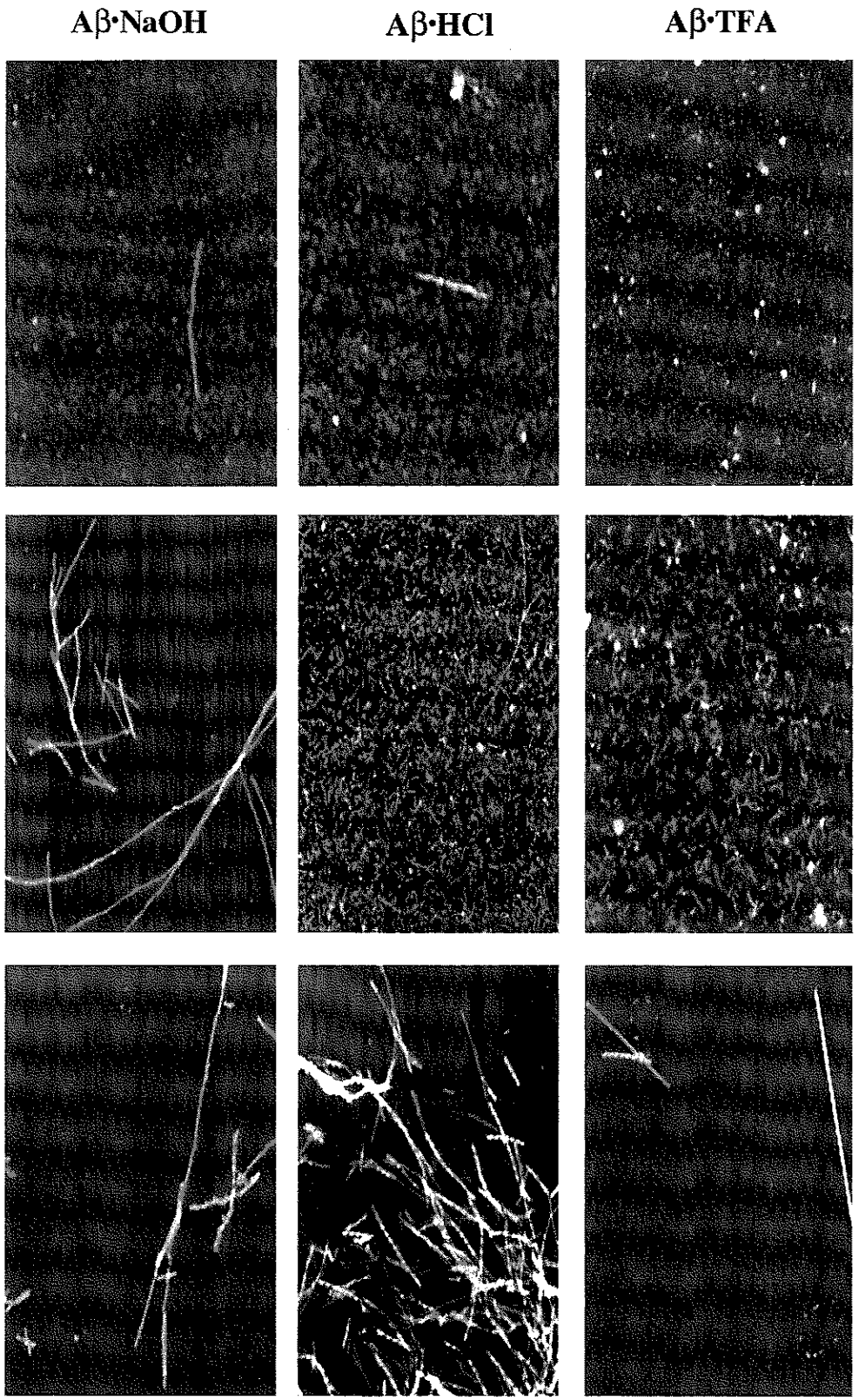

FIGURE 4: Morphology of Aβ assemblies determined by atomic force microscopy. Aliquots of the Aβ·TFA, Aβ·HCl, and Aβ·NaOH samples used for CD experiments were examined by AFM after 0 d, 2 d, or 6 d of incubation. The brightness of the features increases as a function of their height. The image width is 2 μm.

troscopy revealed that in the solid state, each preparation contained significant levels of β-sheet structure. Interestingly, immediately after dissolution in buffer, the predominant secondary structure element in each molecule, determined by CD spectroscopy, was random coil. The β-sheet structure of the peptides in the lyophilized state may thus result primarily from aggregated extended chains, which disaggregate upon solvation. These data suggest that caution should be exercised in correlating the structure of Aβ in the solid state with its structure and behavior in solution. β-sheet structures contained within short fibrils or other ordered Aβ oligomers, and which do not denature upon solvation, may account for the residual β-sheet signals in the CD spectra. Although the absolute levels of β-sheet structure found in each preparation immediately following dissolution were similar, differences in the tertiary and quaternary structures of these assemblies have the potential to produce significant differences in the resulting fibrillogenesis kinetics. Assemblies such as protofibrils, which are "on pathway" for fibrillogenesis, should facilitate fibril formation. In contrast, assemblies which are "off pathway" should impede fibril formation. In fact, CD and AFM studies of the temporal changes in Aβ conformation and morphology occurring during fibrillogenesis did reveal kinetic differences between the NaOH-pretreated peptides and the acid-treated peptides. NaOH-treated peptides consistently displayed accelerated rates of conformational change from random coil to β-sheet. This change in secondary structure correlated with fibril formation, as determined by AFM. Images acquired periodically following peptide dissolution showed that long, ordered fibrils formed within 2 d in the case of the Aβ·NaOH peptide, whereas 6 d were required for similar assemblies to appear in the Aβ·TFA and Aβ·HCl samples.

The increased propensity of Aβ·NaOH peptides for fibril formation, relative to the acid-treated peptides, argues that the conformation and assembly state of the Aβ·NaOH peptides are particularly fibrillogenic. In fact, Congo red positive Aβ·NaOH assemblies were formed earlier in the fibrillogenesis process and bound more Congo red than did their Aβ·HCl or Aβ·TFA homologues. The temporal differences in Congo red binding were consistent with the CD and AFM results. The slower overall kinetics displayed in these latter studies resulted from performing the fibrillogenesis reactions at lower temperature (~22°C versus 37°C) in order to facilitate monitoring of conformational and morphological changes. The fact that the Aβ·NaOH peptides bound more Congo red than did the other peptides could be due to the Aβ·NaOH peptides forming greater numbers of Congo red binding assemblies or to these assemblies having greater intrinsic ability to bind the dye. AFM studies suggested that both possibilities could be true. Aβ·NaOH peptides formed fibrils more rapidly than did acid-treated peptides, and in addition, the relative proportions of different fibril types differed between the Aβ·NaOH peptides and the Aβ·HCl or Aβ·TFA peptides. Aβ·NaOH

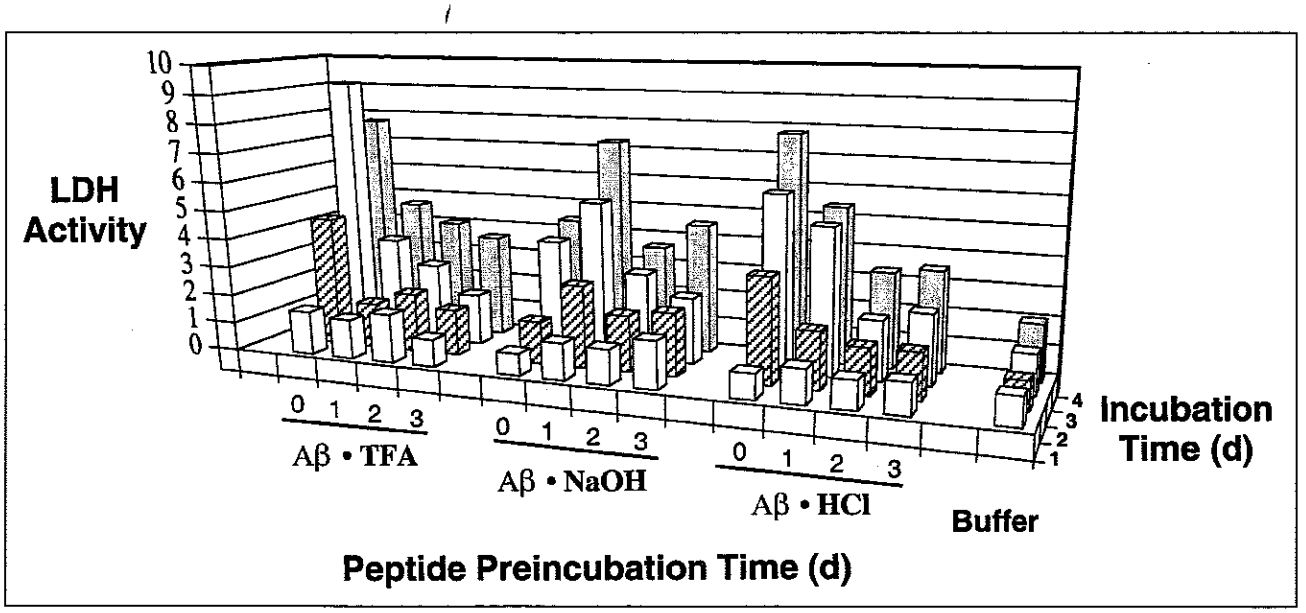

FIGURE 5: Neurotoxicity of Aβ peptides. Primary rat cortical neurons were treated with Aβ peptides that had been preincubated for 0, 1, 2, or 3 days in HEPES buffer. Following addition of peptide, aliquots of conditioned medium were assayed in duplicate for LDH activity each day for four consecutive days. Peptide preincubation time is noted at the bottom of the figure. The interval of peptide incubation with cells is noted at the bottom right. Relative mean LDH activity (see Materials and Methods) is noted at the left. The graph presents results from one experiment, but is representative of results from at least 3 independent experiments.

formed abundant fibrils with heights of ~3-6 nm and lengths which often exceeded 1 mm. Aβ·HCl and Aβ·TFA formed fibrils of similar length but with heights of ~9-10 nm. Recent AFM studies have suggested that rigid fibrils of lesser height occur late in the fibrillogenesis process[31], a fact consistent with the acceleration of fibrillogenesis observed following NaOH pretreatment.

Empirical observations of the solubility behavior of the three peptide preparations also support the idea that NaOH pretreatment facilitates fibril formation. Solutions of Aβ·NaOH peptides in water remain transparent upon addition of neutral buffer. In contrast, when buffer is added to solutions of Aβ·TFA and Aβ·HCl in water, transitory turbidity is observed. This suggests that the pH transition occurring in both Aβ·TFA and Aβ·HCl solutions, from highly acidic to neutral, causes peptide precipitation. In these precipitates, Aβ is likely to exist in a non-fibrillogenic assembly. As the pH of the solution rises past 5.5 (the pI of Aβ), the peptide again becomes soluble as disaggregation occurs. However, it is possible that incomplete repopulation of a fibrillogenic conformational state decreases the fibrillogenesis rate and the final fibril number (relative to Aβ·NaOH peptides).

Although the kinetics of fibrillogenesis differed among the peptides studied here, similar secondary structure transitions occurred during fibril formation. In each case, a predominantly disordered peptide formed a stable β-sheet rich moiety in the context of an ordered polymeric assembly. This process involved formation of an α-helix-containing intermediate. This type of intermediate has recently been described in studies of amyloid protofibril formation[11]. The N-terminus of Aβ contains amino acids with good propensities for α-helix formation and thus this region may be involved in forming the partially helical intermediate[32]. A helical N-terminus could be stabilized through the intermolecular association of Aβ molecules and then convert to β-sheet during the final stages of fibril formation. It is important to emphasize that the structural transitions which we propose mediate the formation of fibrils occur in a concerted manner among monomeric Aβ and its higher order assemblies. In particular, it is unlikely, and there is no evidence extant, that a stable β-sheet structure can be formed in a monomeric Aβ molecule. This type of structure is found only in Aβ molecules within multimeric Aβ assemblies such as fibril nuclei, protofibrils, or mature amyloid-type filaments. This mechanism of "concerted β-sheet formation" differs from hypotheses suggesting that the N-terminal domain of soluble Aβ (amino acids 10-24) is helical and must convert to β-sheet prior to the polymerization of Aβ into fibrils[15, 33].

The outcome of the secondary and higher order structural transitions of Aβ is the formation of fibrils. Each of the three peptide preparations produced a similar series of fibril intermediates. Small globular structures, often 3.5-4.5 nm in height, were commonly observed in the initial stages of fibril formation. These gave rise to typical amyloid protofibrils[11, 22, 31, 34] which formed mature amyloid type fibrils with time.

The biological activity of the fibrils formed by each of the three peptide preparations was examined by measuring the release of LDH from cultured primary rat cortical neurons. Each of the peptides caused similar and substantial increases in LDH release over the 4 d time course of the experiment. Assays of Aβ toxicity often have involved preincubation of peptide preparations prior to addition to cells. This was done to produce fibrils, the Aβ assembly thought to be the key mediator of toxicity. However, recent studies have demonstrated that in addition to mature fibrils, a number of smaller Aβ assemblies are toxic[10-12]. Here, we found that lengthy preincubation is not required to cause toxicity. In fact, maximal toxic effects were consistently observed either with no preincubation or with a 24 h preincubation. Longer periods, e.g., 48-72 h, resulted in decreased toxic effects. A likely explanation for this phenomenon is fibril-fibril interaction, which can result in the lateral association of fibrils to form extensive aggregates and in the orthogonal enmeshing of fibrils as they become very long. These types of macromolecular fibril structures either may not come into contact with susceptible cells, due to precipitation, or if they do contact cells, they may be incapable of inducing toxic effects.

In summary, the results presented here show that NaOH pretreatment of Aβ significantly improves the yield of Aβ during preparation of peptide stocks. In addition, substantially fewer aggregates are found in these stocks, diminishing the variability caused by preexistent seeds. Aβ·NaOH peptides readily form fibrils which are conformationally, tinctorially, and morphologically similar to typical amyloid fibrils. Importantly, these fibrils are toxic to cultured primary neurons. NaOH pretreatment may thus prove to be a useful procedure for improving the reproducibility and reliability of experiments designed to study the biophysical and biological behavior of Aβ.

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