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Substoichiometric inhibition of A β ₁₋₄₀ aggregation by a tandem A β _{40-1-Gly8-1-40} peptide

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Abstract

A β peptides aggregate to form insoluble and neurotoxic fibrils associated with Alzheimer's disease. Inhibition of the aggregation has been the subject of numerous studies. Here we describe a novel, substoichiometric inhibitor of A β ₁₋₄₀ fibrillization as a tandem dimeric construct consisting of A β ₄₀₋₁ (reverse sequence) linked to A β ₁₋₄₀ via an eight residue glycine linker. At molar ratios of the tandem peptide to A β ₁₋₄₀ of 1:10 to 1:25 inhibition of fibrillization, as measured by Thioflavin T, was observed. We postulate that the tandem construct binds to a fibrillar intermediate but the reverse sequence delays or prevents further monomer association.

Keywords

ThioflavinT; fibrillization; reverse sequence; atomic force microscopy

1. INTRODUCTION

The aggregation of amyloid- β (A β) is believed to be associated with the onset of Alzheimer's disease and there have been numerous studies of compounds that affect the aggregation process. A large number of compounds have been observed to inhibit aggregation and these have generally been grouped into three classes: those that inhibit oligomerization, those that inhibit oligomerization and fibrillization and those that inhibit fibrillization only (1-9). Among these studies, several investigators have used small peptides that would be expected to bind to full length A β but would be unable to form any structure that could serve as a template for subsequent monomer (or oligomer) addition (10,11). These latter studies were based on the observation of Tjernberg et al. (12) which suggested that the central region of the A β peptide (residues 16-20) was responsible for the self-association and subsequent aggregation. These inhibitory peptides include those that are N-methylated (13,14) or cross-linked by a disulfide bond (15) or linked to an oligolysine (16). In general, such small peptides are indeed effective in preventing either oligomer formation or

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Supplementary Information

The mass spectroscopy data of the tandem peptide (figure S1) and the AFM image of the Tandem peptide aggregates (figure S2) are provided in the Supplementary materials.

aggregation (17,18) but the concentration of the peptide inhibitors required to prevent aggregation is high suggesting that the binding of the peptide is weak. Recently, Taylor et al. reported a class of retro-peptides which inhibited aggregation of either A β ₁₋₄₀ or A β ₁₋₄₂ at substoichiometric concentrations (19).

Here we report a novel approach to affect aggregation by using a tandem peptide consisting of two A β molecules linked by a glycine linker in such a way to give the tandem peptide construct NH₂-A β ₄₀₋₁-(Gly)₈-A β ₁₋₄₀-COOH. In this construct the N-terminal A β ₄₀₋₁ peptide has the reverse sequence of wild-type A β while the C-terminal A β peptide has the forward sequence.

2. MATERIAL AND METHODS

2.1 Preparation of the Tandem peptide and A β ₁₋₄₀

In our studies both A β ₁₋₄₀ and the tandem peptide were prepared as a fusion proteins as recently described by Garai et al. (20). The fusion protein of the tandem peptide was purified from inclusion bodies after dissolving in 6 M guanidine-HCl (GdnHCl), passed over a Ni²⁺-column and refolded on a Sephacryl S100 column in 25 mM HEPES with 100 mM NaCl at pH 7.5. The fusion protein eluted from the S100 column in two main fractions, one monomeric (60%) and the other multimeric (40%). Both fractions were treated with Factor Xa (New England Biolabs, USA) for 6 hrs at room temperature in 25 mM HEPES, pH 7.5 and 100 mM NaCl, using a ratio of Factor Xa to fusion protein of 1:100 (w/w). After cleavage, the peptide was precipitated with 200 μ M ZnCl₂, dissolved in 6 M GdnHCl and purified by HPLC. The tandem peptide eluted at 33.5-35% in an acetonitrile gradient, was lyophilized and stored at -20 °C. The yield of tandem peptide was 1mg/6L cell culture and showed a typical tyrosine fluorescence spectrum. Mass spectrometry of the purified material showed a single peak at a molecular weight of 9097.64 Da compared to the theoretical expected average isotopic mass of 9098 Da (supplementary figure S1). The peptide concentrations were measured by absorbance using values of 1450 M⁻¹cm⁻¹ and 2900 M⁻¹cm⁻¹ at 276 nm for A β ₁₋₄₀ and the tandem peptide, respectively.

2.2 Preparation of Peptide Solution and ThioflavinT assay

The time course of aggregation in the presence and absence of the tandem peptide was measured by Thioflavin T (Figure1). For these experiments, A β ₁₋₄₀ and the tandem peptide at concentrations of 200 μ M and 50 μ M respectively were dissolved in 5 mM NaOH. A 50 mM borate stock solution at pH 7.8 was then added to this solution to lower the pH to 8. The final concentration of borate buffer was kept at 25 mM. The solution was centrifuged at 14000g for 20-30 minutes and the supernatant solution was used for the experiments. Varying concentration of A β ₁₋₄₀ and the tandem peptide were incubated at 37 °C and the extent of aggregation was measured by Thioflavin T.

For ThioflavinT assay, 20 μ L of the peptide solution was added in 1ml of glycine buffer (50 mM, pH 9.0) containing 10 μ M of ThioflavinT. Solutions were excited at 450 nm and emission was recorded at 490 nm. Before each reading a blank containing no peptide solution was taken to check the baseline. Between each reading the cuvettes were thoroughly rinsed with distilled water and dried to avoid any contamination.

2.3 Atomic Force Microscopy (AFM) of Amyloid fibrils

For AFM studies, 75 μ M A β ₁₋₄₀ in the presence and absence of 3 μ M tandem peptide was incubated for 4 days at 37 °C in 25 mM borate buffer at pH 8.0. 5 μ L of sample was diluted 20-fold with distilled water and allowed to settle on the freshly cleaved mica surface for 30 min. The majority of the solution was drawn off and the samples dried for 10-15 minutes in

vacuo. The images were recorded using AFM MFP-3D-BIO (Asylum Research, Santa Barbara) with a scan resolution of 512×512 points/scan at a scan speed of 1 Hz.

2.4 Circular Dichroism Spectra of Tandem peptide

For secondary structural analysis with CD, a tandem peptide stock solution (50 μM) in 5 mM NaOH solution buffer was diluted 5-fold into 200 mM phosphate buffer at pH 8.0. The CD scans were recorded immediately after dilution using a Jasco J-715 spectropolarimeter. Ten scans from 200 to 260 nm with speed of 20 nm/minute were averaged. A buffer blank was performed under the same conditions and the final spectra were analyzed after buffer blank subtraction.

3. Results

3.1 The ThioflavinT assay

Figure 1A shows that the onset of fibrillization in the presence of the tandem peptide at a tandem peptide: $\text{A}\beta_{1-40}$ molar ratio of 1:25 is delayed about 2-fold. At a tandem peptide: $\text{A}\beta_{1-40}$ molar ratio of 1:10 (Figure 1B), no fluorescence increase is observed after 350 hrs compared to the fibrillization being complete by 175 hrs in the absence of the tandem peptide. The concentrations of $\text{A}\beta_{1-40}$ used in these experiments are 50 μM and 65 μM respectively, while those of tandem peptides are 2 and 6.5 μM , respectively. As a control experiment, two reference samples containing 2 μM and 6.5 μM of tandem peptide alone were kept under the same experimental conditions. Neither increase in ThioflavinT signal nor any precipitation was observed even after 12 days of incubation.

3.2 Atomic force Microscopy

Figure 2 shows AFM images of $\text{A}\beta_{1-40}$ in the absence and presence of tandem peptide at a 1:25 ratio. Figure 2A clearly shows large fibrils (~20 μm in length) in the absence of the tandem peptide after 4 days of incubation while no fibril formation was observed in the presence of the tandem peptide (Figure 2B).

3.3 Circular Dichroism spectra of the Tandem peptide

The CD spectrum of the tandem peptide showed a characteristic minimum at 216 nm suggesting a high β -sheet propensity (Figure 3). Interestingly, when the tandem peptide was kept for 4-5 days under these conditions at concentration ~10 μM no increase in ThioflavinT signal was observed.

4. Discussion

The experimental results clearly indicate a sub-stoichiometric inhibition of $\text{A}\beta_{1-40}$ fibrilization by the tandem peptide. The present study represents a different and unique approach to study the aggregation mechanism of $\text{A}\beta$. The results presented here not only suggest a mechanism for $\text{A}\beta_{1-40}$ aggregation but also a general procedure for inhibiting other aggregation processes. The substoichiometric inhibition suggests first that the tandem peptide is binding to a form, presumably an oligomer, which is on pathway to form fibrils, and which is present in only low concentration. From the CD data, the tandem peptide shows a high β -sheet propensity which might allow specific binding to an intermediate directly involved in fibrillization pathway. On the other hand, it is known that reverse sequence $\text{A}\beta_{40-1}$ has different properties than that of normal $\text{A}\beta_{1-40}$. For example, Giordano et. al., have suggested that both the nature of aggregates formed and the mechanism of toxicity differs between the peptides containing the forward and reverse sequences (21). The reverse sequence $\text{A}\beta_{40-1}$ forms globular aggregates in contrast to the forward sequence

which is fibrillar (21). This is indeed what we see in AFM when the tandem peptide aggregates at higher concentration ($\geq 25\mu\text{M}$) (supplementary figure S2).

Jan et al. (22) have shown that while $\text{A}\beta_{1-40}$ appears to retard the formation of mature $\text{A}\beta_{1-42}$ fibrils, the reverse sequence $\text{A}\beta_{40-1}$ does not affect fibrillization of protofibrillar $\text{A}\beta_{1-42}$ indicating the specificity of the interaction. Thus in the complex formed by tandem peptide and the on pathway oligomer(s) involved in fibrilization, the reverse sequence present in the tandem peptide may not allow monomer addition thereby preventing further aggregation.

5. Conclusion

These studies suggest a new approach for understanding the mechanism of fibrillization of intrinsically disordered proteins. Such studies are first of its kind and various permutation of such sequence attachment in $\text{A}\beta$ is presently being pursued in our lab to check their effects on fibrilization. We are also currently in process of implementing tandem constructs *in vivo* to check its effect on aggregation of $\text{A}\beta$ inside neuronal cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Aggregation of Aβ₁₋₄₀ in the absence and presence of tandem peptide. Aggregation was measured using ThioflavinT. For this assay, 20 μL of the Aβ solution was added to 980 μL of a 10 μM Thioflavin T solution in 50 mM glycine buffer at pH 9.0. The samples were thoroughly mixed and fluorescence spectra were recorded using an excitation wavelength of 450 nm and emission wavelength of 490 nm. The fibrilization conditions were 25 mM sodium borate buffer at pH 8.0 at 37 °C. Figure 1A represents ThioflavinT fluorescence signal as a function of time for 50 μM of Aβ₁₋₄₀ (red circles) and 50 μM Aβ₁₋₄₀ + 2 μM of Tandem peptide (black squares). Figure 1B represents ThioflavinT fluorescence signal as a function of time for 65 μM of Aβ₁₋₄₀ (red circles) and 65 μM Aβ₁₋₄₀ + 6.5 μM of tandem peptide (black squares).

Figure 2. AFM images of fibrils, (A) 75 μM A β_{1-40} in the absence and (B) 75 μM A β_{1-40} in the presence of 3 μM tandem peptide after 4 days of incubation. The scaling bar is 5 μ . The color bar on the right hand side of the image represents the vertical height from the surface.

Figure 3.

The CD spectra of the tandem peptide. The data were obtained in 200 mM phosphate buffer at pH 8.0 at room temperature using a cell of 0.1 cm path-length. The concentration of the tandem peptide used was 10 μ M.