Inhibition of β-Amyloid(40) Fibrillogenesis and Disassembly of β-Amyloid(40) Fibrils by Short β-Amyloid Congeners Containing *N*-Methyl Amino Acids at Alternate Residues†

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ABSTRACT: A potential goal in the prevention or therapy of Alzheimer's disease is to decrease or eliminate neuritic plaques composed of fibrillar β-amyloid (Aβ). In this paper we describe *N*-methyl amino acid containing congeners of the hydrophobic "core domain" of Aβ that inhibit the fibrillogenesis of full-length Aβ. These peptides also disassemble preformed fibrils of full-length Aβ. A key feature of the inhibitor peptides is that they contain *N*-methyl amino acids in *alternating* positions of the sequence. The most potent of these inhibitors, termed Aβ16−22m, has the sequence NH_2-K(Me-L)V(Me-F)F(Me-A)E-$CONH_2$. In contrast, a peptide, NH_2-KL(Me-V)(Me-F)(Me-F)(Me-A)-E-$CONH_2$, with *N*-methyl amino acids in consecutive order, is not a fibrillogenesis inhibitor. Another peptide containing alternating *N*-methyl amino acids but based on the sequence of a different fibril-forming protein, the human prion protein, is also not an inhibitor of Aβ40 fibrillogenesis. The nonmethylated version of the inhibitor peptide, NH_2-KLVFFAE-$CONH_2$ (Aβ16−22), is a weak fibrillogenesis inhibitor. Perhaps contrary to expectations, the Aβ16−22m peptide is highly soluble in aqueous media, and concentrations in excess of 40 mg/mL can be obtained in buffers of physiological pH and ionic strength, compared to only 2 mg/mL for Aβ16−22. Analytical ultracentrifugation demonstrates that Aβ16−22m is monomeric in buffer solution. Whereas Aβ16−22 is susceptible to cleavage by chymotrypsin, the methylated inhibitor peptide Aβ16−22m is completely resistant to this protease. Circular dichroic spectroscopy of Aβ16−22m indicates that this peptide is a β-strand, albeit with an unusual minimum at 226 nm. In summary, the inhibitor motif is that of alternating *N*-methyl and nonmethylated amino acids in a sequence critical for Aβ40 fibrillogenesis. These inhibitors appear to act by binding to growth sites of Aβ nuclei and/or fibrils and preventing the propagation of the network of hydrogen bonds that is essential for the formation of an extended β-sheet fibril.

Alzheimer's disease (AD)[1] is a member of the growing class of amyloid diseases, but its clinical importance is paramount, as it is the fourth leading cause of death in the United States and the most common cause of dementia (*1*). The signature pathology of AD is the presence of extracellular plaques composed primarily of β-amyloid peptides (Aβ) (*2*). Genetic and neuropathologic studies suggest that the accumulation of amyloid plaques is central to the pathogenesis of the disorder. The number of plaques, for example, appears to correlate with the degree or severity of the dementia (*3*, *4*). Furthermore, fibrillar Aβ, but not soluble Aβ, is neurotoxic and causes neuronal death both *in vitro* and *in vivo* (*5*−*8*). Other studies suggest that prefibrillar aggregates of Aβ peptide are neurotoxic (*9*). Mutations in either the amyloid precursor protein (APP), the immediate precursor of the Aβ peptides, or the presenillins (PS), which regulate the processing of Aβ, can lead to elevated production of Aβ and are associated with severe and early-onset forms of AD (*10*, *11*). Prevention of Aβ aggregation, consequently, has emerged as a potential goal in the therapy or prevention of Alzheimer's disease, and similar strategies are possible for related amyloid disorders (*12*).

In this paper, we describe the design, synthesis, and biochemical characterization of rationally designed peptide inhibitors of Aβ fibrillogenesis. These peptides incorporate *N*-methyl amino acids into alternate positions of a short sequence based on a hydrophobic "core domain" of Aβ, i.e., residues 17−22, known to be critical for Aβ fibrillogenesis. *N*-Methyl amino acids were utilized in the design of these peptides because they are predicted to disrupt the peptide−peptide interactions that promote Aβ fibrillogenesis. In

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[1] Abbreviations: Aβ, β-amyloid; AD, Alzheimer's disease; APP, amyloid precursor protein; BCA, bicinchoninic acid; BOC, *tert*-butoxycarbonyl; CD, circular dichroic; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMSO, dimethyl sulfoxide; FMOC, 9-fluorenylmethoxycarbonyl; HATU, 2-(1*H*-9-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HOBt, *N*-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; HFIP, hexafluoroisopropyl alcohol; IC, inhibitory concentration; MALDI-TOF, matrix-assisted laser desorption time of flight; MBHA, methylbenzhydrylamine; PS, presenillin; TFA, trifluoroacetic acid.

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particular, *N*-methyl amino acids (1) replace an amide proton that normally stabilizes the β-sheet through hydrogen-bonding interactions between individual β-strands, (2) prevent the close approach of β-strands in a β-sheet because of steric hindrance, and (3) induce β-strand structure in the peptide itself because of the preference of tertiary amides for the *trans* conformation (*13*−*18*).

N-Methyl amino acids have been used in several systems to control protein and peptide aggregation. An *N*-methyl amino acid was used to block the dimerization of interleukin-8 (*19*). Similarly, *N*-methyl amino acids have been used to control the aggregation of peptide nanotubes (*20*). Doig designed a nonaggregating three-stranded β-sheet peptide containing *N*-methyl amino acids (*21*). Recently, this investigator and co-workers applied this strategy in the synthesis of Aβ(25−35) congeners containing single *N*-methyl amino acids (*22*). In some cases, these peptides were found either to alter the morphology or prevent aggregation and neurotoxicity of Aβ(25−35). *N*-Methyl amino acids have also been used to prevent the aggregation of covalently linked, parallel β-sheet dimers (*23*, *24*).

We will demonstrate, below, that two peptides based on the core domain of Aβ and containing *N*-methyl amino acids in alternate positions inhibit the fibrillogenesis of full-length Aβ40. Moreover, these peptides also disassemble preformed fibrils made of Aβ. We will suggest, furthermore, that these inhibitor peptides operate mainly by binding to Aβ through one hydrogen-bonding face and simultaneously blocking the propagation of the hydrogen-bonded array of the β-sheet with a non-hydrogen bonding face. This will be demonstrated by comparing inhibitors with *N*-methyl amino acids in *alternate* positions to poor inhibitors of the same basic sequence but containing an equal or greater number of *N*-methyl amino acids in *consecutive* positions. Finally, we will demonstrate that the inhibition is sequence specific and that an *N*-methyl peptide from another fibrillar protein, the human prion protein, does not inhibit fibrillogenesis of Aβ.

MATERIALS AND METHODS

Peptide Synthesis, Purification, and Analysis. The human Aβ40 peptide was synthesized using standard 9-fluorenylmethoxycarbonyl chemistry on an Applied Biosystems Model 431A peptide synthesizer: NH_2-DAEFRHDSGY^{10}-EVHHQKLVFF^{20}AEDVGSNKGA^{30}IIGLMVGGVV^{40}-COOH. Peptides with a free acid at the carboxy-terminus were prepared by using preloaded Wang resins (Midwest Biotech). The *N*-methyl peptides were synthesized manually using 9-fluorenylmethoxycarbonyl chemistry and an MBHA amide resin (Midwest Biotech). Amino acids added after *N*-methyl amino acids (Novabiochem) were coupled for 3−5 h using the HATU (PE Biosystems) activating reagent. Other residues were coupled for 1.5 h with HOBt/DCC (PE Biosystems).

The peptides were purified using a reverse-phase, C18 preparative HPLC column (Rainin Dynamax) at 60 °C. Peptide purity was greater than 95% by analytical HPLC (Rainin C18 column). The molecular masses of the peptides were verified with electrospray and MALDI-TOF mass spectrometry.

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman Optima XLA ultracentrifuge equipped with an An60Ti rotor and analytical cells with two-channel, charcoal-filled centerpieces. Peptides were dissolved in 100 mM phosphate buffer, pH 7.4, and 150 mM NaCl at concentrations of 100 μM, 500 μM, and 5 mM. The equilibrium distribution of peptide was measured at 20 °C with rotor speeds of 36 000−54 000. All scans were performed by measuring the UV absorbance at 256 or 220 nm depending on the concentration of the peptide. Fifty or 75 scans were averaged at each point with step sizes of 0.001 cm. Duplicate scans taken 2 or 4 h apart were overlaid to determine whether equilibrium had been attained. Partial specific volume was estimated from amino acid composition, and solvent density was calculated using the SEDNTERP program.

Fibrillogenesis and Fibril Disassembly Assays. Peptides were stored as lyophilized powders at −20 °C and assayed for inhibitory activity using an adaptation of the Lansbury method (*25*). Prior to an experiment, the peptides were dissolved in hexafluoroisopropyl alcohol (HFIP) as concentrated stock solutions (50 mg/mL). For an inhibition assay, the inhibitor peptide was divided into aliquots, and the HFIP was evaporated under a stream of dry nitrogen. The dried peptide was resuspended in 100 mM Tris buffer and 150 mM NaCl, pH 7.4. An aliquot of Aβ40 peptide in HFIP was then added to the solution, containing or not containing an inhibitor peptide. The mixtures were vortexed for approximately 30 s and then incubated at 37 °C for 5−7 days without shaking. The final concentration of Aβ40 in the mixture was either 100 or 200 μM. In some experiments, stock solutions of Aβ40 were also prepared in DMSO to compare the effects of dissolving Aβ in DMSO or HFIP. No significant differences were found between these two solvents in fibrillogenesis assays. Final concentrations of HFIP and DMSO in the assay solutions were less than 2% (v/v), which was found not to inhibit fibrillogenesis (*26*).

For a disassembly experiment, Aβ40 was incubated alone for 7 days to allow fibrils to form, as described in the previous paragraph. An aliquot of the formed fibrils in buffer was then added to inhibitor peptide that had been dried from HFIP. The extent of fibrils remaining intact was assayed using thioflavin T fluorescence, Congo Red binding, and electron microscopy, as described below.

Electron Microscopy. After incubation for the appropriate period of time, an aliquot of each sample was applied to a glow-discharge, 400-mesh, carbon-coated support film and stained with 1% uranyl acetate. Micrographs were recorded using Philips EM300 at magnifications of 17000×, 45000×, and 100000×.

Fluorescence Spectroscopy. Fluorescence experiments were performed essentially as described using a Hitachi F-2000 fluorescence spectrophotometer (*27*). Maximum fluorescence was observed with the excitation and emission wavelengths set to 446 and 490 nm, respectively. The reaction mixture contained 5 μM thioflavin T in 50 mM glycine−NaOH buffer, pH 8.5. A 5 μL aliquot of solution containing fibrils was injected into 1 mL of the thioflavin solution. The solution was mixed vigorously, and the signal was then averaged for 30 s.

Circular Dichroism. The circular dichroic (CD) spectra were recorded using a Jasco P715 spectropolarimeter. Peptides were dissolved in 100 mM phosphate buffer at pH 7.4, at concentrations ranging from 0.1 to 6 mg/mL, as

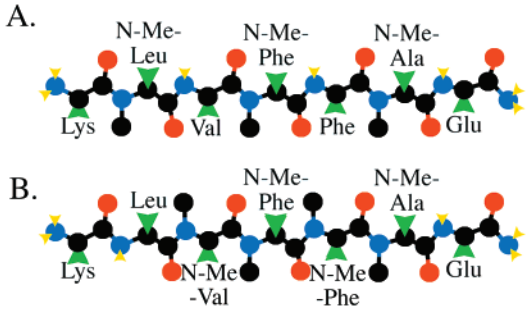

FIGURE 1: Diagram of (A) Aβ16−22m and (B) Aβ16−22m(4) that illustrates the position of the methyl groups when the peptides are arrayed in a β-strand conformation. In the figure, carbon atoms are in black, nitrogen atoms are in blue, oxygen atoms are in red, and hydrogen atoms on amide and amino nitrogen atoms are in yellow; other hydrogen atoms are not shown. In Aβ16−22m or Aβ16−22mR, the methyl groups are aligned on only one face of the β-strand. In contrast, the methyl groups are located on both faces of the Aβ16−22m(4) peptide.

determined with the bicinchoninic acid assay (BCA). A 1 mm or 0.1 mm path length was used for measurements, depending on the concentration of the solution. Six to eight scans were acquired from 250 to 200 nm.

Chymotrypsin Digestion. The peptides were dissolved in 0.5% ammonium bicarbonate at a concentration of 1.0 mg/mL. The pH of the solution was 8.4. Chymotrypsin (Worthington Biochemical Corp.) was added to the peptide solutions so that the final concentration was 0.1 mg/mL. Samples were incubated at 37 °C. After 24 h, the samples were frozen and lyophilized. The samples were analyzed by reverse-phase HPLC using an analytical C18 column (Rainin Microsorb) and eluted using a 60 min gradient from 10% to 70% acetonitrile, containing 0.1% (v/v) TFA. The loss of intact peptide and appearance of fragments were quantitated by integration of the appropriate peaks. Results were expressed as a percent digestion of the peptides. In addition, identities of the peaks were confirmed by electrospray mass spectrometry.

RESULTS

Design of Fibrillogenesis Inhibitor Peptides. The peptides described below are based on the central, hydrophobic core domain of Aβ40 that is critical for fibril formation, since alteration of this domain abrogates fibrillogenesis (*28*, *29*). Our strategy was to incorporate *N*-methyl amino acids into alternate positions of this short peptide. In a β-sheet, alternate amide protons and carbonyl oxygens are oriented to opposite sides of the peptide backbone. Thus, a peptide containing an alternation of ordinary amino acids and *N*-methyl amino acids, when in the β-strand (or extended) conformation, should have one "face" containing ordinary amino acids and one "face" containing *N*-methyl amino acids (Figure 1). We predicted that, in such a peptide, the face containing ordinary amino acids would be able to interact with Aβ, while the face containing *N*-methyl amino acids would not, and would, on the contrary, disrupt forming and/or existing Aβ fibrils.

Accordingly, we synthesized the peptides listed in Table 1. Peptide I (Aβ16−22) consists of amino acids 16−22 of Aβ and an amidated C-terminus but contains no *N*-methyl amino acids. Peptides II and III (Aβ16−22m and Aβ16−22mR, respectively) contain *N*-methyl amino acids at alternate residues; thus these two peptides are predicted to act as

Table 1: Summary of Peptides Synthesized

	peptide	sequence
I	Aβ16−22	NH_2-KLVFFAE-$CONH_2$
II	Aβ6−22m	NH_2-K(Me-L)V(Me-F)F(Me-A)-E-$CONH_2$
III	Aβ16−22mR	NH_2-E(Me-L)V(Me-F)F(Me-A)-K-$CONH_2$
IV	Aβ16−22m(4)	NH_2-KL(Me-V)(Me-F)(Me-F)(Me-A)-E-$CONH_2$
V	PrPm	NH_2-GA(Me-A)AAA(Me-V)V-$CONH_2$
VI	Ac-Aβ16−22	Ac-NH-KLVFF-$CONH_2$

inhibitors of fibrillogenesis. These two peptides differ from each other in the placement of the two charged residues, Aβ16−22m preserving and Aβ16−22mR reversing the positions of these two amino acids found in natural Aβ. Peptides IV and V [Aβ16−22m(4) and PrPm, respectively] also contain *N*-methyl amino acids but are predicted not to act as inhibitors of Aβ fibrillogenesis. Aβ16−22m(4) has the same sequence as the previous three peptides, except that it contains *N*-methyl amino acids at consecutive rather than alternate positions. Consequently, if this peptide formed a β-strand, it would have *N*-methyl amino acids on both faces of the peptide backbone and would be predicted not to interact with Aβ40. PrPm has *N*-methyl amino acids at alternate positions, but the sequence is from an unrelated protein (albeit another fibril-forming one), the human prion protein. In all cases, the peptides were synthesized with amidated C-termini.

Synthesis of Fibrillogenesis Inhibitor Peptides. Yields from syntheses of peptides containing *N*-methyl amino acids are not adequate if coupling reagents from standard FMOC chemistry are used (*30*, *31*). For this reason, the activating reagent HATU was required for the coupling steps immediately after an *N*-methyl amino acid (*30*−*33*). The use of this reagent gave excellent purity and yields of the target peptides.

The *N*-methyl amino acid containing peptides are surprisingly soluble, and solutions could be prepared with peptide concentrations exceeding 40 mg/mL at physiological pH (7.4) and salt concentration (150 mM). In contrast, the corresponding unmethylated peptides are soluble at concentrations up to ≈1−2 mg/mL, i.e., 20−40-fold less soluble under similar conditions. In view of the increased hydrophobicity and the diminished hydrogen-bonding potential of the *N*-methylated peptide, its excellent solubility in water was unexpected.

Inhibition and Disassembly. Two of the *N*-methyl peptides, Aβ16−22m and Aβ16−22mR, prevented fibril formation of Aβ40 in a dose-dependent manner *in vitro*. These are the two peptides containing *N*-methyl amino acids in alternating positions of the sequence. The extent of inhibition was quantitated with the thioflavin assay described in Materials and Methods. Figure 2A shows thioflavin fluorescence as a function of inhibitor concentration; since a constant concentration of Aβ40 peptide was used, this is expressed as the molar ratio of inhibitor:Aβ40 peptide. To compare relative potency of the peptides, data for both inhibition of fibrillogenesis and disassembly of preformed fibrils were fit to a simple equation, that of a hyperbola, i.e., analogous to that used to analyze Michaelis−Menten kinetics or ligand binding to macromolecules:

$$\text{fluorescence (\% of control)} = 100\% - \frac{IC_{max}P}{IC_{50} + P}$$

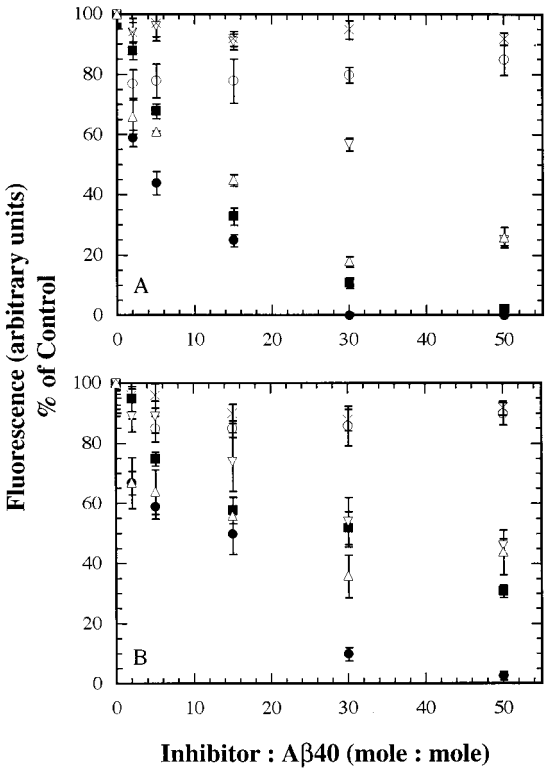

FIGURE 2: Inhibition of fibrillogenesis (A) and disassembly (B) of Aβ40 fibrils by inhibitor and control peptides. In (A), Aβ40 samples were incubated for 1 week at 37 °C in the presence of various concentrations of peptides; thioflavin-induced fluorescence was then measured as described in Materials and Methods. In (B), the peptide inhibitors were added to Aβ40 fibrils which had been preformed by incubating Aβ40 for 1 week at 37 °C. After addition of peptide inhibitors, the mixtures were incubated for an additional 3 days at 37 °C. After incubations, a 5 μL aliquot of peptide solution was diluted into 1 mL of 50 mM glycine, pH 8.5, containing 5 μM thioflavin. Data were collected as described in Materials and Methods. Data are expressed as a percentage of the signal obtained in the absence of inhibitor peptides. Symbols are as follows: (●) Aβ16−22m; (■) Aβ16−22mR; (△) Aβ16−22; (▽) Aβ16−22m-(4); (×) PrPm; (○) Ac-Aβ16−22.

Table 2: Summary of Fibrillogenesis Inhibition and Fibril Disassembly Data

	inhibition of fibrillogenesis		fibril disassembly	
peptide	IC_{50}	IC_{max}	IC_{50}	IC_{max}
Aβ6−22m	4.2	100	6.9	100
Aβ16−22mR	7.8	100	23.7	100
Aβ16−22m(4)	38.9	100	31.6	100
PrPm	6.0	8.6	8.9	10.3
Ac-Aβ16−20	8.4	100	11.3	100
Aβ16−22	1.1	23.0	11.3	89.2

where P is the inhibitor peptides:Aβ ratio and the two parameters are analogous to V_{max} and K_m, respectively. Values of the two parameters for each of the peptides are listed in Table 2. Both Aβ16−22m and Aβ16−22mR are effective inhibitors of fibrillogenesis; the IC_{50} of Aβ16−22m and Aβ6−22mR occurred at inhibitor:Aβ40 ratios of 4:1 and 9:1, respectively. Incubation with greater than a 30-fold molar excess of Aβ16−22m resulted in complete elimination of thioflavin fluorescence; for Aβ16−22mR, this occurred at higher ratios, 50:1. The Aβ16−22m(4) peptide, containing four *N*-methyl amino acids, but in consecutive rather than alternating positions, was a weak inhibitor of Aβ fibrillogenesis, having an IC_{50} ratio in excess of 40:1. The unmethylated control peptide, Aβ16−22, had a relatively modest inhibitory effect on fibril formation. As shown in Figure 2A, at concentrations at which Aβ16−22m inhibited fibrillogenesis completely, the unmethylated Aβ16−22 inhibited fibrillogenesis by approximately 10−20%. The inhibition of fibrillogenesis by Aβ16−22 did not exceed 20% in any of these assays. Finally, an unrelated, methylated peptide, PrPm, had no effect on Aβ40 fibril formation.

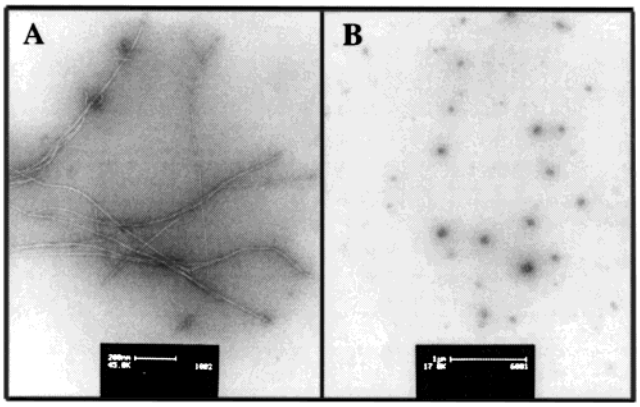

FIGURE 3: Electron microscopic examination of the effect of Aβ16−22m on fibril formation. (A) Electron micrograph of Aβ40 fibrils formed after a 1 week incubation at pH 7.4. Magnification, 42000×. (B) Electron micrograph of Aβ40 incubated with Aβ16−22m (30-fold molar excess) for 7 days. Magnification, 17000×.

These results were confirmed by electron microscopy, which demonstrated a complete lack of fibrils in Aβ40 samples with a 30-fold molar excess of inhibitor (Figure 3); EM showed round particles which may be complexes of Aβ40 and Aβ16−22m. Inhibition of fibril formation was also confirmed with a Congo Red binding solution assay (data not shown).

The inhibitor peptides, Aβ16−22m and Aβ6−22mR, both are also able to disassemble preformed Aβ40 fibrils. After incubation of Aβ40 for 7 days to form fibrils, different concentrations of the inhibitor peptides were added to the fibril solution. The extent of disassembly was then quantitated using the thioflavin assay after an additional 3 days of incubation at 37 °C. The IC_{50} for the disassembly occurred at inhibitor:Aβ40 ratios of approximately 10:1 and 25:1 for Aβ16−22m and Aβ16−22mR, respectively (Figure 2B). As was observed for inhibition of fibril formation, the remaining peptides either disaggregated fibrils weakly or did not do so.

To facilitate comparison of our data with those obtained for other fibrillogenesis inhibitors using different variations of methodology, we synthesized and tested, using the techniques described in Materials and Methods, a known fibrillogenesis inhibitor, that of Tjernberg et al. (*54*), listed as peptide VI (Ac-Aβ16−22) in Table 1. As with the other peptides reported above, we examined a range of inhibitor concentrations, using a standardized concentration of Aβ40 known to lead to fibril formation with predictable yields and kinetics, and expressed the results in terms of an inhibitor:Aβ molar ratio. As shown in Figure 2, Ac-Aβ16−22 did indeed inhibit Aβ(40) fibrillogenesis and disassembled preformed Aβ(40) fibrils. The IC_{50} occurred at an inhibitor:Aβ ratio of 10:1, in basic aggreement with the results of Tjernberg et al. By the criterion of the IC_{50}, Ac-Aβ16−22

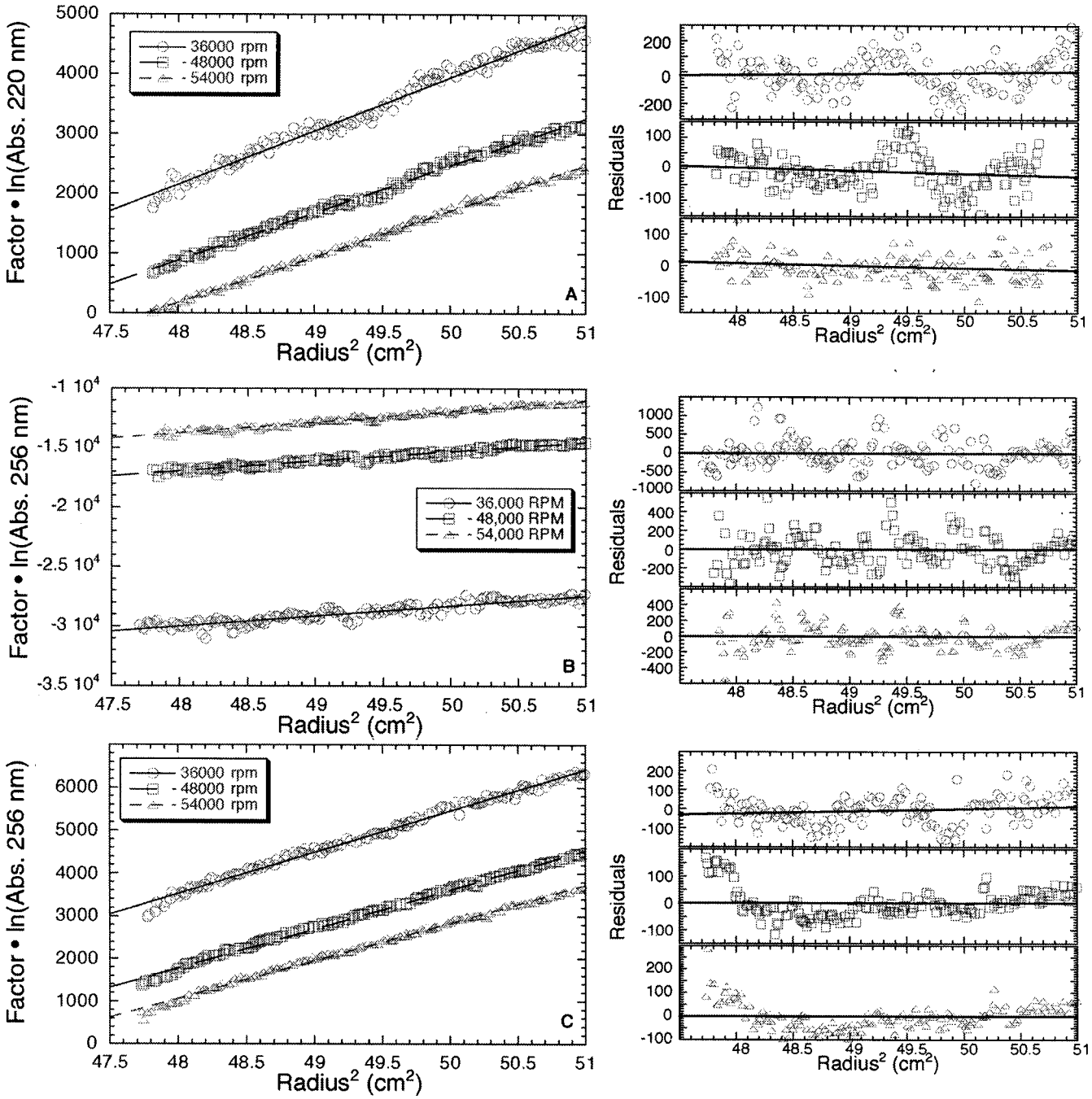

FIGURE 4: Analytical ultracentrifugation sedimentation equilibrium of a 100 μM (A), 500 μM (B), and 5 mM (C) solution of Aβ16−22m in buffer (100 mM phosphate, 150 mM NaCl, pH 7.4) at 36 000, 48 000, and 54 000 rpm. The data are displayed as normalized log plots. A homogeneous sample should exhibit a series of parallel lines with the same slope (MW) for all rotor speeds. The solid lines drawn through the data were obtained by fitting the ln(absorbance) versus r^2 (r = radius) data to an equation of a single ideal species. Higher order fits resulted in poorer agreement with the experimental data. The residual differences between the experimental data and theoretical curves are plotted in the right panels.

was highly effective for inhibiting fibrillogenesis and disassembling preformed fibrils, though slightly less so than Aβ16−22m or Aβ16−22mR.

Analytical Ultracentrifugation. A number of small peptides derived from the full-length Aβ are capable of aggregating and forming fibrils. Analytical ultracentrifugation, consequently, was used to determine if Aβ16−22m aggregates, either as an oligomer or as a fibrillar species. Experiments were conducted at three different peptide concentrations and at three different rotor speeds (Figure 4). Modeling the data as a single ideal species resulted in the best agreement with the theoretical curves. Table 3 summarizes the molecular weight values obtained from the analysis of the different data sets. The average molecular weight is 870 ± 10, which is quite close to the calculated weight of 893.9.

Table 3: Summary of Analytical Ultracentrifugation Data

	36 000 rpm	48 000 rpm	54 000 rpm
100 μm Aβ16−22m	904 ± 9	796 ± 5	767 ± 4
500 μm Aβ16−22m	858 ± 29	825 ± 15	904 ± 14
5 mM Aβ16−22m	969 ± 7	919 ± 4	888 ± 5

Circular Dichroism. Peptides containing *N*-methyl residues are restricted in their backbone conformations; *N*-methyl

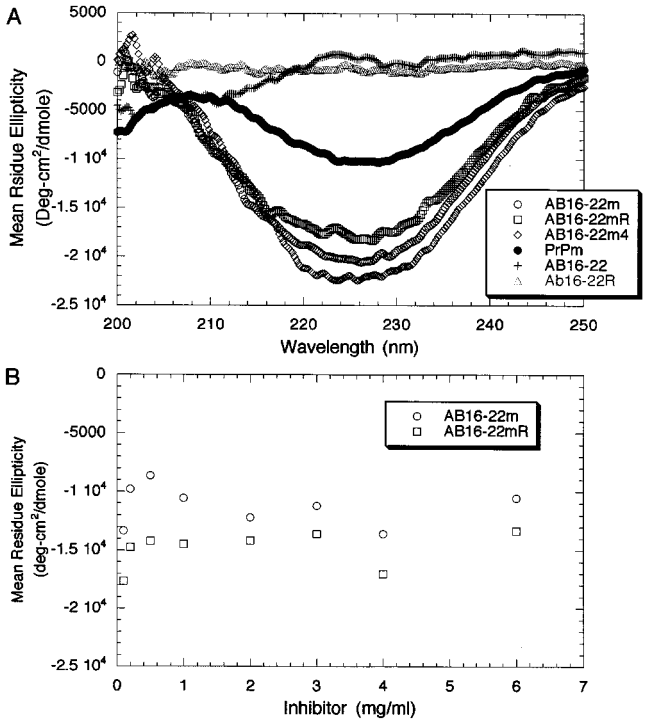

FIGURE 5: Circular dichroic spectra of inhibitor peptides. (A) compares the spectra of Aβ16−22 and Aβ16−22m. (B) examines the concentration dependence of the β-sheet structure as reflected by the mean residue ellipticity at 226 nm. Data were collected as described in Materials and Methods.

amino acids destabilize α-helices and tend to promote the β-sheet (*34*, *60*). The CD spectra of Aβ16−22m and Aβ16−22mR, both of which have three *N*-methyl amino acids, are characteristic of a β-sheet secondary structure except that the minimum is shifted to 226 nm (Figure 5A). Similar red-shifted β-sheet spectra have been observed for a number of other peptides, and this shift has been attributed to the twist of the β-sheet (*35*−*38*). In the case of the inhibitor peptide, however, it is also possible that the methyl groups are affecting the electronic properties of the peptide bond and, hence, their transitions observed by CD spectroscopy. Red-shifted minima have also been observed for other peptides containing *N*-methyl amino acids (*23*, *24*). In contrast to the *N*-methyl peptides, the CD spectrum of the unmethylated, control peptide Aβ16−22 is that of a random coil in solution. The mean residue ellipticity of Aβ16−22m at 226 nm, the minimum observed in the CD spectra, is independent of concentration (Figure 5B) between peptide concentrations of 0.1 and 6 mg/mL. This is consistent with the analytical ultracentrifugation results that demonstrated the peptide is monomeric in solution.

Protease Resistance. Small peptides are often highly sensitive to proteolytic degradation, and this was indeed the case for Aβ16−22. This unmethylated peptide contains a predicted chymotryptic cleavage site and was shown to be cleaved by chymotrypsin (Figure 6C,D). The figure also shows the molecular mass of peptides in the peaks, as determined by mass spectrometry. Peak A, eluting at 16.8 min, had a molecular weight of 505.61, consistent with the predicted molecular weight of 506.4 for NH_2-KLVF-COOH; peak B, eluting at 22 min had a molecular weight of 851.98, consistent with the predicted molecular weight of 852.6 for the intact starting peptide, NH_2-KLVFFAE-$CONH_2$; and

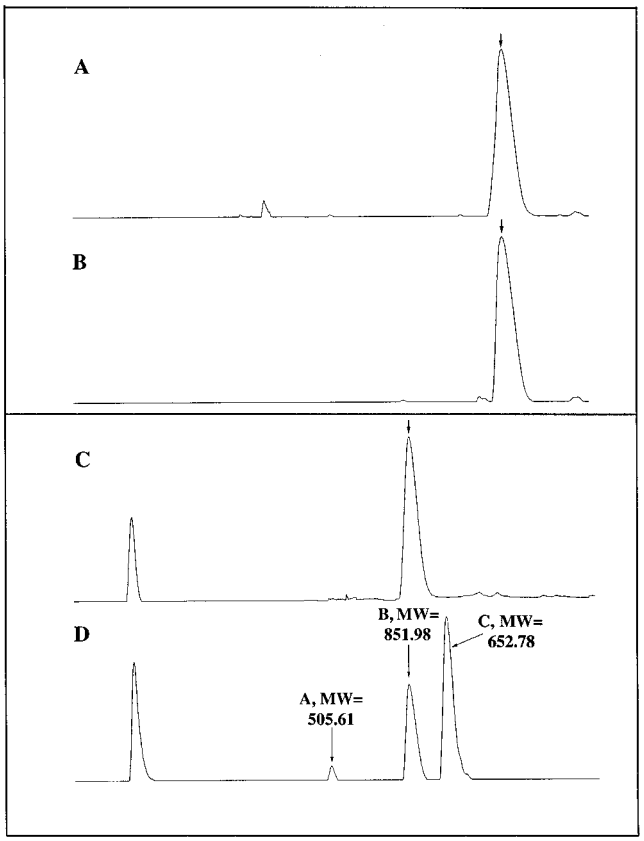

FIGURE 6: Protease resistance of Aβ16−22 and Aβ16−22m. Peptides were incubated for 24 h at 37 °C with 1% (w/v) chymotrypsin. The percentage of undigested peptide was determined by RP-HPLC as described in Materials and Methods. The figure shows chromatographs of Aβ16−22m before (A) and after (B) incubation with chymotrypsin and of Aβ16−22 before (C) and after (D) incubation with chymotrypsin. The arrow marks the position of the intact peptides.

peak C, eluting at 25 min, had a molecular weight of 652.78, consistent with the predicted molecular weight of 653.5 for NH_2-KLVFF-COOH. In contrast, Aβ16−22m exhibited complete resistance to chymotrypsin digestion over a period of 24 h (Figure 6A,B).

DISCUSSION

In this paper we demonstrate that two small, selectively methylated peptides function as inhibitors of Aβ40 fibrillogenesis and induce disassembly of preformed Aβ40 fibrils. The inhibitor peptides Aβ16−22m and Aβ16−22mR were designed to present two faces when in the β-strand (extended) conformation: a "binding face" and a "blocking face". The periodicity of a β-strand makes it an inherently repetitive structure. Amphiphilic β-strand peptides, for example, have alternating hydrophilic and lipophilic amino acids (39). This repetitive nature of β-strands allows for the design of peptides with faces of different characters by the strategic placement of modifications. In the peptides described in this paper, *N*-methyl amino acids were used to form the blocking face because the methyl group removes a backbone hydrogen bond interaction between β-strands in a β-sheet. In addition, the *N*-methyl amino acids are sterically hindered and tend to be restricted in their backbone conformations to the β-sheet geometry (*13*−*18*, *34*). The need for the *N*-methyl amino acids to alternate was shown by the fact that Aβ16−22m-

(4), a homologous peptide containing four consecutive *N*-methyl amino acid residues, was only a weak inhibitor. Furthermore, the fact that PrPm also was not an inhibitor suggests that alternate spacing of *N*-methyl amino acids was not sufficient to form an inhibitor, i.e., there is also a need for the inhibitor to have sequence homology to the fibril-forming peptide.

The Aβ16−22m and Aβ16−22mR peptides fulfill the design requirements for a fibrillogenesis inhibitor. In addition to inhibiting fibrillogenesis, these peptides also cause disassembly of preformed Aβ40 fibrils. The latter feature is in common with some well-studied inhibitors of fibrillogenesis or crystallization [e.g., polymerization of hemoglobin S (*39*) and calcium oxalate crystallization (*40*), among others] and suggests reversibility of many of the steps of Aβ fibrillogenesis.

Aβ16−22m and Aβ16−22mR also possess two other traits of potential relevance to the development of a therapeutic agent. First, they are highly soluble in aqueous solutions. This may be surprising in view of the added hydrophobicity attributable to the *N*-methyl group and due to the removal of one potential site of hydrogen bonding between the peptide and water. Nevertheless, the *N*-methyl peptides are 20−40 times more soluble than the unmethylated congeners. Indeed, this appears to be a trait in common for all *N*-methyl peptides we have studied, as both Aβ16−22m(4) and PrPm were also highly soluble in water. Second, Aβ16−22m is highly resistant to proteolytic digestion. Although the unmethylated congener, Aβ16−22, contains a scissile peptide bond, the methylated peptide was completely resistant to chymotryptic digestion. Protease resistance has been observed for other *N*-methyl amino acid containing peptides (*42*, *43*) and may be a general trait.

Although the CD spectra of Aβ16−22m and Aβ16−22mR were most consistent with a β-sheet conformation, an unusual feature is the minimum at 226 nm, instead of the more usual minimum at 218 nm. Red-shifted minima have been observed for other β-sheet peptides and may be attributed either to the unusual pitch of the β-sheet (*23*, *24*) or, in the case of *N*-methyl amino acids containing peptides, possibly also to differences in the electronic structure of the groups undergoing the transition.

Both of the peptides with alternating *N*-methyl residues, Aβ16−22m and Aβ16−22mR, were inhibitors of fibrillogenesis, but the former peptide was consistently observed to be the more effective inhibitor. The same rank order was observed for disassembly of preformed Aβ40 fibrils. While these data can be accommodated by the assumption of either a parallel or antiparallel orientation of either inhibitor with respect to the Aβ40 peptide, the antiparallel orientation appears somewhat more likely for the more potent of these two inhibitory peptides, Aβ16−22m. In the case of Aβ16−22m, an antiparallel orientation would minimize unfavorable charge interactions between the Lys and Glu side chains of Aβ16−22m and Aβ40. For the less potent inhibitor, Aβ16−22mR, two possibilities would then seem to exist: (1) It too might align with the Aβ40 peptide in an antiparallel orientation, but this would result in an unfavorable charge interactions between side chains; such unfavorable charge interactions could account for its lesser potency as an inhibitor peptide. (2) Alternatively, to avoid such unfavorable charge interactions, this peptide could be aligned parallel to the Aβ40 peptide. However, if this latter possibility were true, the decreased potency of Aβ16−22mR would then suggest that, in the absence of unfavorable side chain interactions, an antiparallel orientation between Aβ and inhibitors is inherently more stable than the parallel orientation. In support of this contention, Tycko and co-workers have recently observed in solid-state NMR experiments that while full-length Aβ peptides exist in a parallel, in-register alignment in the fibril, similar to Aβ(10−35) fibrils (*45*−*47*), Aβ16−22 peptides form fibrils with antiparallel β-sheets (*44*). Similarly, Tjernberg et al. have demonstrated that a similar peptide, Aβ16−20 (KLVFF), binds stereospecifically to the homologous region of Aβ (*48*). On the basis of modeling studies, these authors propose an antiparallel orientation between the inhibitor and full-length peptide. Further data are needed to examine the orientation with respect to Aβ40 of the two inhibitor peptides reported in this paper.

The ability to inhibit the formation of Aβ fibrils, or to disassemble preformed Aβ fibrils, has potential therapeutic significance. Guided by this precept, many investigators have either searched for natural inhibitors of fibrillogenesis or designed and synthesized inhibitors of Aβ fibrillogenesis. A number of small nonpeptide molecules have been shown to inhibit amyloid formation. Nicotine, melatonin, rifampicin, and hexadecyl-*N*-methlypiperidinium bromide, for example, block either Aβ aggregation or toxicity (*29*, *49*−*51*). The mechanism of inhibition of these unrelated compounds, however, is not clear.

Peptides homologous to regions of Aβ are also frequently used as inhibitors of fibril formation. Most of these studies have focused on the central hydrophobic core domain of Aβ (^{17}LVFF^{21}A) that is critical for fibrillogenesis (*28*). Ghanta et al. (*52*) and Pallitto et al. (*53*), for example, designed an inhibitor peptide derived from residues 15−25 that also contains an oligolysine disrupting element. Although this peptide prevented Aβ toxicity in cell culture, it did not block aggregation or fibrillogenesis of Aβ40, and the mechanism by which it blocks toxicity is not certain. Tjernberg et al. reported an acetylated hexapeptide corresponding to this central region that is an effective, equimolar inhibitor of Aβ40 aggregation (*48*, *54*). A significant problem with this peptide, however, is that it aggregates and forms fibrils by itself. In addition, it has modest solubility in aqueous media and is susceptible to proteases, both of which could limit its potential as a therapeutic agent. Soto and co-workers have utilized the unique structural properties of the amino acid proline in the design of "β-sheet breaker" peptides derived from the same hydrophobic region but containing nonconservative amino acid substitutions (*55*−*58*). Notably, these peptides incorporate prolines into sequences of Aβ fragments and are effective inhibitors of fibrillogenesis *in vitro* and *in vivo*. The similar *in vitro* activity of the two inhibitor peptides presented in the present paper and the β-sheet breaker peptides provides hope that Aβ16−22m and Aβ16−22mR will also function in vivo. Findeis et al. has prepared a series of peptides with modified N-termini, some of which are potent inhibitors of Aβ nucleation and polymerization (*59*). Most recently, Hughes et al. studied congeners of Aβ25−35 that were N-methylated at single residues (*22*). Of these, one peptide (Aβ25−35, N-methylated at Gly33) blocked the aggregation into fibrils and the toxicity of Aβ25−35. Another

peptide (Aβ25−35, N-methylated at Gly25) formed fibrils and was neurotoxic like nonmethylated (Aβ25−35), while a third peptide (Aβ25−35, N-methylated at Leu34) had reduced toxicity and altered fibril morphology but did not eliminate fibril formation by Aβ25−35. Tests of the ability of these singly N-methylated peptides to inhibit fibrillogenesis by full-length Aβ40 were not reported.

A comparison of peptides assessed by different investigators, using even slightly different assays, is by no means straightforward. Variations in fibrillogenesis kinetics can be marked (compare, for example, refs *54* and *59*). Several investigators (*54*, *56*, *59*) report inhibition of fibrillogenesis when inhibitor is added to Aβ peptides at a molar ratio of 1:1. This does not, however, necessarily indicate a 1:1 stoichiometry of an inhibitor:Aβ complex, although some have inferred this to be the case. To facilitate a comparison between our peptides and a peptide known to be an effective inhibitor—as positive control—we tested Ac-Aβ16−22 (*54*). As shown in Figure 2 and Table 2, this peptide did indeed inhibit Aβ40 fibrillogenesis. To estimate relative potency of the peptides as fibrillogenesis inhibitors and disassemblers of fibrils, data were fit to a simple equation, that of a hyperbola (i.e., analogous to that used to analyze Michaelis−Menten kinetics or ligand binding to macromolecules). Using the criteria of the two parameters generated by this analysis, the IC_{50} and IC_{max}, the potency of Aβ16−22m and Aβ16−22mR was at least as great as or slightly greater than that of a known fibrillogenesis inhibitor.

The goal of the studies presented in this paper was the rational design and synthesis of peptide inhibitors of fibrillogenesis. These peptides also may serve as potential structural probes of Aβ fibrillogenesis. Further experiments are needed to examine the mode of association between these inhibitor peptides and Aβ40, as well as the structure and pharmacodynamics of the inhibitor peptides themselves.

The strategy of using *N*-methyl amino acids in inhibitor peptides may be applicable to other diseases that involve aberrant protein aggregation and can, in theory, be applied to any self-associating proteins for which a site of peptide−peptide interaction is known. Preliminary studies suggest that an *N*-methyl amino acid containing peptide directed at aggregation of the prion protein may also be an effective aggregation inhibitor. Thus, we believe that the *N*-methyl amino acid containing peptide inhibitor may offer potential therapeutic benefit in Alzheimer's disease and a host of other amyloid diseases.

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