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ACCELERATED COMMUNICATION

Secondary Structure of Amyloid β Peptide Correlates with Neurotoxic Activity *In Vitro*

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SUMMARY

Amyloid β peptide (Aβ), the major protein constituent of senile plaques in patients with Alzheimer's disease, is believed to facilitate the progressive neurodegeneration that occurs in the latter stages of this disease. Early attempts to characterize the structure-activity relationship of Aβ toxicity *in vitro* were compromised by the inability to reproducibly elicit Aβ-dependent toxicity across different lots of chemically equivalent peptides. In this study we used CD spectroscopy to demonstrate that Aβ secondary structure is an important determinant of Aβ toxicity. Solubilized Aβ was maximally toxic when the peptide adopted a β-sheet conformation. Three of the four Aβ lots tested had a random coil conformation and were weakly toxic or inactive, whereas the single Aβ lot exhibiting toxic activity at low peptide concentrations had significant β-sheet structure. Incubation of the weakly toxic Aβ lots in aqueous stock solutions for several days before use induced a time-dependent conformational transition from random coil to β-sheet and increased Aβ toxicity in three different toxicity assays. Furthermore, the secondary structure of preincubated Aβ was dependent upon peptide concentration and pH, so that β-sheet structures were attenuated when peptide solutions were diluted or buffered at neutral and basic pH. Our data could explain some of the variable toxic activity that has been associated with Aβ in the past and provide additional support for the hypothesis that Aβ can have a causal role in the molecular neuropathology of Alzheimer's disease.

AD is a slowly progressing neurodegenerative disorder of the elderly that culminates in dementia and death (1). Pathologically, AD is characterized by numerous deposits of amyloid in brain senile plaques and cerebrovasculature, intraneuronal neurofibrillary tangles, selective neuronal cell loss, and gliosis (2). A major component of senile plaques is Aβ, a 39–43-residue peptide that aggregates as a β-pleated sheet to form the fibrillar congophilic deposits characteristic of AD brain (3). Aβs are derived from the proteolysis of APP, a widely expressed membrane-bound glycoprotein with multiple isoforms generated by differential splicing of a gene localized on chromosome 21 (4). The carboxy terminus of Aβ encompasses part of the putative membrane-spanning region of APP, and the amyloidogenic properties of this peptide are largely determined by this hydrophobic stretch of residues (5, 6).

Several lines of evidence suggest that plaque formation may have a causal role in the molecular pathogenesis of AD. Firstly, classical senile plaques are typically surrounded by a network of dystrophic neurites (2). This regional colocalization of senile plaques and morphologically abnormal neurons has been cited as evidence for Aβ involvement in AD neuropathology. Secondly, patients with Down's syndrome (trisomy 21) develop amyloid deposits, neuronal cell loss, and dementia at an early age that are indistinguishable from AD (7). In this disorder, abnormal Aβ deposition is believed to be a consequence of APP overexpression resulting from an extra copy of the APP gene. Thirdly, the most conclusive evidence to date that Aβ deposition may be causal in AD comes from genetic linkage studies of patients with familial AD. In certain cases this autosomal dominant form of the disease has been linked to point mutations in and around the Aβ domain of the APP gene (8, 9).

Given these findings, there is increasing support for the hypothesis that the neuronal pathology of AD is due to an abnormal accumulation of Aβ in brain, presumably caused by

ABBREVIATIONS: AD, Alzheimer's disease; Aβ, amyloid β peptide; XPP, amyloid precursor protein; ED, embryonic day; LDH, lactate dehydrogenase; XTT, sodium 3′-[1-(phenylamino)carbonyl]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate; HPLC, high performance liquid chromatography; MES, 2-(*N*-morpholino)ethanesulfonic acid.

a disease-related perturbation of APP processing. As a result, APP metabolism and Aβ neurotoxicity have become important therapeutic targets for drug discovery. Although our understanding of the cellular pathways involved in the processing of APP has increased dramatically in the past 2 years, less is known about the cellular mechanisms that underly Aβ neurotoxicity. This is partially due to the confusion that occurred early on, when conflicting data from different laboratories indicated that Aβ was inactive, directly toxic, indirectly toxic (potentiating other forms of neurotoxicity), or trophic in a number of *in vitro* model systems of AD (10–14).

The inability to reproducibly elicit Aβ-dependent neurotoxicity has given rise to a careful reassessment of the importance of assay conditions and peptide structure in determining toxic activity. Although some of the variability can be attributed to the different protocols used to assay Aβ activity *in vitro*, we recently reported variable toxic activity across chemically equivalent Aβ lots within one laboratory (14). These findings suggest that there is an intrinsic variability in Aβ neurotoxic potency that cannot be attributed to differences in experimental protocols. Very recently the importance of peptide conformation in determining Aβ toxic bioactivity became apparent when it was discovered that the *in vitro* neurotoxic potency of Aβ increased with peptide aggregation (15–17). Because Aβs are postulated to aggregate as β-sheet structures (18), in the present study we used CD spectroscopy to investigate the relationship between Aβ secondary structure and neurotoxic potency across different lots of chemically equivalent peptides. We demonstrate that increased β-sheet structure correlates with enhanced neurotoxic activity in three different neurotoxicity assays using rat embryonic neuronal cell cultures. Moreover, incubation of the weakly toxic peptide lots in water for several days induces a conformational transformation from random coil to β-sheet and increases Aβ neurotoxic potency. Finally, we show that peptide concentration and pH can influence Aβ conformation and toxicity *in vitro*.

Materials and Methods

CD Spectroscopy. Four lots (ZI682, ZI960, ZJ209, and ZK052) of synthetic Aβ(1–40), comprising the first 40 amino acid residues of the natural peptide, lyophilized as trifluoroacetate salts were purchased from Bachem. The peptides were solubilized at 1 mM or 250 μM concentrations in double-distilled deionized water as stock solutions and then diluted in water to the experimental concentrations for CD spectroscopy. Peptide concentration was confirmed by amino acid analysis or by measurement of the UV absorbance spectra of the different solutions at 278 nm and conversion to concentration values using an extinction coefficient of 0.3062 $(mg/ml)^{-1}$ cm^{-1}. In some experiments, Aβ pH values were adjusted with a buffer mixture consisting of 2 mM Tris·HCl, 2 mM acetate, and 2 mM MES. An AVIV model 62DS CD spectrometer was used to record spectra in the far-UV (260-200-nm) range. Using a cuvette of 0.2-cm path length, spectra were collected at room temperature with step intervals of 1.5 nm and an average time of 3 sec. Three scans were averaged and plotted as mean residue ellipticity (θ), in units of deg cm^2/dmol, after subtraction of the background (water) spectrum (19). Curves were fit with a smoothing algorithm that performed a least squares polynomial fit of the original data.

Primary rat neuronal cultures. ED 18 rat hippocampal cells were plated at a density of 150,000/cm^2 onto plastic dishes coated with polyethyleneimine. Cells were maintained in serum-containing tissue culture medium for 10 days *in vitro* before incubation with different concentrations of freshly prepared or aged Aβ in a serum-free defined medium (as described in Ref. 14). After a 4-day incubation period, aliquots of culture medium were assayed for LDH levels with a standard 340-nm LDH assay (Sigma). ED 18 rat cortical cells were used to study the effects of Aβ on neuronal cell responses to an excitotoxic challenge. Cells seeded into polyethyleneimine-coated dishes at a density of 250,000/cm^2 were exposed to 25 μM freshly prepared or aged Aβ in serum-containing medium at 3 days *in vitro*. After a 2-day incubation period, changes in cytosolic Ca^{2+} concentrations elicited with 25 μM glutamate were assayed with the fluorescent calcium dye fluo-3 (as described in Ref. 20). Cell viability was assayed in sister cultures plated at 125,000 cells/cm^2, by measuring the reduction of the tetrozolium salt sodium XTT in metabolically active cells, after 4 days of incubation with freshly prepared or aged Aβ. XTT metabolism forms a colored soluble formazan product that can be quantified spectrophotometrically (as described in Ref. 21).

Results

Synthetic Aβs comprising up to the first 43 amino acid residues of the natural peptide are intrinsically quite soluble in aqueous solutions (6). Before forming fibrillar aggregates that are qualitatively similar to Aβ deposits in senile plaques (18), the longer peptides residues (1-28, 1-39, 1-40, and 1-42) slowly assemble as soluble β-sheet structures (6, 22–25). At a qualitative level CD spectra of solubilized peptides can reveal the presence or absence of defined peptide secondary structure. A peptide without defined secondary structure elicits a negative peak near 199 nm (random coil). α-Helical conformations produce a doublet of negative peaks near 208 and 222 nm, and β-sheet structures have a single negative peak around 217 nm. With this method spectral intensities at the different wavelengths reflect the proportional contribution of the different secondary structures to a given peptide conformation (19).

Aβ lots ZI960 and ZI682 exhibited different *in vitro* neurotoxic potencies in an earlier study (14). Whereas 10 μM ZI682 was significantly neurotoxic to rat hippocampal cells, 10 μM ZI960 was inactive. Aqueous stock solutions (250 μM) of both lots were diluted to 10 μM in water immediately before CD spectroscopy. Although both peptides had equivalent primary structures, a qualitative comparison of the CD spectra generated with the two lots revealed significant differences in peptide secondary structure (Fig. 1). The spectra of the toxic peptide ZI682 had a negative peak near 218 nm, indicating that this

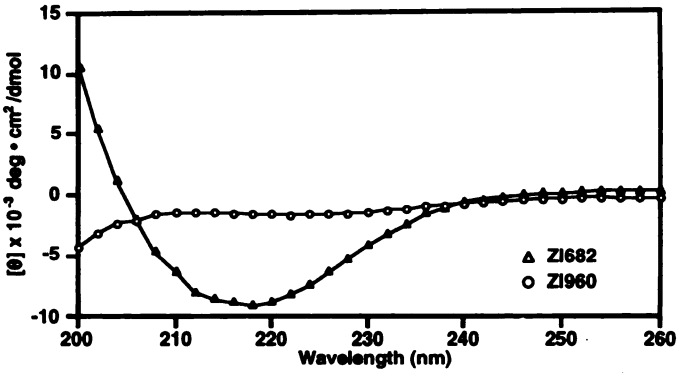

Fig. 1. Secondary structures of two chemically equivalent Aβ lots that exhibited different toxic activities in a previous study. CD spectra of 10 μM ZI682 and ZI960 diluted from 250 μM water stock solutions that had been stored at −20°. ZI682, which was toxic to embryonic rat hippocampal cells, had significant β-sheet structure at this concentration. Conversely, 10 μM ZI960, which was nontoxic, had a random coil conformation. pH values for 10 μM ZI682 and ZI960 were 4.5 and 4.6, respectively.

peptide had significant β-sheet structure. Conversely, the spectra elicited with ZI960 had a negative peak near 200 nm, resembling a random coil conformation.

Incubating Aβ for several days in aqueous solutions before use ("aging") enhances Aβ toxicity and promotes peptide aggregation (15–17). Because aggregation is dependent upon β-sheet formation, we investigated whether changes in peptide secondary structure could account for the age-induced increases in neurotoxic activity. Stock solutions of Aβ lot ZI960 (1 mM) in water were either used immediately or allowed to age for 7 days at 37°. After a 4-day incubation period with freshly prepared or aged Aβ diluted into the tissue culture medium, hippocampal cell death was assayed by measuring LDH levels in the conditioned medium. Although freshly prepared ZI960 was not toxic below 50 μM, aging increased peptide toxicity so that 25 μM ZI960 became routinely toxic (Fig. 2A). Consistent with the aging-induced increase in LDH efflux, large numbers of degenerating neurons and glia were observed by phase-contrast microscopy (data not shown). CD spectra of 25 μM dilutions (into water) from the aged and fresh Aβ stock solutions used in the LDH neurotoxicity assay are illustrated in Fig. 2B. At this concentration, fresh ZI960 was a random coil and aged ZI960 was a β-sheet.

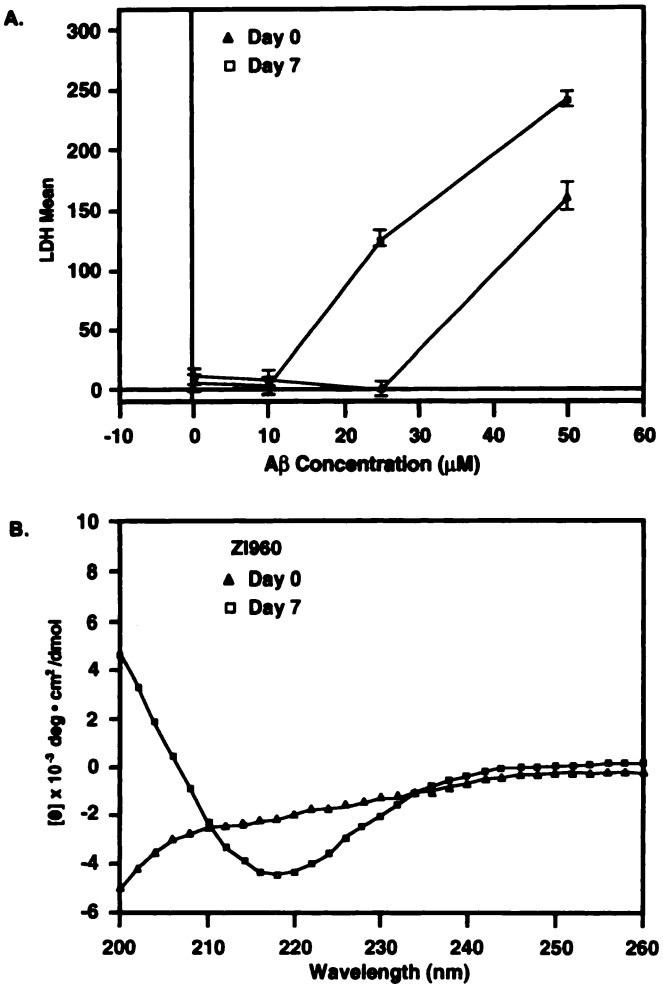

Fig. 2. Aging enhances the neurotoxic potency of Aβ lot ZI960 and promotes β-sheet formation. A, LDH levels in conditioned medium of hippocampal cultures incubated with different concentrations of freshly prepared or 7-day-aged Aβ lot ZI960 diluted from 1 mM aqueous stock solutions. Data are plotted as units/ml LDH; *bars*, 1 SEM (four experiments). Results were replicated in three independent experiments. B, CD spectra of 25 μM dilutions of ZI960 (into water) from the same stock solutions used in A. The pH of freshly prepared or aged Aβ stock solutions was 2.4–2.5.

To establish whether the correlation between β-sheet structure and toxic potency could be extended to different peptide lots and neurotoxicity assays, we examined the effect of Aβ lot ZJ209 on glutamate-induced increases in neuronal cytosolic Ca^{2+} levels. Aβ has previously been shown to disrupt intracellular Ca^{2+} homeostasis, thereby causing sustained increases in cytosolic Ca^{2+} levels (13) that can precede by up to several days any morphological evidence of neurodegeneration (17). After 2 days of incubation with 25 μM ZJ209 diluted from 1 mM freshly prepared stock solutions, ED 18 rat cortical cells displayed normal elevated intracellular Ca^{2+} responses to glutamate (Fig. 3A). In contrast, cortical cultures incubated with 25 μM ZJ209 diluted from 7-day aged stock solutions exhibited larger sustained increases in cytosolic Ca^{2+} levels after a glutamate pulse, relative to control cultures or cultures exposed to fresh peptide. Aging also increased the neurotoxic activity of ZJ209, as assessed by XTT metabolism in sister cultures. Freshly prepared ZJ209 was weakly toxic at 50 μM. Aging resulted in a substantial potentiation of neurotoxic potency so that 12.5 μM ZJ209 became significantly toxic (Fig. 3B). The CD spectra for 25 μM fresh and aged ZJ209 (Fig. 3C) demonstrated that the transition from random coil (freshly prepared) to β-sheet (aged) coincided with the enhanced toxicity observed after aging.

Aβs aggregate as oligomeric β-sheet structures; therefore, the rate of aggregation is dependent upon the peptide concentration (6, 22, 24). Consistent with these findings, aging Aβ at higher concentrations reduced the incubation time necessary for Aβ to acquire significant β-sheet structure (Fig. 4A). A 50 μM dilution of Aβ lot ZJ209 from 250 μM stock solutions required 6 days of aging before exhibiting any β-sheet structure (Fig. 4A, *left*), whereas 50 μM ZJ209 diluted from 1 mM stock solutions had significant β-sheet structure after only 3 days of aging (Fig. 4A, *right*). Freshly prepared ZJ209 had a random coil conformation that did not exhibit any obvious concentration dependence (Fig. 4B, *left*). However, once Aβ adopted a β-sheet structure, peptide conformation could be influenced by concentration. Aβ serially diluted from aged 1 mM stock solutions of ZJ209 immediately before CD spectroscopy contained progressively less β-sheet structure, eventually reverting to a random coil conformation near 20 μM (Fig. 4B, *right*).

pH is an important determinant of Aβ secondary structure, aggregate formation, and solubility (6, 22–26). At pH 2.5 (pH of unbuffered 1 mM stock solutions of Aβ trifluoroacetate salt) we routinely observed a time-dependent increase in β-sheet formation across different Aβ lots (Figs. 2B, 3C, and 4). To investigate the pH dependence of Aβ secondary structure, 25 μM aliquots of Aβ lot ZK052 diluted from freshly prepared or 5-day aged 1 mM stock solutions were adjusted to different pH values immediately before CD spectra were collected. Freshly prepared ZK052 was mostly a random coil across the tested pH range (Fig. 5A). However, a comparison of the peak spectral intensities elicited with 25 μM aged ZK052 at pH 3.81 and 7.32 (Fig. 5B) revealed that the β-sheet structure acquired with aging under acidic conditions could be reduced simply by increasing solution pH. At pH 9.14 aged ZK052 was again a random coil. Like the concentration-dependent conformational changes described above, the pH-induced changes in Aβ sec-

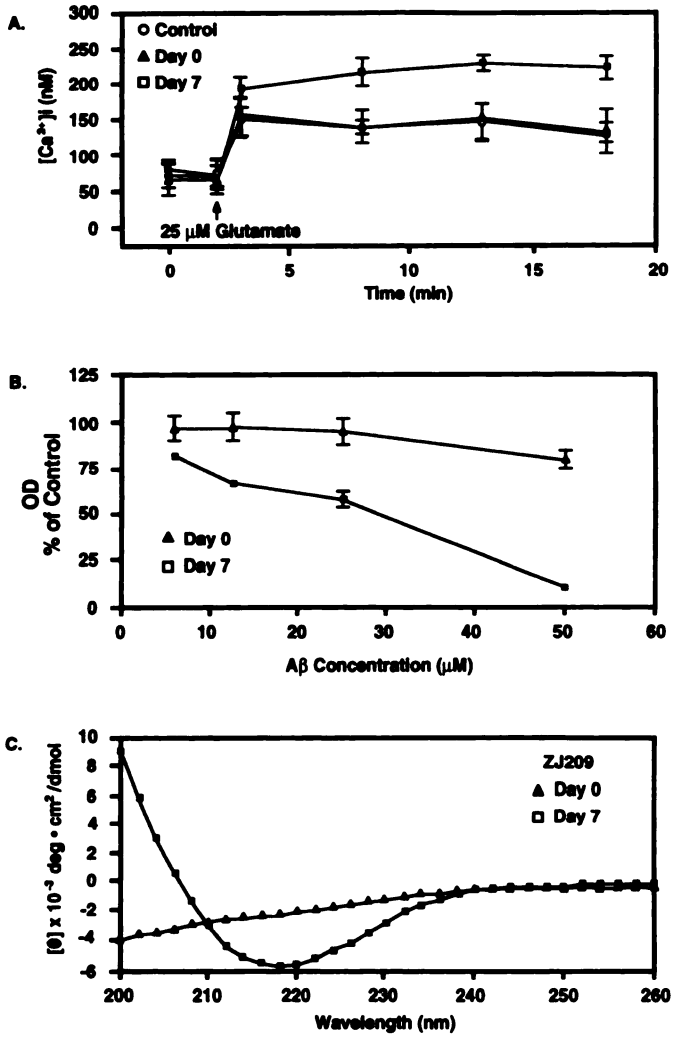

Fig. 3. Time-dependent potentiation of Aβ lot ZJ209 toxicity parallels the change in peptide secondary structure. A, Enhanced intracellular Ca^{2+} evoked with 25 μM glutamate in cortical cell cultures. Cells were incubated without peptide or with 25 μM ZJ209 diluted from 1 mM freshly prepared or 7-day-aged water stocks. Values represent the mean and standard deviation (four experiments). These results were replicated in five independent experiments. B, Cell viability of cortical cell cultures as a function of exposure to different concentrations of freshly prepared or aged ZJ209. Decreases in XTT metabolism reflect increased toxic activity. The absorbance of this metabolic reaction is expressed as a percentage of values measured in control cultures. *Bars*, mean ± 1 SD (four experiments). Similar results were observed in three independent experiments. C, CD spectra for the peptides (25 μM) used in these neurotoxicity assays. The pH of stock solutions remained constant (2.4–2.5) throughout the experiment.

ondary structure occurred immediately, within the time necessary to collect a CD spectrum (15 min). Even when Aβ was aged in a nonacidic environment, which is reportedly not optimal for β-sheet formation (6, 22, 26), significant β-sheet was formed. Fig. 5C depicts the change in Aβ secondary structure that was observed when ZK052 was aged for 7 days at pH 7.42.

Peptide characterization by amino acid sequencing, reverse phase HPLC, and electrospray mass spectroscopy did not reveal any significant differences between Aβ lots or between fresh and aged peptides (data not shown). Whereas the HPLC profiles of the aged peptides did exhibit several extra small peaks that summed to <1% of the dominant Aβ peak, peptide concentration and pH of the stock solutions remained constant over a 7-day period.

Discussion

We have characterized the relationship between Aβ secondary structure and neurotoxic activity in primary neuronal cell cultures. Our findings demonstrate that the secondary structure of solubilized Aβ(1-40) can be highly predictive of *in vitro* neurotoxic activity. Within the time constraints of our neurotoxicity assay protocols, Aβ that had a β-sheet structure was maximally toxic to rat embryonic neuronal cell cultures, whereas Aβ that was predominantly a random coil was either nontoxic or weakly toxic. Aβ lot ZI682, the only peptide that exhibited neurotoxic activity below 20 μM without aging, assumed a β-sheet structure immediately after going into solution. Under similar conditions the less toxic lots (ZI960, ZJ209, and ZK052) adopted a random coil structure. It would seem surprising that different lots of chemically equivalent peptides would adopt such different secondary structures. However, slight variations in the synthesis, purification, and/or lyophilization of these peptides and/or the presence of trace contaminants could significantly alter peptide conformation. Some of the initial confusion in attempting to demonstrate Aβ toxicity using low concentrations of freshly prepared peptide may be explained by this lot-to-lot variability in peptide secondary structure.

Aging Aβ in 1 mM aqueous solutions at 37° for 3–7 days evoked a time-dependent conformational transistion and significantly enhanced Aβ toxicity. After aging, weakly toxic lots displayed increased β-sheet structure, became more toxic (as determined by enhanced LDH levels and decreased XTT metabolism), and potentiated neuronal cellular responses to excitoxic concentrations of glutamate. However, at high concentrations of freshly prepared peptide we occasionally observed a dissociation between β-sheet formation and toxic activity. Although 77 μM freshly prepared Aβ did not exhibit any obvious β-sheet structure, 50 μM freshly prepared Aβ displayed moderate toxic activity in one of the neurotoxicity assays (Fig. 2A). It is possible that freshly prepared Aβ has some underlying β-sheet structure that our qualitative CD spectroscopy analyses fail to reveal. A more likely alternative is that conformational changes initiated during incubation in water continue in tissue culture medium, so that toxicity becomes apparent with long (2–4-day) incubation protocols.

The longer Aβs are reported to have a mixture of α-helical and β-sheet structures in solution, especially in the presence of solvents that stabilize Aβ α-helical formation (6, 22, 25). In this study the only ordered secondary structures observed when Aβ(1-40) was solubilized in water were β-sheets. It is possible that our qualitative CD analysis and/or lack of spectral information below 200 nm mask a relatively small α-helical component (19). However, Tomski and Murphy (23), using quantitative CD spectroscopy to scan in the far-UV (190–240-nm) range also concluded that Aβ(1-40) solubilized in water did not have any α-helical structure.

Although freshly prepared Aβ did not exhibit any obvious change in secondary structure as a function of concentration or pH, once an Aβ sample adopted a β-sheet structure the peptide conformation was sensitive to subsequent changes in concentration and pH. The conformational changes induced with aging were easily reversed by diluting the peptide concen-

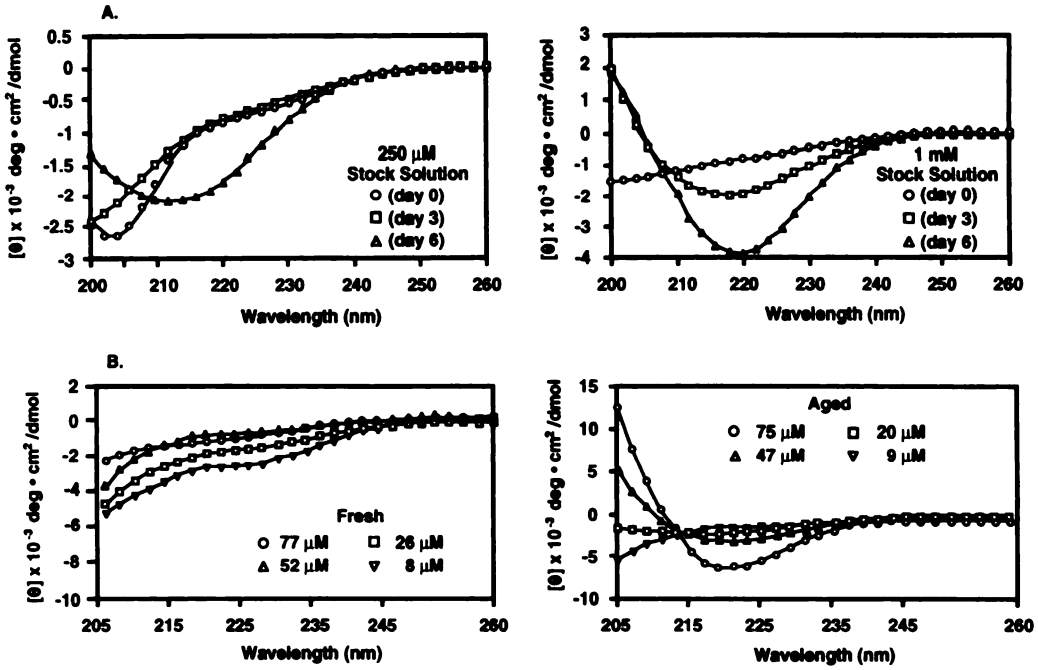

Fig. 4. Peptide concentration in aqueous stock solutions influences the rate of β-sheet formation and modulates the secondary structure of aged Aβ. A, Rate of β-sheet formation as a function of aging for 250 μM (*left*) or 1 mM (*right*) stock solutions. Aβ lot ZJ209 was aged for 3 or 6 days at 37° and then diluted to 50 μM for CD spectroscopy. pH values were 3.0 for 250 μM solutions and 2.5 for 1 mM solutions. B, CD spectra of freshly prepared (*left*) or aged (3 day) (*right*) ZJ209 samples made up as 1 mM stock solutions, which were serially diluted to different concentrations immediately before CD analysis. The pH of 1 mM stock solution was 2.5.

tration. Likewise, β-sheet structures acquired with aging were attenutated when Aβ was buffered at basic pH values. Also, although β-sheet formation of the longer Aβs is facilitated between pH 4 and pH 7 (6, 22), we invariably observed a progressive increase in β-sheet formation when Aβ was aged at pH 2.5 or 7.42. This discrepancy probably reflects the different incubation and solubilization protocols used. We routinely aged Aβ in water for longer periods of time (3–7 days), at higher concentrations (1 mM), and at elevated temperatures (37°). All of these factors could compensate for the less suitable pH environment, so that we were still able to observe a time-dependent increase in β-sheet formation with our aging protocols.

Peptide solubility decreases as Aβ aggregates, particularly in solutions of high ionic strength (6, 22–25). β-Sheet formation facilitates peptide aggregation; therefore, decreases in peak spectral intensities as determined by CD spectroscopy could reflect loss of spectral information as aggregated Aβ precipitates from solution. However, in pure aqueous solutions, within the time constraints of our aging protocols, we observed increased β-sheet formation without a concomitant change in Aβ solubility. Although light microscopic inspection of aged 1 mM stock solutions revealed the presence of a small particulate fraction that was not present in freshly prepared Aβ stock solutions, peptide concentration (as assessed by amino acid analysis or by measurement of the UV absorbance spectra of stock solutions and diluted Aβ samples) remained constant with aging. In contrast, 1 mM Aβ solutions became increasingly viscous after aging for 10–12 days and exhibited a visible "gel" after 1 month (data not shown).

Pike *et al.* (16) reported recently that the only Aβs exhibiting toxic activity formed stable aggregates *in vitro*. This is in agreement with our findings showing a correlation between β-sheet secondary structure and Aβ neurotoxicity. Because Aβ aggregation is believed to require intermediate conformational changes that include β-sheet formation and stacking of β-sheet structures (18), aging may stabilize and/or accelerate the appearance of a conformation that facilitates Aβ aggregation. However, we cannot rule out the possibility that the toxic conformation is a β-sheet and/or a lower-order soluble aggregate. β-Sheet formation and aggregation are closely coupled events that are difficult to separate, especially using neurotoxicity assays that require incubation periods lasting from several hours to 4 days before overt signs of neurodegeneration are elicited. It is possible that a soluble toxic Aβ conformation triggers a slow degenerative process that does not become apparent until after the peptide has proceeded to the stable aggregate stage. In light of this fact, it is interesting to note that Pike *et al.* (16) concluded that, although toxicity was dependent upon the formation of stable aggregates, the degree of peptide aggregation could not be correlated with neurotoxic potency.

If a discrete secondary structure and/or aggregate state is important for Aβ toxic activity, certain predictions about conditions that would modulate Aβ toxicity can be tested *in vitro*. For example, "overaging" of Aβ could diminish neurotoxic potency. We have data suggesting that there is an optimal incubation duration for maximizing Aβ toxicity (27). The enhanced neurotoxic potencies elicited with aging reach a maximum and then disappear with longer aging protocols, even before changes in solution turbidity indicate significant peptide precipitation. Also, recently tested Aβ lots that are significantly toxic and have a β-sheet structure when freshly prepared at low concentrations (similar to lot ZI682) become less toxic with aging.[1] Under these conditions the reduced neurotoxic poten-

[1] Patrick C. May, Kimberly S. Fuson, Wei Ying Li, and Linda K. Simmons, unpublished observations.

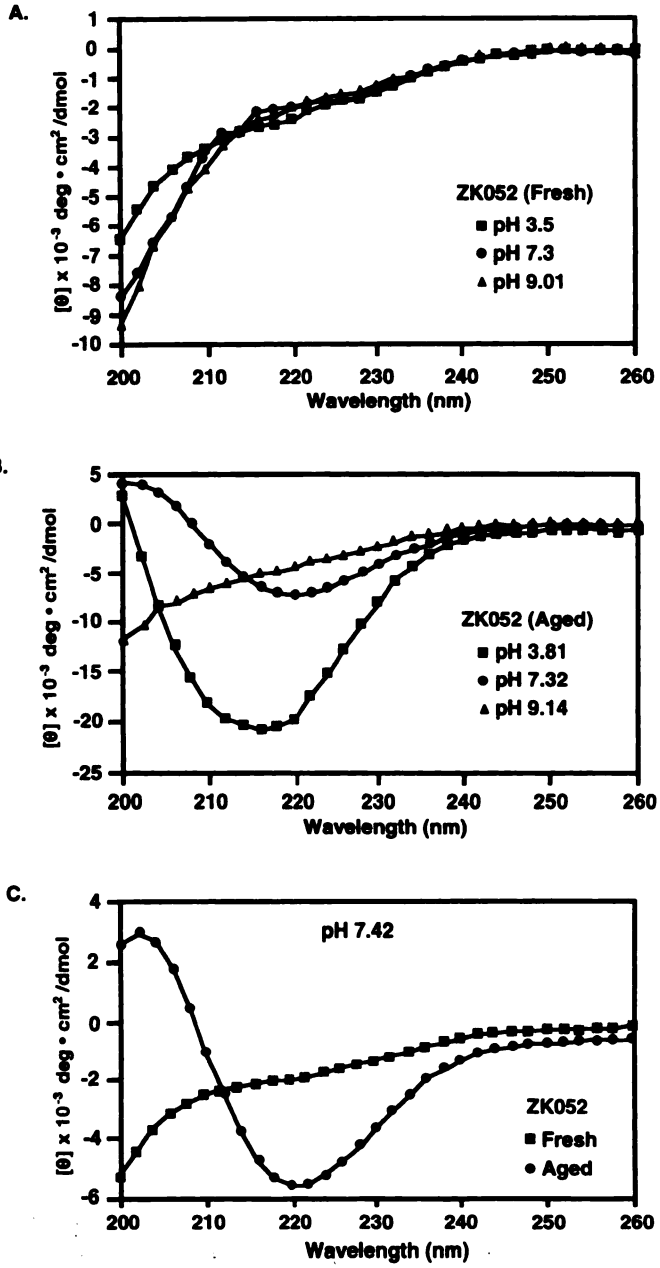

Fig. 5. pH dependence of Aβ lot ZK052 secondary structure. A, CD spectra of freshly prepared ZK052 diluted to 25 μM from 1 mM stock solutions. The unbuffered pH of 25 μM ZK052 was 3.5. The pH of 25 μM ZK052 was adjusted to pH 7.3 and 9.01 immediately before spectra were collected. B, β-Sheet structure of 25 μM 5-day-aged ZK052. Peptide secondary structure was analyzed without buffering (pH 3.81) or with the pH adjusted to 7.32 or 9.14. C, Time-dependent increase in β-sheet formation when ZK052 was aged for 7 days in 1 mM stock solutions buffered at pH 7.42. The CD spectrum was collected at 25 μM.

cies could be explained by a specific toxic conformation/aggregate state that is masked by subsequent conformational changes as Aβ continues to form larger aggregates and fibril-like structures in tissue culture medium. It is also possible that the β-sheet structures formed during aging could facilitate aggregation to such an extent that Aβ precipitates upon being added to tissue culture medium, thereby limiting peptide interaction with large numbers of cells.

Quite recently a heterogeneous collection of Aβ fragments have been detected in cerebrospinal fluid of normal individuals and patients with AD (28). Because these findings raise the possibility that circulating Aβ could contribute to plaque formation and neurodegeneration in AD pathology, characterizing the conformational and aggregational states of solubilized Aβ becomes even more important, especially if Aβ conformation is an important factor for eliciting toxic activity. Consistent with previous reports, we demonstrate that Aβ conformation can be influenced by conditions in the surrounding microenvironment. Moreover, the ease with which β-sheet formation can be reversed does not appear to be an artifact of solubilizing Aβ in water. Stable aggregates of Aβ formed in salt-containing solutions at physiological pH are in equilibrium with solubilized Aβ, so that resuspension of sedimentable Aβ in solution increases peptide solubility (24). These studies suggest that the conformational behavior of soluble and insoluble Aβ could be manipulated *in vitro* to systematically determine the conditions that promote or decrease toxic activity. Furthermore, drugs that stabilize the random coil conformation and/or disrupt β-sheet secondary structures and aggregation could be tested *in vitro* for neuroprotective activity. Given the paucity of *in vivo* animal models for AD, *in vitro* testing of potentially therapeutic compounds could prove to be a viable alternative.

Although the mechanisms underlying Aβ neurotoxicity *in vitro* are still poorly understood, converging data from different laboratories suggest that peptide conformation may have an important role in determining toxic activity. Mattson *et al.* (17) have proposed that aggregated Aβs elicit toxic activity by disrupting intracellular Ca^{2+} remove buffering homeostasis. Arispe *et al.* (29) have demonstrated that Aβ can form Ca^{2+}-permeable ion channels in artificial membranes, providing a possible mechanism whereby Aβ conformation could dictate the ease with which Aβ could form membrane-spanning pores that would compromise cell viability. Using whole-cell voltage-clamp protocols (30), we have observed increases in hippocampal neuron membrane conductances with 1 μM Aβ that had significant β-sheet structure, whereas 1 μM freshly prepared Aβ (random coil) was inactive. These findings suggest that the β-sheet conformation may be required to elicit Aβ-dependent increases in biological membrane permeability. Whatever mechanisms are involved, it is becoming increasingly apparent that the structure-activity relationship for Aβ neurotoxicity might be a common motif for peptides that aggregate as a β-sheet structures. For example, Forloni *et al.* (31) have recently described neurotoxic activity *in vitro* with a pathogenic prion protein that aggregates to form amyloid fibrils.

Any attempt to model AD *in vitro* should take into consideration the fact that Aβ aggregation and fibril formation are prominent features of AD pathology. To date, little is known about the relationship between potentially toxic soluble Aβ conformations and the insoluble Aβ deposits found in senile plaques. It is possible that other plaque components, such as heparan sulfate proteoglycans (32), α_1-antichymotrypsin (33), apolipoproteins (34), and trace metals (35), can act as "chaperones" to facilitate precipitation of plaque amyloid. Of particular interest is the recent report describing the identification of an apolipoprotein (apolipoprotein J) that selectively binds soluble Aβ in cerebrospinal fluid (36). Given the accumulating evidence for conformationally dependent Aβ toxicity, it will be important to investigate plaque-dependent factors that could

have an impact upon Aβ secondary structure, aggregation, solubility, and neurotoxic activity.

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References

1. Katzman, R. Alzheimer's disease. *N. Engl. J. Med.* **314**:964–972 (1986).
2. Selkoe, D. J. Biochemistry of altered brain proteins in Alzheimer's disease. *Annu. Rev. Neurosci.* **12**:463–490 (1989).
3. Glenner, G. G., and C. W. Wong. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem. Biophys. Res. Commun.* **120**:885–890 (1984).
4. Kang, J., H.-G. Lemaire, A. Unterbeck, J. M. Salbaum, C. L. Masters, K. H. Grzeschik, G. Multhaup, K. Beyreuther, and B. Muller-Hill. The precursor of Alzheimer's disease amyloid A4 protein resembles a cell-surface receptor. *Nature* (*Lond.*) **325**:733–736.
5. Jarrett, J. T., E. P. Berger, and P. T. Lansbury, Jr. The carboxy terminus of the β amyloid protein is critical for the seeding of amyloid formation: implications for the pathogenesis of Alzheimer's disease. *Biochemistry* **32**:4693–4697 (1993).
6. Barrow, C. J., A. Yasuda, P. T. M. Kenny, and M. G. Zagorski. Solution conformations and aggregational properties of synthetic amyloid β-peptides of Alzheimer's disease: analysis of circular dichroism spectra. *J. Mol. Biol.* **225**:1075–1093 (1992).
7. Wisniewski, K., H. Wisniewski, and G. Wen. Occurrence of neuropathological changes and dementia of Alzheimer's disease in Down's syndrome. *Ann. Neurol.* **17**:278–282 (1985).
8. Murrell, J., M. Farlow, B. Ghetti, and M. Benson. A mutation in the amyloid precursor protein associated with hereditary Alzheimer's disease. *Science* (*Washington D. C.*) **254**:97–99 (1991).
9. Goate, A., M.-C. Chartier-Harlin, M. Mullan, J. Brown, F. Crawford, L. Fidani, L. Giuffra, A. Haynes, N. Irving, L. James, R. Mant, P. Newton, K. Rooke, P. Roques, C. Talbot, M. Pericak-Vance, A. Roses, R. Williamson, M. Rossor, and J. Hardy. Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. *Nature* (*Lond.*) **349**:704–706 (1991).
10. Yankner, B. A., L. K. Duffy, and D. A. Kirschner. Neurotropic and neurotoxic effects of amyloid β protein: reversal by tachykinin neuropeptides. *Science* (*Washington D. C.*) **250**:279–282 (1990).
11. Whitson, J. S., D. J. Selkoe, and C. W. Cotman. Amyloid β protein enhances the survival of hippocampal neurons *in vitro*. *Science* (*Washington D. C.*) **243**:1488–1490 (1989).
12. Roher, A. E., M. J. Ball, S. V. Bhave, and A. R. Wakade. β-Amyloid from Alzheimer disease brains inhibits sprouting and survival of sympathetic neurons. *Biochem. Biophys. Res. Commun.* **174**:572–579 (1991).
13. Mattson, M. P., B. Cheng, D. Davis, K. Bryant, I. Lieberburg, and R. Rydel. β-Amyloid peptides destabilize calcium homeostasis and render human cortical neurons vulnerable to excitotoxicity. *J. Neurosci.* **12**:376–389 (1992).
14. May, P. C., B. D. Gitter, D. C. Waters, L. K. Simmons, G. W. Becker, J. S. Small, and P. M. Robison. β-Amyloid *in vitro* toxicity: lot-to-lot variability. *Neurobiol. Aging* **13**:605–607 (1992).
15. Busciglio, J., A. Lorenzo, and B. A. Yankner. Methodological variables in the assessment of *beta*-amyloid neurotoxicity. *Neurobiol. Aging* **13**:609–612 (1992).
16. Pike, C. J., D. Burdick, A. J. Walencewicz, C. G. Glabe, and C. W. Cotman. Neurodegeneration induced by β-amyloid peptides *in vitro*: the role of peptide assembly state. *J. Neurosci.* **13**:1676–1687 (1993).
17. Mattson, M. P., K. J. Tomasselli, and R. E. Rydel. Calcium-destabilizing and neurodegenerative effects of aggregated β-amyloid are attenuated by basic FGF. *Brain Res.* **621**:35–49 (1993).
18. Halverson, K., P. E. Frazier, D. A. Kirschner, and P. T. Lansbury, Jr. Molecular determinants of amyloid deposition in Alzheimer's disease: conformational studies of synthetic β-protein fragments. *Biochemistry* **29**:2693–2844 (1990).
19. Woody, R. M. Circular dichroism of peptides, in *The Peptides* (V. J. Hruby, ed.), Vol. 7. Academic Press, New York, 15–115 (1985).
20. Wahl, P., A. Schousboe, T. Honore, and J. Drejer. Glutamate-induced increase in intracellular Ca^{2+} in cerebral cortex neurons is transient in immature cells but permanent in mature cells. *J. Neurochem.* **53**: 1316–1319 (1989).
21. Roehm, N. W., G. H. Rodgers, S. M. Hatfield, and A. L. Glasebrook. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods* **142**:257–265 (1991).
22. Barrow, C. J., and M. Zagorski. Solution structures of β peptide and its constituent fragments: relation to amyloid deposition. *Science* (*Washington D. C.*) **253**: 179–182 (1991).
23. Tomski, S. J., and R. M. Murphy. Kinetics of aggregation of synthetic β-amyloid peptide. *Arch. Biochem. Biophys.* **294**:630–638 (1992).
24. Burdick, D., B. Soreghan, M. Kwon, J. Kosmoski, M. Knauer, A. Henschen, J. Yates, C. Cotman, and C. Glabe. Assembly and aggregation properties of synthetic Alzheimer's A4/β amyloid peptide analogs. *J. Biol. Chem.* **267**:546–554 (1992).
25. Hilbich, C., B. Kisters-Woike, J. Reed, C. L. Masters, and K. Beyreuther. Aggregation and secondary structure of synthetic amyloid βA4 peptides of Alzheimer's disease. *J. Mol. Biol.* **218**:149–163 (1991).
26. Frazier, P. E., J. T. Nguyen, W. K. Surewicz, and D. Kirschner. pH-dependent structural transitions of Alzheimer amyloid peptides. *Biophys. J.* **60**:1190–1201 (1991).
27. Fuson, K. S., B. D. Gitter, G. Becker, and P. C. May. Time-dependent alterations in neurotoxicity across multiple lots of amyloid β peptide (Aβ). *Neurosci. Soc. Abstr.* **19**:832 (1993).
28. Seubert, P., C. Vigo-Pelfry, F. Esch, M. Lee, H. Dovey, D. Davis, S. Sinha, M. Schlossmacher, J. Whaley, C. Swindlehurst, R. McCormack, R. Wolfert, D. Selkoe, I. Lieberburg, and D. Schenk. Isolation and quantification of soluble Alzheimer's β-peptide from biological fluids. *Nature* (*Lond.*) **359**: 325–327 (1992).
29. Arispe, N., E. Rojas, and H. B. Pollard. Alzheimer disease amyloid β protein forms calcium channels in bilayer membranes: blockade by tromethamine and aluminum. *Proc. Natl. Acad. Sci USA* **90**:567–571 (1993).
30. Li, W. Y., and L. K. Simmons. Increased ionic conductance elicited with β amyloid peptide is dependent upon peptide secondary structure. *Soc. Neurosci. Abstr.* **19**:1037 (1993).
31. Forloni, G., N. Angeretti, R. Chiesa, E. Monzani, M. Salmona, O. Bugiani, and F. Tagliavini. Neurotoxicity of a prion protein fragment. *Nature* (*Lond.*) **362**:543–546 (1993).
32. Snow, A. D., H. Mar, D. Nochlin, R. T. Sekiguchi, K. Kimata, Y. Koike, and T. N. Wight. Early accumulation of heparan sulfate in neurons and in the *beta* amyloid protein containing lesions in Alzheimer's disease and Down's syndrome. *Am. J. Pathol.* **137**:1253–1270 (1990).
33. Abraham, C. R., T. Shirahama, and H. Potter. The protease inhibitor α_1-antichymotrypsin is associated solely with amyloid deposits containing the β-protein and is localized in specific cells of normal and diseased brain. *Neurobiol. Aging* **11**:123–129 (1990).
34. Strittmatter, W. J., K. H. Weisgraber, D. Y. Huang, L. Dong, G. S. Salvesen, M. Pericak-Vance, D. Schmechel, A. M. Saunders, D. Goldgaber, and A. D. Roses. Binding of human apoliprotein E to synthetic amyloid β peptide: isoform-specific effects and implications for late-onset Alzheimer disease. *Proc. Natl. Acad. Sci USA* **90**:8098–8102 (1993).
35. Mantyh, P., J. Ghilardi, S. Rogers, E. DeMaster, C. Allen, E. Stimson, and J. Maggio. Aluminum, iron and zinc ions promote aggregation of physiological concentrations of β-amyloid peptide. *J. Neurochem.* **61**:1171–1174 (1993).
36. Ghiso, J., E. Matsubara, A. Koudinov, N. Choi-Miura, M. Tomita, T. Wisniewski, and B. Frangione. The cerebrospinal-fluid soluble form of Alzheimer's amyloid *beta* is complexed to SP40,40 (apolipoprotein J), an inhibitor of the complement membrane-attack complex. *Biochem. J.* **293**:27–30 (1993).

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