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Quantitative determination of the topological propensities of amyloidogenic peptides

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Abstract

One of the interesting puzzles of amyloid beta-peptide of Alzheimer's disease (Aβ) is that it appears to polymerize into amyloid fibrils in a parallel beta sheet topology, while smaller subsets of the peptide produce anti-parallel beta sheets. In order to target potential weak points of amyloid fibrils in a rational drug design effort, it would be helpful to understand the forces that drive this change. We have designed two peptides CHQKLVFFAEDYNGKDEAFFVLKQHW and CHQKLVFFAEDYNGKHQKLVFFAEDW that join the significant amyloidogenic Aβ (14–23) sequence HQKLVFFAED in parallel and anti-parallel topologies, respectively. (Here, the word "parallel" refers only to residue sequence and not backbone topology). The N-termini of the hairpins were labeled with the fluorescent dye 5-((((2-iodoacetyl)amino)ethyl)amino)naphthalene-1-sulfonic acid (IAEDANS), forming a fluorescence energy transfer donor–acceptor pair with the C-terminus tryptophan. Circular dichroism results show that the anti-parallel hairpin adopts a beta-sheet conformation, while the parallel hairpin is disordered. Fluorescent Resonance Energy Transfer (FRET) results show that the distance between the donor and the acceptor is significantly shorter in the anti-parallel topology than in the parallel topology. The fluorescence intensity of anti-parallel hairpin also displays a linear concentration dependence, indicating that the FRET observed in the anti-parallel hairpin is from intra-molecular interactions. The results thus provide a quantitative estimate of the relative topological propensities of amyloidogenic peptides. Our FRET and CD results show that beta sheets involving the essential Aβ (14–23) fragment, strongly prefer the anti-parallel topology. Moreover, we provide a quantitative estimate of the relative preference for these two topologies. Such analysis can be repeated for larger subsets of Aβ to determine quantitatively the relative degree of preference for parallel/anti-parallel topologies in given fragments of Aβ.

Keywords: Amyloid; FRET; Flourescent resonance energy transfer; Alzheimer's disease; Circular dichroism; Electron microscopy; Hairpin peptides

1. Introduction

Increasing evidence supports the hypothesis that the amyloid beta-peptide (Aβ) is of central importance in the pathogenesis of Alzheimer's disease (AD) [1,2]. It is widely believed that the aggregation of Aβ into amyloid fibrils initiates a cascade of events that lead to dementia and death [3,4]. The implicit strategy of aggressively inhibiting aggregation is being pursued in hopes of curing or preventing AD [5–7]. Because Aβ fibrillization is entirely pathological, this strategy is especially attractive in that therapeutic intervention targeting aggregation is expected to produce few side effects. In spite of the universal need for a systematic, structure-based program for designing amyloid aggregation inhibitors, little progress has been made towards this goal. No high-resolution, three-dimensional structure of an amyloid fibril is known, and the forces that drive Aβ fibril formation are not understood.

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This lack of structural knowledge is underscored by a series of papers using solid-state NMR (SSNMR) techniques to determine structural constraints for Aβ fibrils. The first application of SSNMR to Aβ fibrils examined the nine-residue C-terminus fragment Aβ (34–42) [8,9], which is argued to nucleate full Aβ fibrils [10]. By combining their rotational resonance data utilizing molecular modeling techniques, a molecular model of the fibrils was proposed. The model prominently includes an anti-parallel beta-sheet conformation [11,12]. SSNMR studies [13] of the seven-residue fragments Aβ (16–22) also find an anti-parallel beta-sheet.

However, SSNMR measurements on Aβ (10–35) [14,15] and Aβ (1–40) [16,17] find that the fibrils formed from peptides including the central 10–35 section are, most remarkably and unexpectedly, organized in a parallel beta sheet. The specific forces that drive this change of topology are not known, but are likely to be central to the problem. A parallel beta-sheet juxtaposes hydrophobic segments (residues 17–21 and 30–42) of Aβ. The hypothesis that residues in the 17–21 region and their neighbors are critically involved in fibril formation is supported by the fact that residue substitution in the 17–20 region of Aβ can affect or eliminate fibril formation [18] in Aβ (1–40) and Aβ (1–42) [19].

Systematic consideration of larger Aβ fragments including this region led to the discovery of a small fragment, Aβ (14–23)=HQKLVFFAED, capable of producing fibrils whose EM morphology was similar to that of full-length Aβ [20]. The SSNMR results indicated that Aβ aggregation is sensitive to the residue sequence of the molecule considered. A necessary step towards understanding the residue-specific forces that determine fibril formation is to measure the relative attraction between sets of residues believed to be essential to aggregation. This will assist in the location of the structural weak points of fibrils, and provide targets for structure-based drug design.

Fluorescence resonance energy transfer (FRET) is widely used in studies of biomolecular structure and dynamics [21]. It provides information about distances on the order of 10 to 100 Å and is thus suitable for investigating spatial relationship of interest in biochemistry [22]. Fluorescence resonance energy transfer [23,24] is a distance dependent interaction between the electronic excited states of two dye molecules in which excitation is transferred from a donor chromophore group to an acceptor chromophore group without emission of a photon.

In this work, we use FRET and circular dichroism spectroscopy (CD) to determine quantitatively the relative degree of attraction between two copies of the decapeptide Aβ (14–23), arranged in anti-parallel and parallel topologies. We have designed two peptides CHQKLVFFAEDYNGKDEAFFVLKQHW and CHQKLVFFAEDYNGKHQKLVFFAEDW that contain the Aβ (14–23) HQKLVFFAED decapeptide in such parallel and anti-parallel topologies, respectively. They are separated by a four-residue sequence, YNGK, frequently found in Type 1 beta-turns [25]. A schematic picture of the expected hairpin conformations appears in Fig. 1. (It should be noted that the backbone conformation of the parallel hairpin, is, in fact, an anti-parallel conformation. However, the residue sequence that is presented would occur in a purely parallel beta sheet. For this reason, this hairpin is referred to as a "parallel" hairpin).

2. Materials and methods

2.1. Design and labeling of the parallel and anti-parallel hairpins

In these experiments, we designed peptide structures containing the decapeptide Aβ 14–23 sequence, structurally constrained so as to encourage the appearance of the anti-parallel and parallel topologies. The hairpin peptide contained two copies of Aβ 14–23 connected by four residues, YNGK, that are frequently found in β-turns [25,31]. A cysteine residue was added at the N-terminus of the peptide, and tryptophan was added at the C-terminus to form a 26 residue peptide. The specific amino-acid sequences were:

CHQKLVFFAEDYNGKDEAFFVLKQHW (parallel), and
CHQKLVFFAEDYNGKHQKLVFFAEDW (antiparallel).

The schematic structures of the designed hairpins are shown in Fig. 1. (Here, the word "parallel" refers only to residue sequence and not backbone topology).

2.2. Sample preparation

Peptides used in the studies were custom ordered from AnaSpec Inc. (San Jose, CA). Among FRET thiol-reaction

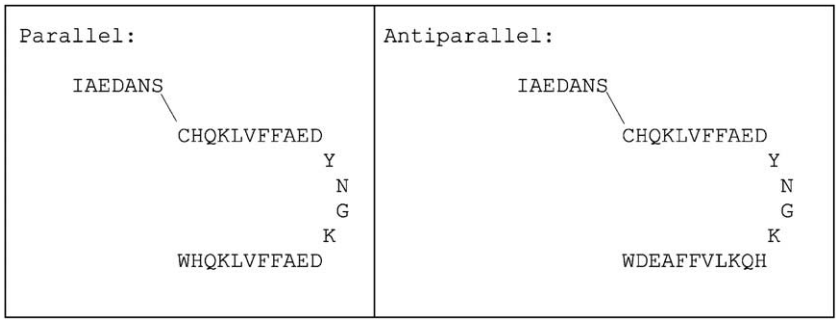

Fig. 1. The designed anti-parallel and parallel hairpin. The N-terminus of the peptide is labeled by fluorescent dye IAEDANS that forms the acceptor–donor pair with the C-terminus tryptophan in a FRET experiment.

probes, we chose 5-((((2-iodoacetyl)amino)ethyl)amino) naphthalene-1-sulfonic acid (IAEDANS) [26–28] as an acceptor, and tryptophan as a donor. The dye IAEDANS was obtained from Molecular Probes, Inc. (Eugene, OR). The hairpin peptides were dissolved at 50–100 uM in a 50 mM ammonium bicarbonate (NH4HCO3), pH 7.8.

About 5.3 mg peptide was used to react with IAEDANS. IAEDANS and peptide were mixed at 4:1 molar ratio. The reaction was under oxygen free environment. IAEDANS was allowed to react with thiol group of the cysteine residue at the N-termini of the anti-parallel and parallel hairpins for 1 to 2 h at room temperature. The labeled peptides were purified by HPLC on a Vydac reverse phase C8 column, with specifications of 300 Å, 5 μM, 10 mm ID × 250 mm L, with solvent (A) 100% water, 0.01% TFA and solvent (B) 100% acetonitrile, 0.01% TFA. Purity and identity were verified by utilizing an analytical C8 column, 300 Å, 5 μM, 4.6 mm ID × 150 mm L and MALDI TOF Mass Spectrometer. The purity was higher than 95%. The IAEDANS labeled parallel and anti-parallel hairpin peptides were dissolved in hexafluoro-2-propanol at 1 mg/ml for 0.5 h to disperse any possible aggregations. HFIP was then removed by speed vacuum. About 3.6 mg labeled peptide was obtained. The yield is about 62%.

2.3. FRET experiments

Before FRET experiments, the fluorescence dye labeled peptides were dissolved in DMSO at peptide concentrations of 5 mM, and then were diluted to a 50 μM stock solution using a buffer containing 50 mM Tris-HCl, and 150 mM NaCl, pH7.4. The stock solution was aliquoted and diluted to different concentrations with the same buffer. Peptides without the IAEDANS label served as controls. The FRET experiments were conducted in aqueous solutions containing 0.02–0.5% DMSO.

FRET experiments were conducted in a cuvette with a 1.0 cm path-length using a Perkin-Elmer Luminescence Spectrometer model LS50B. The excitation wavelength was 280 nm. The fluorescence signal was recorded graphically from 300 nm to a final wavelength of 540 nm. In all cases, 2 scans were performed and averaged. The instrument's excitation slit was set to 3.0 mm. The emission slit was 6.0 mm. The scan speed was 200 nm/min. The experiments were conducted at 25 °C.

2.4. Distance estimation from FRET

FRET is a distance-dependent interaction between electronic excited states of a fluorescence donor and acceptor. IAEDANS (maximal absorption wavelength λmax=336 nm, emission λmax=490 nm) at the N-terminus of the hairpin is the acceptor, while tryptophan (absorption λmax=280 nm; emission λmax=350 nm) at the C-terminus is a donor.

For a single donor–acceptor pair, the efficiency of energy transfer E is related to the distance R between the donor and acceptor by [30]:

$$E = (R_0/R)^6 / \left[1 + (R_0/R)^6\right].$$

Where R_0 is the distance at which energy transfer is 50% efficient. For the tryptophan–IAEDANS pair, R_0 is 22 Å [22,29].

The FRET efficiencies E were determined by:

$$E = 1 - F_{D-A}/F_D$$

where F_{D-A} and F_D are the fluorescence intensities of the donor in the presence and in the absence of the acceptor, respectively. Given the above equation, we can estimate the distance R between the donor–acceptor pair [28].

For concentration dependence studies IAEDANS labeled parallel and anti-parallel hairpins peptides were aliquoted as follows: 0.05, 0.1–0.5 μM (in increasing intervals of 0.1 μM), and 1.0–10.0 μM (in increasing intervals of 1.0 μM) in 50 mM Tris-HCl, and 150 mM NaCl, pH7.4. Measurements were taken on the Perkin-Elmer Luminescence Spectrometer model LS50B, at absorption of 480 nm (the observed maximum absorption).

2.5. Circular Dichroism spectroscopy (CD)

For FRET experiments, the peptides were first dissolved in DMSO and then diluted to aqueous buffer for FRET experiments. However, for CD experiments, DMSO interferes with CD absorbence causing significant amount of noise. We therefore chose to dissolve the peptides in 50 mM Tris-HCl, and 150 mM NaCl, pH7.4 containing 50% methanol in which the peptides did not precipitate. The EM experiments were also conducted in 50% methanol buffer solution.

CD experiments were conducted on a JASCO Model J-810 Spectropolarimeter. The sample cuvette pathlength was 0.1 cm, the wavelength varied from 195 to 260 nm. The scanning mode was continuous, and the scanning speed was 50 nm/min. Four scans were collected and averaged at 25 °C. Samples were measured at time of start (T=0), and incubated in the cuvette at room temperature 6 days (T=6 days). The same samples in the same buffer conditions were also examined by EM.

The peptide was denatured by diluting the 1 mM peptide/DMSO solution into 2 μM in 6 M Guanidine-HCl, 50 mM Tris-HCl, 150 mM NaCl, at pH7.4 to disrupt the structures of parallel and antiparallel peptides. The FRET and CD signals of the denatured peptides were compared with those of the antiparallel and parallel peptides.

2.6. Electron Microscopy (EM)

All samples were incubated for 6 days at room temperature in 50 mM Tris-HCl, 150 mM NaCl at pH7.4. 10 μL samples were placed on specimen grids covered by a formvar/carbon support film. Excess fluid was wicked off after 2 min, and the grids were negatively stained with 4 mg/mL uranyl acetate in water. The stained grids were then examined and photographed with JEOL JEM-100CXII electron microscope.

3. Results

The designed anti-parallel and parallel hairpins can both form fibrils at 50 uM concentrations (Figs. 2 and 3). The

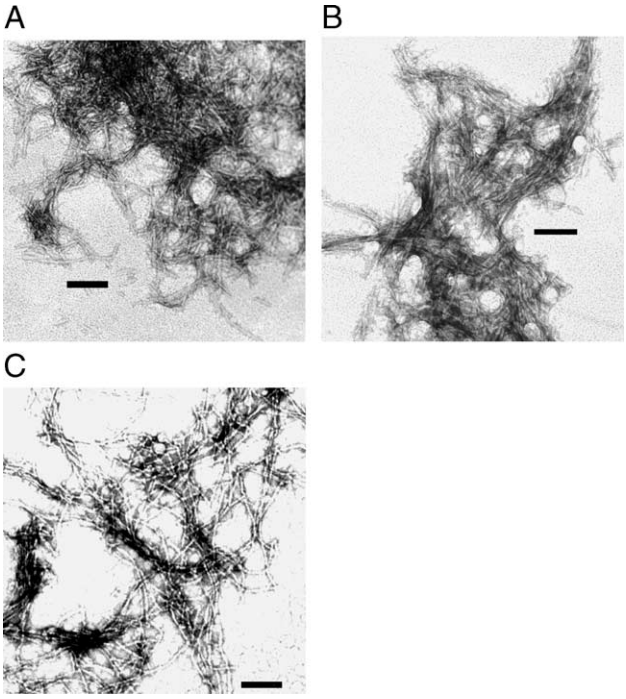

Fig. 2. The negative-stained electron microscopy photographs of the fibril filaments formed by (A) 50 μM anti-parallel IAEDANS peptides, (B) 50 μM parallel AEDANS peptide, and (C) 50 μM Aβ (1–40) used as a control. The fibrils are formed in 50 mM Tris-HCl, 150 mM NaCl at pH7.4, incubated for 6 days at room temperature. Bars shown in the photographs correspond to 100 nm. The EM photographs suggest that the morphology of the fibrils formed by the designed and the fluorescent dye labeled peptides and normal Aβ fibrils are similar.

morphology of fibrils formed by the IAEDANS labeled peptides (Fig. 2(A) and (B)) is similar to that of the fibrils formed by Aβ1-40 as shown in the native-stained EM (Fig. 2 (C)).

3.1. The anti-parallel hairpin shows significant beta-sheet content while the parallel hairpin is disordered

In the CD experiments (Fig. 4), the parallel hairpin adopted a disordered conformation at the starting time $T=0$. In contrast, the anti-parallel hairpin displayed significant β-sheet content at $T=0$, as the spectrum showed a positive band around 200 nm and a negative band at 217 nm [32]. As expected, the control Aβ1-40 exhibited β-sheet content. When incubated at room temperature for six days, Aβ1-40 displayed increased β-sheet percentage, the anti-parallel hairpin remained unchanged, while the negative band of the parallel hairpin moved from 230 to 225 nm, see Fig. 4(a), (b) and (c).

3.2. The FRET distance in the anti-parallel hairpin is shorter than that of the parallel hairpin

Fluorescence resonance energy transfer (FRET) was used to estimate the distance between the N-terminus and the C-terminus of the two hairpin peptides [33,34]. Under denaturing conditions both the anti-parallel hairpin and the parallel hairpin displayed little or no FRET (Fig. 5(A)), indicating that the C-terminus and N-terminus of the two hairpins were relatively far apart, compared to the Förster distance.

Fig. 5(B) shows the FRET of the designed hairpin in 50 mM Tris-HCl, 150 mM NaCl at pH7.0 buffer. It can be seen in Fig. 5(B) that the anti-parallel hairpin displays a larger energy transfer than the parallel hairpin. A greater energy transfer corresponds to a shorter distance between the hairpin termini being studied. This indicates that the anti-parallel hairpin has a shorter distance from the donor to the acceptor, i.e., the C-terminus is closer to the N-terminus in the anti-parallel hairpin. The average FRET distances are 20.2 ± 0.5 Å for the anti-parallel hairpin and 27.0 ± 1.8 Å for the parallel hairpin (errors are the standard deviation of the mean, with three measurements per point). It should be pointed out that the fluctuation in measured value likely has contributions from the relative stability of the two conformations, as well as from statistical fluctuations.

3.3. The anti-parallel hairpin energy transfer has linear concentration dependence, indicating an intramolecular interaction effect

In order to study the topological propensities of the designed hairpins, it is necessary to determine whether the energy transfer stems from intramolecular interactions within the hairpin, i.e., that the effect is caused by interactions between probes in the same molecule. This can be determined by measuring the dependence of the effect on concentration. If the

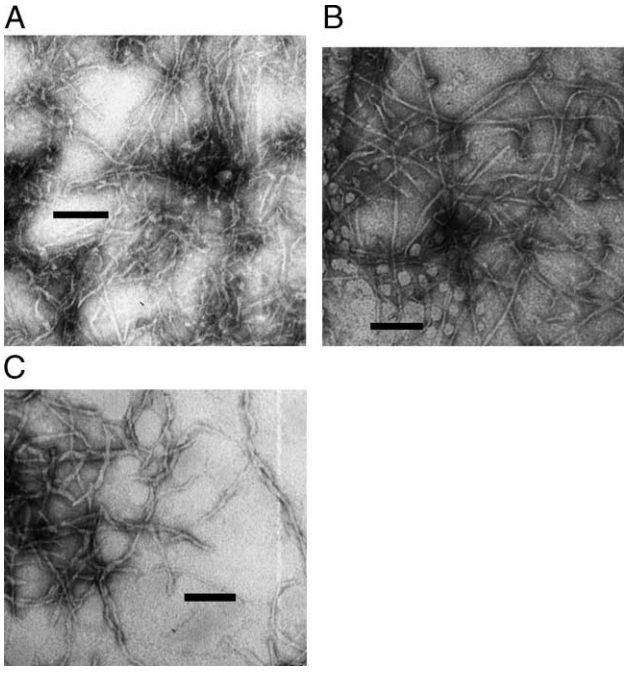

Fig. 3. The negative-stained electron microscopy photographs of the fibril filaments formed by (A) 50 μM anti-parallel IAEDANS peptides, (B) 50 μM parallel AEDANS peptide, and (C) 50 μM Aβ (1–40) used as a control. The fibrils are formed in 50% methanol at pH7.4, incubated for 6 days at room temperature. Bars shown in the photographs correspond to 100 nm. The EM photographs suggests that the morphology of the fibrils formed by the designed and the fluorescent dye labeled peptides and normal Aβ fibrils are similar.

measured energy transfer depends linearly on concentration, it implies that the effect is intramolecular. If, by contrast, the effect is quadratic or higher as a function of concentration, an intermolecular process that is second or higher order is

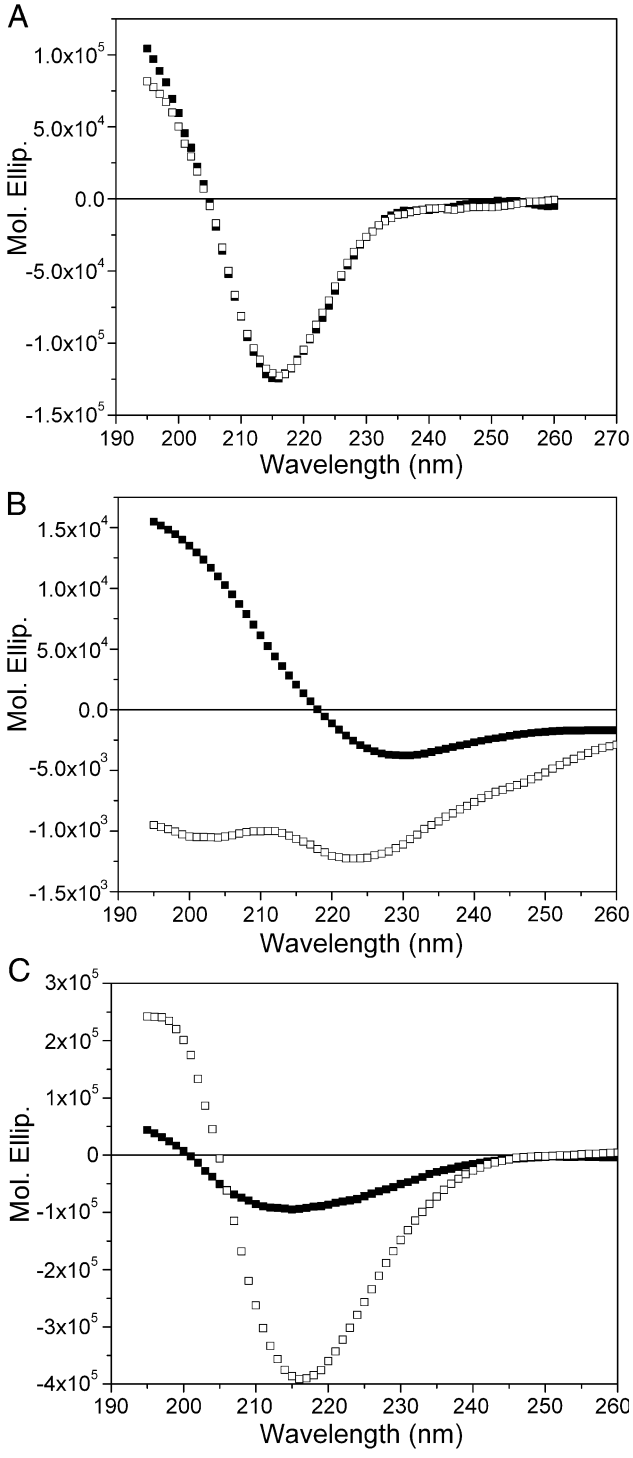

Fig. 4. CD spectra for (A) an 50 μM anti-parallel hairpin labeled with IAEDANS measured at $T=0$ day (■) and when incubated 6 days (□) at room temperature. (B) 50 μM parallel hairpin labeled with IAEDANS measured at $T=0$ day (■) and when incubated 6 days (□) at room temperature. (C) 50 μM amyloid beta peptide 1–40 measured at $T=0$ day (■) and when incubated 6 days (□) at room temperature. All the samples were dissolved in 50% methanol, pH7.4 (adjusted by NaOH). Note that the vertical scales are different.

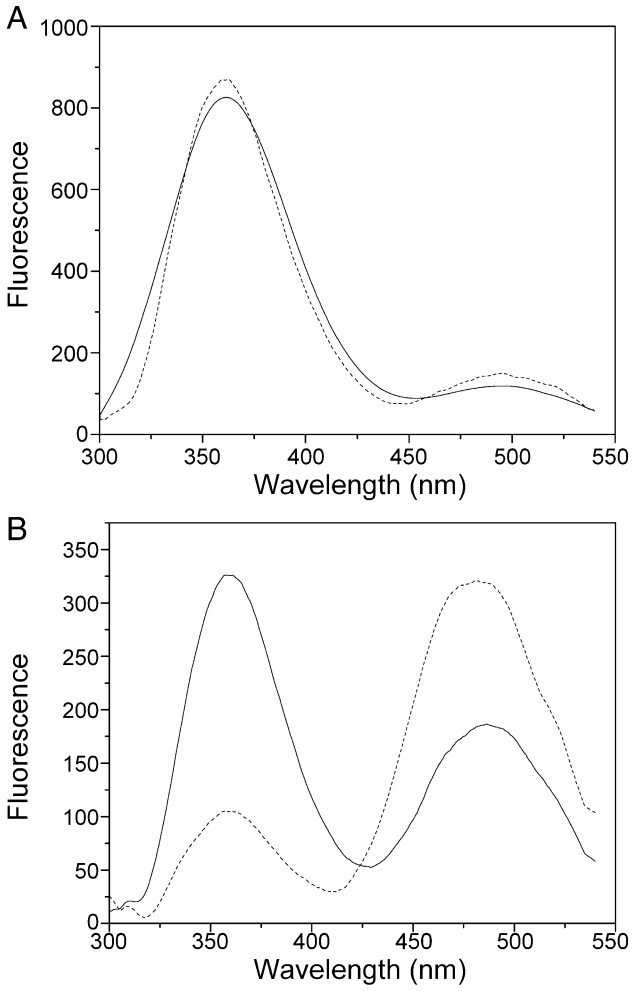

Fig. 5. FRET spectrum of the designed peptide (A) 2 μM IAEDANS labeled anti-parallel (dashed line) and parallel hairpin (solid line) in 6M GuHCl, 50 mM Tris-HCl, 150 mM NaCl, pH7.4. Under denaturing conditions, there is no FRET. (B) 2 μM IAEDANS labeled anti-parallel (dashed line) and parallel hairpin (solid line) in 50 mM Tris-HCl, 150 mM NaCl, pH7.4, immediately after the peptide/DMSO solution was added in the buffer. There is FRET in both the anti-parallel and parallel hairpins, but donor quenching is more pronounced in the anti-parallel hairpins. Background subtraction was done with unlabeled peptide.

implicated. We have measured the concentration dependence of the hairpin-IADEANS emission intensity at 480 nm. The data collected for the study demonstrates a direct linear relationship between concentration and intensity of absorbance for the anti-parallel hairpin. The anti-parallel hairpin also displayed an emission intensity nearly four times as large at a given concentration than that of the parallel hairpin (Fig. 6), providing further evidence that the anti-parallel hairpin forms more readily, with closer proximity, and with greater stability than the parallel hairpin.

4. Discussion

In order to determine allowable topologies in amyloid fibril formation one need to measure quantitatively the relative propensity for two peptides to align in a beta-sheet conformation. In this work we have performed such a comparative

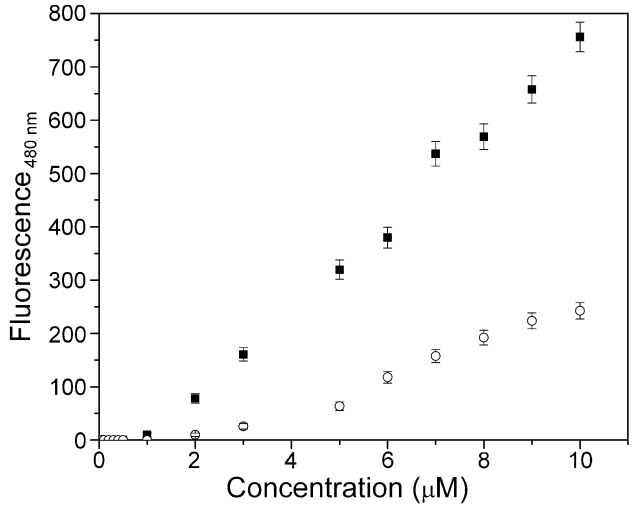

Fig. 6. Concentration dependence of FRET intensity of the anti-parallel (■) and the parallel (O) peptides measured at the emission wavelength 480 nm. Measurements were conducted immediately after the 100 μM stock solution was aliquoted to the Tris buffer at the desired concentrations, as well as after incubating the samples at room temperature for overnight.

analysis using FRET techniques. We used this technique to determine the relative degree of attraction between an important set of residues of Aβ, and have shown that an anti-parallel residue topology is preferred for this fragment. (Here, the word "parallel" refers only to residue sequence and not backbone topology).

This result is in accord with previous work on constructing anti-parallel versus parallel constrained peptide hairpin "bricks" [35]. It was shown that bricks with a parallel topology could not subsequently assemble into fibrils. Here, we probe the effect further, and show that, even in the solution phase, the anti-parallel topology is significantly preferred over the parallel hairpin topology (Figs. 7 and 8).

A reasonable explanation for the preference for an anti-parallel topology over a parallel topology follows from

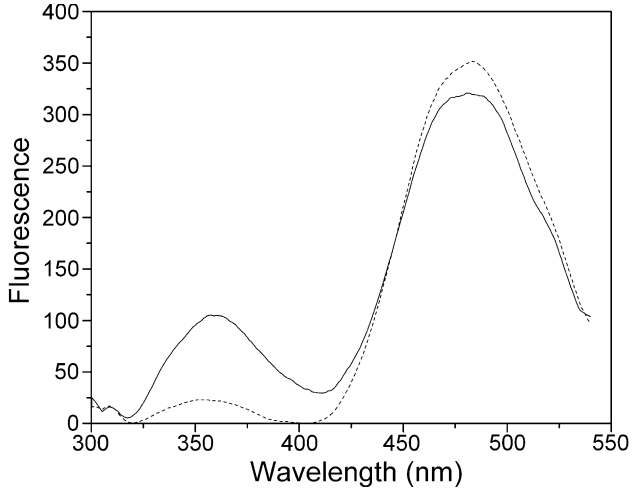

Fig. 7. Time course of FRET signal of the antiparallel hairpin. FRET of 2 μM AEDANS labeled antiparallel hairpin Aβ14–23 on $T=0$ day (solid line) and on $T=6$ day (dashed line) in buffer 50 mM Tris-HCl, 150 mM NaCl, pH 7.4 at room temperature.

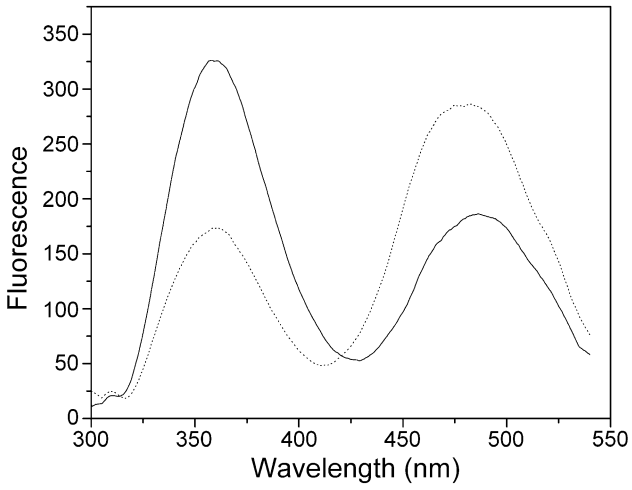

Fig. 8. Time course of FRET signal of the parallel hairpin. FRET of 2 μM AEDANS labeled parallel hairpin Aβ14–23 in buffer 50 mM Tris-HCl, 150 mM NaCl, pH7.4) on $T=0$ day (solid line) and on $T=6$ day (dashed line) at room temperature.

consideration of Coulombic repulsion. In a parallel topology, the charged residues (Lys^+, Glu^-, and Asp^-) will be aligned with their equally charged counterparts in a beta sheet conformation. No such Coulombic repulsion occurs for the anti-parallel loop; indeed, if the His residue were to become protonated, a pair of salt bridges between the protonated His^+ and the Asp^- could be formed. As pKa values depend upon environmental factors, this effect may contribute to the relative degree of attraction.

In order to demonstrate that the FRET experiment provides an estimate of an intramolecular distance (in this case, between the N- and C- termini of a hairpin), it is necessary to establish that the FRET signal is essentially intramolecular. This point was established by demonstrating that the concentration dependence of the FRET signal in the anti-parallel hairpin was linearly proportional to the concentration of the hairpin peptide.

One potential source of error in FRET distance measurements is the lack of an independent measurement of the orientation factor k^2, which is normally taken to be 2/3, the value appropriate for free isotropic motion by the FRET probes. It has been argued that this is a reliable assumption [27]. Because of the particular geometry chosen here, this source of error is further minimized. Although the orientation factor k^2 can, in principle, range from 0 to 4, for a pair of dipoles that are fixed adjacent and parallel (as would be found in a rigid beta strand topology), k^2 equals 1 [23]. Thus, the values expected to occur for k^2 range from 2/3 (for free isotropic motion) to 1 (for a rigid beta strand). The distance determined between FRET groups thus could possess a systematic error due to orientation factor effects of at most $1-(2/3)*(1/6)=7\%$. Thus, because of the specific topology chosen here, systematic error due to orientation factor effects is minimized.

We have used FRET techniques to determine directly and quantitatively the relative degree of attraction between segments of the Alzheimer amyloid beta-peptide in anti-parallel and quasiparallel topologies. We have also demonstrated that for an important fragment of this peptide, the anti-parallel

topology is strongly preferred. Although for this Aβ (14–23) fragment, an anti-parallel topology was preferred, the solid-state NMR results reviewed above show that, as longer and longer fragments are considered, a shift in topology occurs. This shift in topology can be explored by the FRET methodology developed here, and will provide information about the details of which specific amino-acid residues cause full-length Aβ to aggregate as it does. This information, in turn, can provide detailed structural targets for the design of potential therapeutics that target the aggregation pathway of Aβ [preliminary screening of this nature in our laboratory has identified small molecules that inhibit Aβ aggregation [36]]. The present study addresses the relative topological propensity of Aβ fragments to associate in solution. As it is not known whether the toxic species of Aβ is a soluble prefibrillar intermediary, or the insoluble fibrils themselves, this solution-state study thus addresses a complementary issue to the previously addressed solid-state assembly question [35]. Further studies of longer fragments are underway in our laboratory.

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