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Zinc-induced Alzheimer's Aβ1–40 Aggregation Is Mediated by Conformational Factors*

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The heterogeneous precipitates of Aβ that accumulate in the brain cortex in Alzheimer's disease possess varying degrees of resistance to resolubilization. We previously found that Aβ1–40 is rapidly precipitated *in vitro* by physiological concentrations of zinc, a neurochemical that is highly abundant in brain compartments where Aβ is most likely to precipitate. We now present evidence that the zinc-induced precipitation of Aβ is mediated by a peptide dimer and favored by conditions that promote α-helical and diminish β-sheet conformations. The manner in which the synthetic peptide is solubilized was critical to its behavior *in vitro*. Zinc-induced Aβ aggregation was dependent upon the presence of NaCl, was enhanced by α-helical-promoting solvents, but was abolished when the peptide stock solution was stored frozen. The Aβ aggregates induced by zinc were reversible by chelation, but could then be reprecipitated by zinc for several cycles, indicating that the peptide's conformation is probably preserved in the zinc-mediated assembly. In contrast, Aβ aggregates induced by low pH (5.5) were not resolubilized by returning the pH milieu to 7.4. The zinc-Aβ interaction exhibits features resembling the gelation process of zinc-mediated fibrin assembly, suggesting that, in events such as clot formation or injury, reversible Aβ assembly could be physiologically purposive. Such a mechanism is contemplated in the early evolution of diffuse plaques in Alzheimer's disease and suggests a possible therapeutic strategy for the resolubilization of some forms of Aβ deposit in the disease.

The pathological hallmark of Alzheimer's disease is the abundant accumulation in the brain of Aβ, a 39–43-amino acid peptide, as morphologically heterogeneous deposits in the neuropil (senile plaques) and cerebral blood vessels (congophilic angiopathy) (1, 2). Aβ is a soluble component of cerebrospinal fluid where it is found in concentrations in the low nanomolar range (3–5). Many studies now indicate that synthetic Aβ becomes toxic to cultured neuronal cells when in a specific β-sheet conformation that involves incubating the synthetic peptide in a aqueous solution over time periods of days to weeks (6–14). Whereas the β-sheet conformation of the peptide is found in highly insoluble fibrillar amyloid deposits in Alzheimer's disease, a substantial proportion of Aβ precipitates into nonfibrillar deposits that can be resolubilized by extraction into aqueous solvents (15).

We have recently reported that Aβ itself specifically and saturably binds zinc, manifesting high affinity binding (K_D = 107 nM) with a 1:1 (zinc:Aβ) stoichiometry and low affinity binding (K_D = 5.2 μM) with a 2:1 stoichiometry (16). This binding is probably histidine-mediated since it is abolished by acidic pH (no binding at pH 6). The zinc-binding site was mapped to a stretch of contiguous residues between positions 6–28 of the Aβ sequence. Occupation of the zinc-binding site, which straddles the lysine 16 position of α-secretase cleavage (17, 18), inhibits α-secretase type (tryptic) cleavage, and so may influence the generation of Aβ from amyloid protein precursor (APP) and may increase the biological half-life of Aβ by protecting the peptide from proteolytic attack (16).

We also found that concentrations of zinc ≥1 μM rapidly destabilize human Aβ1–40 solutions, inducing rapid Aβ precipitation (19) that is highly specific for zinc, although both copper(II) and iron(II) can induce partial aggregation at equivalent concentrations (20). Meanwhile, rat Aβ1–40 (with substitutions of Arg → Gly, Tyr → Phe, and His → Arg at positions 5, 10, and 13, respectively) binds zinc less avidly (K_a = 3.8 μM, with 1:1 stoichiometry) and is unaffected by zinc at these concentrations, perhaps explaining the scarcity with which these animals form cerebral Aβ amyloid (21, 22). In the absence of zinc, the solubilities of the rat and the human Aβ species are indistinguishable (19). We observed that iodinating the peptide on the 10th residue of tyrosine attenuated zinc-mediated precipitation (19), and since this residue is substituted with a phenylalanine in the rat species, we concluded that this tyrosine is critical in coordinating zinc to the human peptide. These observations are important because zinc is abundant in the same neocortical regions where Aβ deposits are most commonly found, and high micromolar zinc concentrations are achieved during glutamatergic neurotransmission (24, 25), suggesting an explanation for the propensity of Aβ to deposit close to the neocortical synaptic vicinity. Recently, we have also found evidence that zinc mediates the assembly of a significant fraction of Aβ deposits in Alzheimer-affected postmortem brain tissue.[1] Hence, an elaboration of the interactions between Aβ and zinc *in vitro* may be germane to the pathology of Alzheimer's disease.

The concentration of zinc required to precipitate Aβ1–40 *in*

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[1] R. A. Cherny, K. Beyreuther, R. E. Tanzi, C. L. Masters, and A. I. Bush, personal communication.

vitro has been in disagreement with results reported recently by Esler *et al.* (26), who have claimed that concentrations no less than 100 μM are required to demonstrate appreciable precipitation of the peptide. In particular, the validity of the filtration assay for Aβ aggregation used in our previous study was challenged by these workers who contended that ^{125}I-Aβ1–40 is a suitable tracer for monitoring the interaction of Aβ with zinc, at variance with our findings (26). Rodriguez *et al.* (27) have recently reported that zinc concentrations below 100 μM induce the abundant and immediate precipitation of soluble Aβ1–40, in agreement with our initial reports (16, 19, 20) and in disagreement with the findings of Esler *et al.* (26). This debate is important for two reasons. First, if the concentration of zinc required to precipitate Aβ is, in fact, over 100 μM, then it is very unlikely that this interaction is of any neurobiological significance. Much of the zinc that is released by synaptic transmission (24, 25) may not be available for exchange with Aβ since it will in large part be sequestered by macromolecules and other ligands (28). If >100 μM zinc is required to induce Aβ precipitation, the majority of the zinc released during neurotransmission would need to exchange with Aβ if synaptic zinc were to be a factor in the peptide's accumulation. This is unlikely. If, on the other hand, low micromolar concentrations of zinc are required to precipitate Aβ, then only ≈1% of the total zinc released during glutamatergic neurotransmission would be required to induce Aβ assembly, making such an interaction far more likely. Second, our findings (19) questioned the use of ^{125}I-Aβ as a valid tracer for unmodified Aβ behavior, and in our hands, the effect of low micromolar zinc upon the precipitation of Aβ differentiated the iodinated peptide from unmodified Aβ. This is important since radiolabeling of Aβ by iodination is a common means of creating a marker for the peptide.

To explore the reasons for the variance between our findings and those of Esler *et al.* (26), we studied physicochemical factors that influence the interaction of zinc with synthetic Aβ1–40, and used turbidometry as a highly specific, although relatively insensitive, method for monitoring peptide aggregation. We now report that the striking precipitation of Aβ1–40 by low micromolar concentrations of zinc is sensitive to complex factors in the buffer milieu that impact upon the peptide's conformation and polymerization state. These factors may explain the variance that exist between our earlier findings and the report of Esler *et al.* (26). Our findings also indicate that the conformational state of the peptide may not be perturbed by precipitation with zinc. These data may provide insights into neurobiological factors that influence the solubility of Aβ in the pathophysiological environment of the brain in Alzheimer's disease.

EXPERIMENTAL PROCEDURES

Reagents and Preparation—Human Aβ1–40 amyloid peptide was used in all experiments, synthesized, purified, and characterized by HPLC[2] analysis, amino acid analysis and mass spectroscopy by W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT). The HPLC elution profile of Aβ1–40 peptide in preparations used in these experiments was identified as a sole peak in the eluate. Amino acid analysis of the synthetic peptide indicated that there were no apparent chemical modifications at amino acid residues. Mass spectroscopy was performed on each batch of peptide as a further confirmation.

Milli-Q water (Millipore Corp., Milford, MA) was used for Aβ solubilization and other stock reagent dilution. Buffers were treated with Chelex-100 resin (Bio-Rad) to minimize trace metal contamination, and filtered through a 0.22-μm cellulose acetate filter unit (Corning Costar Corporation, Cambridge, MA). The background zinc concentrations in the buffers were measured at <0.1 μM by ion-coupled plasma-atomic emission spectroscopy. Standard zinc stock solution (10 mg/ml in 10% HCl, U. S. National Institute of Standards and Technology, Gaithersburg, MD) was used in all experiments. All other reagents were at least analytical grade.

Synthetic Aβ peptide solutions were prepared on the day of the experiment according to the protocol of Evans *et al.* (29), except where indicated. Lyophilized peptide was first solubilized in water to reach 500 μM and then indirectly sonicated for 3 min (30 s on, 10 s off) through a water bath to avoid frothing. The peptide preparation was then filtered through a water-washed Spin-X cellulose acetate filter unit (0.22 μ, Corning Costar Corporation). Sonication and filtration are considered to be critical procedures to remove any trace of peptide microparticulate matter. Concentrations of Aβ1–40 were determined by BCA protein assay (Pierce). The validity of the BCA assay for the measurement of Aβ peptide concentrations in these solutions was confirmed by amino acid analysis.

Aβ1–40 Gel-filtration Chromatography—Experiments were performed using Waters model 650E system (Millipore Corporation, Milford, MA) connected to a column (Bio-Rad Econo-Column, 30 × 1.0 cm) prepacked with Superdex 30 (Pharmacia Biotech AB, Uppsala, Sweden). Aβ1–40 (0.5 ml, 2.3 μM) in Tris-HCl-buffered saline (TBS, 20 mM Tris, 150 mM NaCl, pH 7.4) was injected into the column preequilibrated with TBS at room temperature and eluted at 0.5 ml/min. The column was calibrated with combined gel-filtration molecular mass markers (Bio-Rad and Sigma), vitamin B_{12} (1.35 kDa), aprotinin (6.5 kDa), cytochrome C (12.4 kDa), equine myoglobin (17 kDa), and carbonic anhydrase (29 kDa). The total volume and void volume of the column were determined by elution volumes of dichromate anion (0.22 kDa) and blue dextran (2000 kDa), respectively. The Aβ1–40 elution peak was monitored at 214-nm absorbance, and the amount of Aβ1–40 eluting from the column was estimated by calibrating the absorbance of Aβ at 214 nm against known peptide concentrations.

Turbidometric Assay of Zinc-induced Aβ1–40 Aggregation—Turbidity measurement as an assay for aggregation was performed according to established protocols (29–32) with minor modifications. The reactions were performed at room temperature in a flat-bottom 96-well microtiter plate (Corning Costar Corporation), and absorbances (405 nm) were measured using a V_{max} kinetic microplate reader directed by Softmax version 2.32 software (Molecular Devices Corporation). Automatic 30-s plate agitation mode was selected for the plate reader to evenly suspend the aggregates in the wells before all readings.

In most experiments, Aβ1–40 was brought to 10 μM (300 μl) in either 50 mM HEPES buffer, 150 mM NaCl, 0–300 μM zinc, pH 7.4, ±chelator, or 50 mM MES buffer (150 mM NaCl, pH 5.5), and incubated at 37 °C before absorbance measurements were taken at room temperature.

To investigate the reversibility of zinc-induced Aβ1–40 aggregation, 25 μM zinc and 25 μM Aβ1–40 were mixed in 150 mM NaCl, 50 mM HEPES, pH 7.4 (200 μl), and turbidity measurements were taken at four 1-min intervals using a 96-well plate reader. Subsequently, 20-μl aliquots of 10 mM EDTA or 10 mM zinc (prepared in incubation buffer) were added into the wells alternately, and following a 2-min delay, a further four readings were taken at 1-min intervals. After the final EDTA addition and turbidity reading, the mixtures were incubated for an additional 30 min before taking final readings.

To investigate the reversibility of pH 5.5-induced Aβ1–40 aggregation, Aβ1–40 was brought to 25 μM in 150 mM NaCl, 50 mM HEPES, pH 7.4 (200 μl), and its absorbance was read at 405 nm as the background reading. The pH of the solution was then brought to 5.5 by the addition of concentrated HCl (5.5 μl), and turbidity measurements were taken at four 1-min intervals. Subsequently, concentrated NaOH (7.5 μl) was added into the wells to adjust the pH back to 7.4, and following a 2-min delay, a further four measurements were taken at 1-min intervals. These cycles were repeated as indicated, and the pH of the mixture was constantly monitored with a pH probe.

To further determine the state of aggregation of the incubated peptides in the reversibility experiments, replicate samples (300 μl) representing various zinc-containing or chelated conditions were removed from the incubation tray at various time points, pelleted (10,000 × *g* for 15 min), and either the supernatant was measured for remaining peptide content before and after centrifugation using the BCA assay, or the pellet was stained with 50 μl of Congo Red (1% in 50% ethanol for 5 min). Pellets were washed twice with 50% ethanol (100 μl) before being resuspended in 20 μl of HEPES buffer. An aliquot (3 μl) of each resuspension was placed on a microscope slide uniformly for microscopic analysis under polarized light.

[2] The abbreviations used are: HPLC, high performance liquid chromatography; TBS, Tris-buffered saline; MES, 4-morpholineethanesulfonic acid; TFE, trifluoroethanol; HFIP, 1,1,1,3,3,3-hexafluoro-2-propanol; ZBD, zinc binding domain; MOPS, 4-morpholinepropanesulfonic acid; APP, amyloid protein precursor.

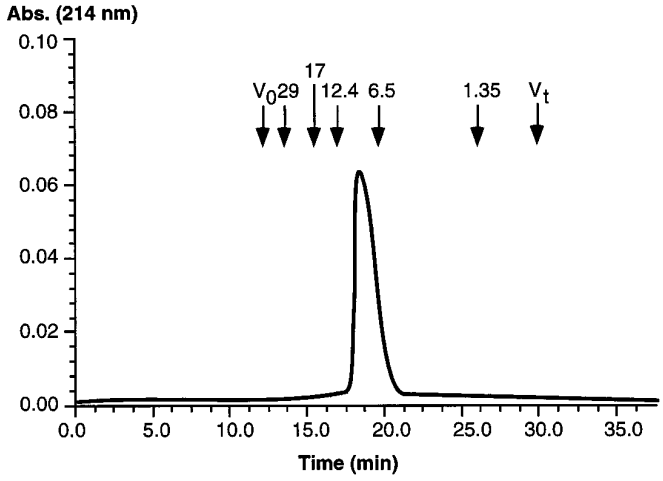

FIG. 1. **Gel-filtration chromatography of Aβ1–40.** Aβ1–40 (0.5 ml, 2.3 μM) in TBS, pH 7.4, was injected into a TBS-equilibrated, precalibrated Superdex 30 column. The elution (0.5 ml/min) was monitored at 214-nm absorbance. The estimated concentration of Aβ1–40 eluting from the column was approximately 470 nM. The relative positions for void (V_0) and total (V_t) volumes of the gel-filtration column, and various molecular mass standards are indicated. A single sharp peak with a molecular size of 8.6 kDa was observed. The results are typical of three experiments.

Studies of the Effects of Solvents upon Zinc-mediated Aβ1–40 Aggregation—Stock solutions of Aβ1–40 (0.2 mM) were prepared by dissolving lyophilized peptide in either 20 mM Tris-HCl, pH 7.4, or 10–30% trifluoroethanol (TFE), or 75% dimethyl sulfoxide (Me_2SO), 25% 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) (v/v), on the day of the study. Other stocks of Aβ1–40 dissolved in 75% Me_2SO, 25% HFIP or water were stored at −20 °C for up to 2 months to determine the effects of storage upon zinc-induced aggregation. All peptide solutions were centrifuged (10,000 × *g* for 20 min) prior to use to remove aggregates. The Aβ1–40 stock solutions in Tris buffer and Me_2SO/HFIP were brought to 2.3 μM with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, ±zinc (0, 10, and 30 μM) and incubated (30 min, 37 °C). The Aβ1–40 stock solution dissolved in 10–30% TFE was brought to 2.3 μM with 150 mM NaCl, 20 mM Tris-HCl, 10–30% TFE, pH 7.4, ±zinc (0, 10, and 30 μM) and incubated (30 min, 37 °C). Following incubation, the mixtures were filtered through a 0.22-μm cellulose acetate filter, and the amount of peptide entering the filtrate was determined by micro BCA protein assay (Pierce), according to a modification of the Aβ aggregation assay developed in our group (19).

RESULTS AND DISCUSSION

To characterize synthetic Aβ1–40 in neutral buffered saline, gel-filtration chromatography was performed. Aβ1–40 solution (2.3 μM) was freshly prepared in TBS from its lyophilized powder (see "Experimental Procedures") on the day of the experiment. The peptide solution was loaded onto the gel-filtration column within 1 h of preparation, and eluted as a single sharp peak corresponding to 8.6 kDa compared to molecular size markers, and at an estimated concentration of 470 nM (Fig. 1), compatible with the peptide being in a dimeric state. Other groups have reported the presence of dimeric Aβ using gel-filtration chromatography (33, 34), and our data are in agreement with the recent report that Aβ1–40 is predominantly dimeric upon gel-filtration in neutral buffered saline (27). However, in the absence of NaCl, we found that the peptide's elution profile became too broad to allow its relative molecular size to be resolved (data not shown), suggesting that the apparent dimerization of the peptide is dependent upon the presence of NaCl. Since we wished to achieve a basic data set that describes the behavior of the most abundant species of Aβ under conditions that approach a physiologically plausible milieu, we proceeded to study the behavior of the peptide in isotonic neutral buffered saline, mindful that the concentration of NaCl appears to enact significant conformational effects upon the peptide.

Some studies have reported chromatographic profiles of Aβ peptides that appear nondimeric (monomeric or oligomeric). Apart from differences in the composition of the solvent system used for the chromatographic procedure, these alternative results may have been due to differences in the behaviors of the specific subspecies of Aβ peptide that were studied, reported differences in the preparation of the Aβ peptide, and variations in the experimental procedure (35–37). For example, in our previous study of Aβ1–40 by gel-filtration chromatography, we observed that the peptide migrated mainly as an apparent dimer (65%) together with minor apparent polymer (30%) and monomer (5%) peaks (16). The difference between our current result and the previous result can be explained as due to two newly introduced variables. First, we have introduced sonication in the preparation of Aβ1–40 peptide solution, which may have contributed to the dissolution of Aβ1–40 polymer into dimeric or monomeric species. Second, in the current study only 5 μg of Aβ1–40 were applied to the column compared with 55 μg in the earlier study. Therefore, absorbance readings at 214 nm in this study are much closer to the base-line buffer absorbance reading, and the absorbance may not be sufficient to demonstrate a significant monomeric peak, since, based upon our earlier data, its proportion is expected to be small in the total Aβ peptide eluent. Therefore, in the current study the presence of a small proportion of monomeric peptide cannot be excluded.

To confirm whether the concentration of zinc required to induce Aβ1–40 precipitation is in the low or high micromolar range, we studied the behavior of the peptide by turbidometry, because it is a well established method that has been used to study the aggregation state of Aβ (29–32). We first determined that the absorbance (405 nm) value of freshly prepared Aβ1–40 (10 μM) in TBS was equivalent to the absorbance of the experimental buffers used (data not shown), indicating that the presence of the soluble Aβ peptide does not contribute to turbidity in this system at the time frame studied. We chose the minimal zinc binding domain (ZBD) at the amino terminus of APP, which has a K_a for zinc binding of ≈750 nM (ZBD, residues 179–189 of APP) (38), as a zinc-binding control peptide for comparison to the behavior of Aβ1–40 in the experiments. The background absorbance turbidity of ZBD (10 μM in TBS, pH 7.4) was found to be of the same as that of Aβ1–40 in TBS alone (data not shown), and incubation of ZBD solutions with the zinc concentrations used in this study did not increase their turbidity, indicating that the ZBD peptide does not aggregate in the presence of zinc.

We proceeded to study conditions representing concentrations of peptide (10 μM) and zinc (<100 μM) for which Esler *et al.* (26) could find no evidence of aggregation. There were negligible changes of absorbance readings for solution of 10 μM Aβ1–40 mixed with 0.1, 0.5 and 1 μM zinc, compared with 10 μM Aβ1–40 alone. However, the absorbance increases of Aβ1–40 with 5 and 10 μM zinc were substantially above background (Fig. 2*A*), corroborating our earlier findings (16, 19, 20). Although we had previously found a significant degree of Aβ1–40 aggregation induced by 1 μM zinc as measured by an absorbance-monitored filtration assay (19), the turbidometry assay used here did not detect Aβ1–40 aggregation at zinc concentrations below 5 μM. This result may be due to the sensitivity limitation of the turbidometry technique. After Aβ solutions were incubated with zinc concentrations below 5 μM, the reaction may have produced particles of insufficient caliber to alter the light scattering properties of the starting solution. Whereas a strength of the turbidometric approach is that a

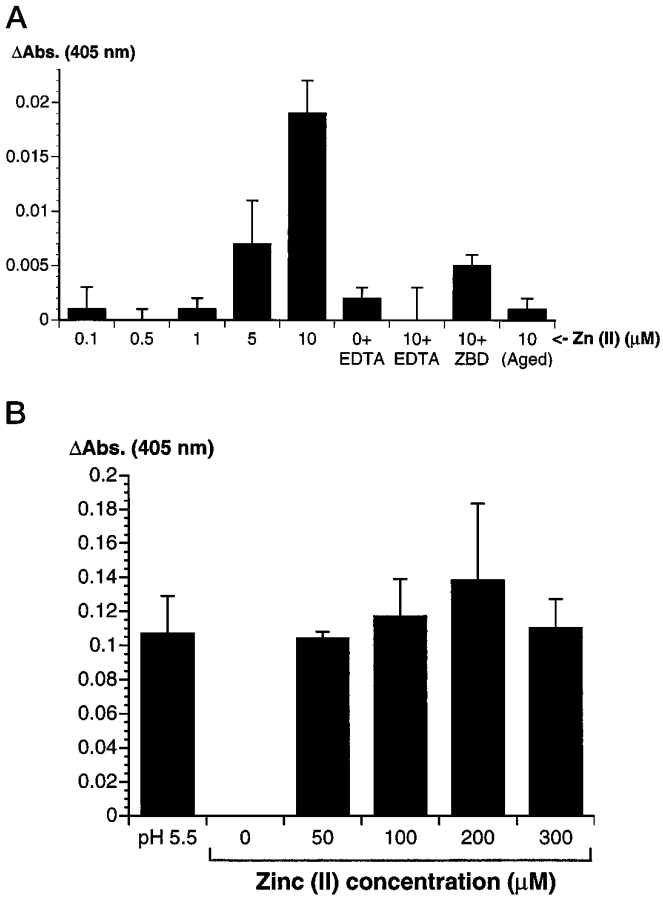

FIG. 2. **Turbidometric analysis of zinc-induced Aβ aggregation.** *A*, effects of low micromolar concentrations of zinc. Aβ1–40 was brought to 10 μM in 150 mM NaCl, 50 mM HEPES (pH 7.4), and mixed with various concentrations of zinc with or without 1 mM EDTA or 10 μM ZBD, as indicated. The data indicate the mean (±S.D., $n = 3$) absorbance (405 nm) changes against the absorbance reading of the incubation buffer alone. *B*, effects of pH 5.5 and high micromolar concentrations of zinc. Aβ1–40 was brought to 10 μM in 150 mM NaCl, 50 mM HEPES (pH 7.4), and mixed with various concentrations of zinc, or incubated in 150 mM NaCl, 50 mM MES (pH 5.5), as indicated. The data indicate the mean (±S.D., $n = 3$) absorbance (405 nm) changes against the absorbance reading of the incubation buffer alone.

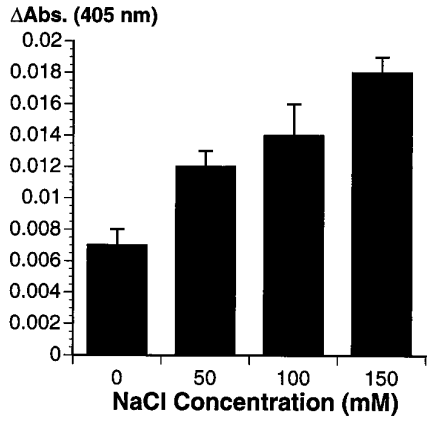

FIG. 3. **Effect of NaCl on zinc-induced Aβ1–40 aggregation.** Aβ1–40 was brought to 10 μM in 50 mM HEPES buffer (pH 7.4) with various concentrations of NaCl, as indicated, and mixed with 10 μM zinc. The data indicate the mean (±S.D., $n = 3$) absorbance (405 nm) changes against the absorbance reading of the incubation buffer alone.

positive signal is a valid indicator of aggregation, a weakness of the approach is its lack of sensitivity in that a negative signal (no change in absorbance at 405 nm) does not necessarily indicate that the solution is free of microparticles.

Zinc-induced Aβ aggregation was instantaneous, and remained constant for 24 h (data not shown). Zinc-induced turbidity was abolished by the presence of the divalent metal ion chelator EDTA (1 mM), and was substantially reduced by the presence of 10 μM ZBD (Fig. 2*A*), indicating that the ZBD effectively acts as a chelator in this system. Since these data show that concentrations of zinc less than or equal to 10 μM clearly induce significant Aβ aggregation, they are in disagreement with the conclusions of Esler *et al.* (26), who did not employ turbidometry in their assays.

Incubation of Aβ1–40 (10 μM) with higher (50–300 μM) zinc concentrations indicated that the degree of turbidity engendered by incubation with zinc matched the degree of turbidity engendered by incubating the peptide at pH 5.5 (Fig. 2*B*). pH 5.5 is the calculated pI of the peptide, and incubation of the peptide at this pH is a precipitation stress for Aβ (39) that has been previously validated by turbidometry (40), and induces β-sheet formation (41). Turbidity changes induced by pH 5.5 and by zinc concentrations ≥50 μM, saturated at approximately 0.12 absorbance unit. As a further corroborative study, the corresponding mixtures were pelleted by centrifugation (10,000 × *g*, 10 min), and the proportion of the peptide remaining in the supernatant as determined by protein assay was <10%, indicating that most of the starting peptide had precipitated (data not shown).

We investigated factors in the experimental incubation conditions to determine which variables could affect the response of synthetic Aβ to zinc. We found that "aged" Aβ1–40, which has been kept in water at −20 °C for more than 2 months, was strikingly unable to be precipitated by zinc (Fig. 2*A*), despite exhibiting no increase in turbidity after being thawed, gently mixed, and refiltered. Aβ stock solutions that were stored for as little as 7 days were also found to have an attenuated response to zinc. These data indicate that to appreciate the maximum effects of low micromolar concentrations of zinc upon the solubility of Aβ, freshly prepared peptide should be used for every experiment, a procedural practice that was not employed by Esler *et al.* (26), who stored their stock peptide solutions at −20 °C for unreported lengths of time before use. Since time-dependent loss of helical content of Aβ within an unstable region of the peptide that is within the zinc-binding site, which spans residue 6 to 28 (16), has been previously described (42), we surmised that the loss of interaction with zinc that we observed upon peptide storage may be due to a conformational change, so we proceeded to study other conformation-influencing factors in the microenvironment of the peptide that may impact upon Aβ interaction with zinc.

To investigate effects of NaCl upon zinc-induced Aβ1–40 aggregation, 10 μM zinc and 10 μM Aβ were incubated in HEPES buffers in the presence of various NaCl concentrations. We observed that the turbidity of these solutions strongly depended upon the concentration of NaCl, so that in the absence of NaCl, zinc-induced precipitation of Aβ was markedly attenuated (Fig. 3). The degree of precipitation induced under these conditions was also confirmed by centrifugation of the samples (10,000 × *g*, 10 min) and measuring the amount of peptide remaining in the supernatant by protein assay. These findings are again at variance with those of Esler *et al.* (26), who reported that the presence of NaCl had no effect upon the degree of Aβ aggregation induced by zinc. Since the presence of 150 mM NaCl increases the ionic strength of the solution, these data again support the likelihood that a change in structure of the peptide is responsible for the variance between our reports and that of Esler *et al*. Because the apparent dimerization of the peptide (see Fig. 1) and the zinc-mediated precipitation of

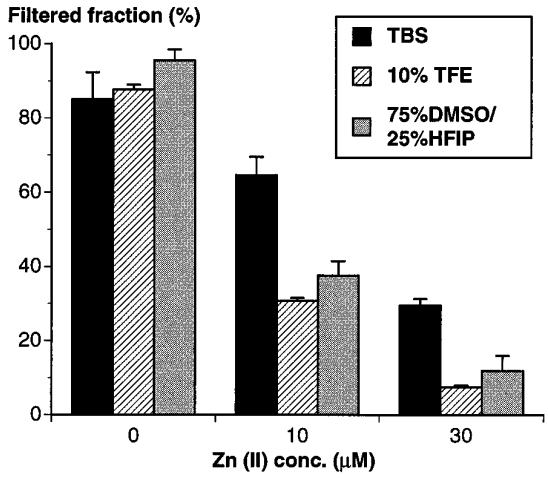

FIG. 4. **Effect of solvents that modulate secondary structure upon zinc-induced Aβ1–40 aggregation.** Stock solutions of Aβ1–40 (0.2 mM) were prepared by dissolving lyophilized peptide in either 20 mM Tris-HCl, pH 7.4, or 10% TFE, or 75% Me_2SO (*DMSO*), 25% HFIP (v/v), as indicated. The Aβ1–40 stock solutions in Tris buffer and Me_2SO/HFIP were brought to 2.3 μM with 150 mM NaCl, 20 mM Tris-HCl, pH 7.4, ±zinc (0, 10, and 30 μM) and incubated (30 min, 37 °C). The Aβ1–40 stock solution dissolved in 10% TFE was brought to 2.3 μM with 150 mM NaCl, 20 mM Tris-HCl, 10% TFE, pH 7.4 ± zinc (0, 10, and 30 μM) and incubated (30 min, 37 °C). Following incubation, the mixtures were filtered through a 0.22-μm cellulose acetate filter, and the amount of peptide entering the filtrate was measured. The data indicate the amount of peptide entering the filtrate expressed as the proportion (filtered fraction) of the starting amount of peptide (means ± S.D., $n = 3$). In all conditions, less peptide enters the filtrate following incubation with zinc. The presence of 30% TFE achieved results similar to those achieved with 10% TFE (data not shown).

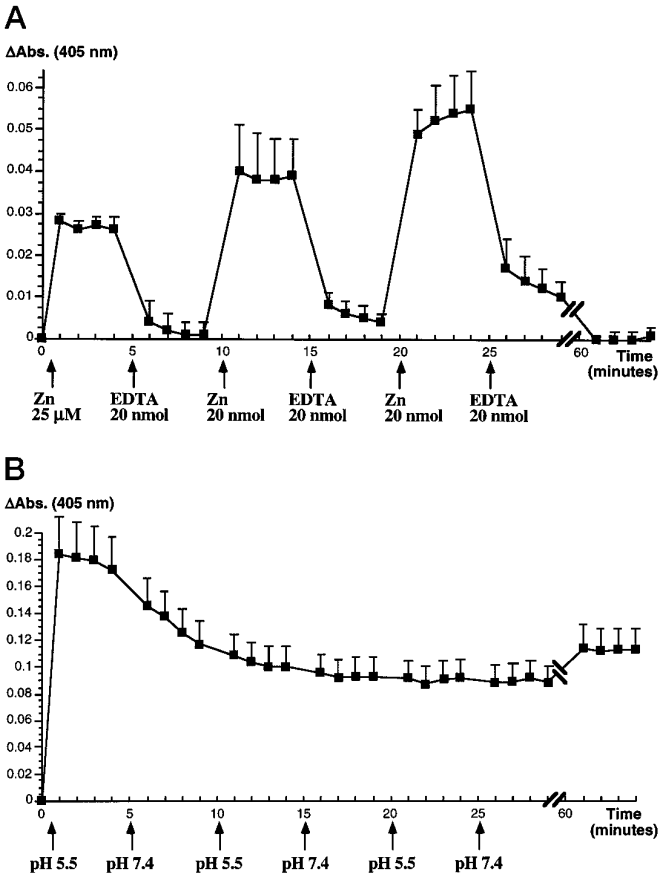

FIG. 5. *A*, reversibility of zinc-induced Aβ1–40 aggregation. 25 μM zinc and 25 μM Aβ1–40 were mixed in 150 mM NaCl, 50 mM HEPES, pH 7.4 (200 μl), and turbidity measurements were taken at four 1-min intervals. Subsequently, 20-μl aliquots of 10 mM EDTA or 10 mM zinc (prepared in incubation buffer) were added into the wells alternatively, and following a 2-min delay, a further four readings were taken at 1-min intervals. After the final EDTA addition and turbidity reading, the mixtures were incubated for an additional 30 min before taking final readings. The data indicate the mean (±S.D., $n = 3$) absorbance (405 nm) changes against the absorbance reading of the incubation buffer alone. *B*, reversibility of pH 5.5-induced Aβ1–40 aggregation. Aβ1–40 was brought to 25 μM in 150 mM NaCl, 50 mM HEPES, pH 7.4 (200 μl), and its absorbance read at 405 nm as the background reading. The pH of the solution was then brought to 5.5 by the addition of concentrated HCl (5.5 μl), and turbidity measurements were taken at four 1-min intervals. Subsequently, concentrated NaOH (7.5 μl) was added into the wells to adjust the pH back to 7.4, and, following a 2-min delay, a further four measurements were taken at 1-min intervals. These cycles were repeated as indicated. The data indicate the mean (±S.D., $n = 3$) absorbance (405 nm) changes against the absorbance reading of the initial incubation buffer.

Aβ are both dependent upon the presence of NaCl, the likelihood exists that zinc-mediated Aβ assembly may be coordinated by the peptide as a dimeric subunit.

Because our data pointed toward a specific conformation of the peptide as mediating the interaction with zinc, we next studied the effect of solvents that promote helical conformation (TFE) or reduce β-sheet conformation (Me_2SO) upon zinc-mediated Aβ aggregation. The effects of these solvents upon Aβ secondary structure have been characterized (43, 44). We observed that the presence of these solvents either within the reaction (10–30% TFE) or limited to the preparation of the stock solution (75% Me_2SO, 25% HFIP), induced a substantial increase in zinc-induced aggregation of Aβ1–40 compared with the amount of precipitation induced by zinc in the absence of solvent (Fig. 4). Although these solvents would normally attenuate the fibrillization of the peptide (43, 44), we find that zinc-induced Aβ aggregation is promoted by preserving the α-helical conformation.

We tested the zinc-induced Aβ aggregates for reversibility by chelation to determine whether precipitation by zinc causes the peptide to preserve its secondary structure or changes it into a thermodynamically irreversible β-sheet conformer. We observed that zinc-induced turbid aggregates are totally reversed by removal of the zinc and that the peptide may alternate between a precipitated and nonprecipitated state for many cycles of fluctuating zinc concentration (Fig. 5*A*). This result indicates that the peptide does not lose conformational energy, once it interacts with zinc, and probably retains its original conformation without entering into a lower energy β-sheet conformation. To test this hypothesis further, we exposed the peptide to incubation at pH 5.5, an environment known to induce β-sheet formation (41). Under these circumstances the peptide solutions became turbid, and the turbidity of the preparation would not return to baseline even after the pH of the incubation was returned to pH 7.4 (Fig. 5*B*). One previous study indicated that Aβ1–28 and Aβ1–39 but not Aβ1–42 exhibit reversible pH-induced aggregation. In that study, 0.1 M sodium acetate buffer at pH 5.0 was used to induce the aggregation of iodinated Aβ species (Aβ1–28, Aβ1–39, and Aβ1–42), the peptide aggregates were resuspended in 0.1 M MOPS buffer of pH 7.4, and their reversibility was quantified by radioactivity counting (39). In the current study, pH-induced Aβ1–40 aggregation was appraised by turbidometry using nonmodified Aβ1–40 in HEPES-buffered saline. Two important variables may explain the discrepant results between the two studies. First, we studied the behavior of an Aβ species, Aβ1–40, that is more hydrophobic than the Aβ1–28 and Aβ1–39 studied by Burdick *et al.* (39). The solubility of Aβ and its propensity to form the irreversible β-sheet is related to the length of its hydrophobic carboxyl terminus (30, 32). More likely, however, is the possibility that the modification of the Aβ peptide by

iodination has rendered the peptide more easily resolubilized by alkalinization, perhaps by making it more difficult for the peptide subunits to align in an antiparallel β-sheet conformation that might be expected as the conditions induce the peptide to adopt the β-sheet conformation. Importantly, our findings that irreversible aggregation of Aβ1–40 is induced by low pH are in agreement with another study that employed the same turbidometric technique (40). Our experiment was conducted with only a 5-min precipitation phase (pH 5.5), which induced instantaneous turbidity, and a 30-min resolubilization phase (pH 7.4), which may have been insufficient to achieve sufficient equilibrium to promote resolubilization. However, the study of Wood *et al.* (40) showed that similar pH-induced aggregation of Aβ1–40 cannot be reversed, even after returning the pH to neutral for 2 days, supporting the likelihood that irreversible precipitation of the peptide differentiates zinc-induced from pH-induced Aβ aggregation. Taken together, these reports indicate that irreversibility of pH-induced Aβ aggregation varies between different Aβ species and different peptide modifications. Results of Aβ1–40 aggregation induced by pH show that, once the Aβ1–40 peptide has lost conformational energy and precipitated in a β-sheet conformation, the thermodynamic barrier to a soluble conformation is difficult to reverse, again suggesting that zinc-mediated aggregation is not due to β-sheet formation since it is readily reversed by chelation.

To confirm that zinc-mediated Aβ aggregation is not due to β-sheet formation, we sampled the precipitate formed in these reactions, stained it with Congo Red, and visualized the product by microscopy under polarized light. Although the peptide formed abundant congophilic precipitates as a consequence of both zinc and pH 5.5 incubations, only the Aβ precipitate formed at pH 5.5 exhibited positive birefringence typical of the β-sheet conformation of amyloid. Therefore, the Aβ precipitate induced by zinc under these specific conditions is not tinctorial amyloid. To confirm that the loss of turbidity that occurred when the zinc-induced Aβ suspension was treated with chelator was, in fact, due to resolubilization rather than due to an alteration in the light-scattering properties of a suspension, a sample of zinc-induced Aβ suspension that had been treated with EDTA was stained with Congo Red, pelleted, and visualized by microscopy. No particulate matter was seen, suggesting that the reversal of turbidity caused by zinc chelation reflected resolubilization of the peptide precipitates (data not shown).

We had previously observed that incubation of Aβ1–40 with zinc induced a congophilic precipitate that exhibited positive birefringence under polarized light, meeting one of the criteria for the morphology of amyloid (19). In that report, we also demonstrated that up to 32% of the zinc-induced Aβ1–40 precipitate could be resolubilized by EDTA chelation. In the current report, we demonstrate that, under these conditions, 100% of the zinc-induced Aβ1–40 precipitate could be resolubilized by EDTA chelation. We hypothesized that this variance was due to a procedural difference for Aβ1–40 peptide preparation implemented since our earlier report. In the current report, we prepared Aβ1–40 solutions by sonication of the lyophilized peptide suspension, so generating a solution that migrates as an apparent dimer in TBS, pH 7.4 (Fig. 1). In the previous report, we did not sonicate the peptide, although in both reports the peptide stocks were filtered. Nonsonicated Aβ1–40 migrates on gel-filtration chromatography as both an apparent dimer and a multimer (16). We consider that the presence of multimeric Aβ1–40 probably reflects incomplete dissolution and that the propagated energy provided to the peptide through thermal heating by sonication has most likely facilitated the complete dissolution of the peptide into dimeric units, allowed the peptide to assume a high energy α-helical conformation, and is probably a better reflection of the physiological state of the peptide since the α-helical conformer is most stable in solution. The presence of multimeric Aβ in the peptide solution may alter the thermodynamics of Aβ assembly and possibly help bring about conditions that favor irreversible fibrillization. To test this hypothesis, we repeated the study of the effects of zinc and chelation cycles upon Aβ aggregation (Fig. 5*A*) using peptide that had been freshly solubilized and filtered, but not sonicated. This preparation, while readily exhibiting turbidity when precipitated by zinc, did not then appreciably resolubilize with EDTA chelation, and staining with Congo Red revealed the presence of tinctorial amyloid particles (data not shown), confirming our earlier report (19). These data confirm that the presence of multimeric Aβ1–40 in the starting peptide preparation favors the irreversible formation of amyloid-containing particles by zinc by a mechanism that is still unclear.

The degree of zinc-induced turbidity in the chelation reversibility experiment increased following subsequent cycles of chelation (Fig. 5*A*), despite the stoichiometric ratio of free zinc to chelator remaining ≤1 in each zinc-induced cycle. Therefore, it is possible that the ionic interaction with zinc may induce conformational changes in the peptide that subsequently alter further interaction with zinc. Conversely, the reversal of the turbidity of zinc-treated peptide suspensions induced by EDTA chelation exhibited a time-dependence in the order of 30 min to reach complete resolubilization (Fig. 5*A*). Hence, although the association of zinc with Aβ to form insoluble assemblies is near-instantaneous, the dissociation appears to possess slower kinetics. This may be due, in part, to the time required for the EDTA to access the interstices of the zinc-induced aggregates.

Our data, in agreement with those of Garzon-Rodriguez *et al.* (27), suggest that the major interacting soluble Aβ1–40 species recruited during zinc-induced aggregation are dimeric. The obligatory binding sequence for zinc mapped to a contiguous stretch of residues between 6 and 28 (19). However, if Aβ dimerization is a prerequisite for zinc binding, this domain (residues 6–28) may actually reflect a region of the peptide responsible for homotypic dimerization. Our data also show that zinc-induced assembly of Aβ is not mediated by β-sheet formation, suggesting that the assemblies of zinc and Aβ, which precipitate in 1:1 stoichiometry (19), are likely to be integrated by ionic interactions within the charged region of the zinc-binding site. This site is within an unstable region of the peptide that is in equilibrium between α-helix and β-sheet (42). The variance between our findings and the findings of Esler *et al.* (26) may therefore be explained by factors, such as those we have described in this report, which could alter the conformational equilibrium in this region.

The striking versatility of the solubility state of Aβ is unexpected, since the presence of the peptide as a collection in Alzheimer's disease pathology has always suggested that its precipitation is pathological. However, Aβ also collects as diffuse deposits, which presumably are more readily resolubilized than mature amyloid, in the brains of individuals who do not have Alzheimer's disease (45) and following head injury (46). We hypothesize that the presence of Aβ precipitates in the brain in these conditions may reflect a physiological purpose. The zinc-mediated reversible precipitation of Aβ can be compared with the interaction of zinc with fibrin whose solubility state is dependent upon zinc for physiological purposes (47). Aβ is found in platelets (48) where it may colocalize with high concentrations (>300 μM) of zinc as well as the APP (49), fibrinogen, and other coagulation factors (50) in the α-secretory granule. Platelet zinc release modulates the propagation of the coagulation cascade through its action on protease activities, such as the effect of zinc at micromolar concentrations in se-

lectively enhancing the inhibition of coagulation factor XIa by protease nexin-2/APP (51), probably through binding to the zinc-binding site on the amino terminus of APP (38). Zinc release also modulates the assembly of clotting structural elements such as fibrin in the vicinity of the activated platelet (47). In the current report we have found that zinc appears to mediate a reversible aggregation response from Aβ, similar to the gelation response of fibrin incubated with low micromolar concentrations of zinc (47). Aβ could conceivably be recruited by high regional concentrations of zinc into the hemostatic plug or a reversible assembly that may play a role in maintaining tissue integrity in injury.

The abundance of zinc that is released by neurons (24), and housed by neuroglia (52), could potentially interact with Aβ during an injury response to induce the diffuse assembly of the peptide while maintaining the α-helical conformation of the peptide. Under these conditions, Aβ assemblies would not be expected to be neurotoxic since they would lack the β-sheet conformation.

The instantaneous assembly of 10 μM Aβ1–40 by 10 μM zinc in neutral buffered saline is such a robust feature of the peptide's behavior that we suggest this provocation as a test of the patency of the synthetic peptide and its α-helical content. The propensity of Aβ to precipitate in the brain neocortex and not peripherally may reflect the high regional zinc concentrations in the neocortex (28), and we contemplate a possible role for zinc-mediated Aβ assembly in the evolution of Alzheimer's disease plaque pathology. Reversible zinc-mediated Aβ assembly may be reflected in the Aβ that is extractable by aqueous solvent from Alzheimer's disease-affected cortex (15); however, if these precipitates undergo a further physicochemical stress that induces a loss of α-helical content, they may then evolve into the more insoluble amyloid deposits. The enrichment of Aβ1–42 in cortical amyloid may reflect a liability that the more insoluble Aβ1–42 species has to develop the irreversible β-sheet conformation. We have previously found that soluble Aβ1–42 is as sensitive to zinc-mediated precipitation as Aβ1–40 (data not shown), but the reversibility of zinc-induced Aβ1–42 aggregates remains to be determined.

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Zinc-induced Alzheimer's Aβ1–40 Aggregation Is Mediated by Conformational Factors

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