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Specific Binding of a β-Cyclodextrin Dimer to the Amyloid β Peptide Modulates the Peptide Aggregation Process

Anna Wahlström,[†] Risto Cukalevski,[‡] Jens Danielsson,[†] Jüri Jarvet,[§] Hideki Onagi,[∥] Julius Rebek, Jr.,[⊥] Sara Linse,[‡] and Astrid Gräslund*[,†]

[†]Department of Biochemistry and Biophysics, Stockholm University, Svante Arrhenius väg 16, SE-106 91 Stockholm, Sweden

[‡]Chemistry Department and Molecular Protein Science, Lund University, P.O. Box 124, SE-221 00 Lund, Sweden

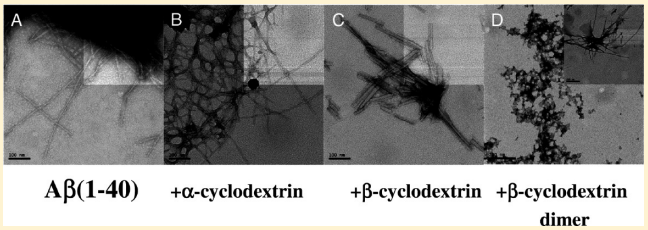
[§]The National Institute of Chemical Physics and Biophysics, 126 18 Tallinn, Estonia

[∥]Research School of Chemistry, The Australian National University, Canberra, ACT 0200, Australia

[⊥]Department of Chemistry, The Skaggs Institute for Chemical Biology, The Scripps Research Institute, La Jolla, California 92037, United States

Supporting Information

ABSTRACT: Alzheimer's disease involves progressive neuronal loss. Linked to the disease is the amyloid β (Aβ) peptide, a 38−43-amino acid peptide found in extracellular amyloid plaques in the brain. Cyclodextrins are nontoxic, cone-shaped oligosaccharides with a hydrophilic exterior and a hydrophobic cavity making them suitable hosts for aromatic guest molecules in water. β-Cyclodextrin consists of seven α-D-glucopyranoside units and has been shown to reduce the level of fibrillation and neurotoxicity of Aβ. We have studied the interaction between Aβ and a β-cyclodextrin dimer, consisting of two β-cyclodextrin monomers connected by a flexible linker. The β-cyclodextrin monomer has been found to interact with Aβ(1−40) at sites Y10, F19, and/or F20 with a dissociation constant (K_D) of 3.9 ± 2.0 mM. Here $^1H-^{15}N$ and $^1H-^{13}C$ heteronuclear single-quantum correlation nuclear magnetic resonance (NMR) spectra show that in addition, the β-cyclodextrin monomer and dimer bind to the histidines. NMR translational diffusion experiments reveal the increased affinity of the β-cyclodextrin dimer (apparent K_D of 1.1 ± 0.5 mM) for Aβ(1−40) compared to that of the β-cyclodextrin monomer. Kinetic aggregation experiments based on thioflavin T fluorescence indicate that the dimer at 0.05−5 mM decreases the lag time of Aβ aggregation, while a concentration of 10 mM increases the lag time. The β-cyclodextrin monomer at a high concentration decreases the lag time of the aggregation. We conclude that cyclodextrin monomers and dimers have specific, modulating effects on the Aβ(1−40) aggregation process. Transmission electron microscopy shows that the regular fibrillar aggregates formed by Aβ(1−40) alone are replaced by a major fraction of amorphous aggregates in the presence of the β-cyclodextrin dimer.



Alzheimer's disease is a form of dementia and leads to neurodegeneration and loss of cognitive function as the disease progresses. Because the average human life expectancy is increasing, the social and economical problems associated with the disease are becoming more widespread.[1,2] Despite intense research, there is still no cure, even though there are drugs that relieve the symptoms.[3] The detailed molecular mechanisms behind the disease are poorly understood, although there is strong evidence of the involvement of the amyloid β (Aβ) peptide.[4−6] The peptide consists of 38−43 amino acid residues and is a cleavage product from the transmembrane protein amyloid precursor protein (APP).[7] It forms long threadlike structures that assemble in extracellular amyloid plaques in the brain,[8] but numerous studies have shown that it is not the amyloid fibrils that are the main cause of the neurodegeneration, but rather soluble oligomeric structures of the peptide.[9]

There are different strategies being used in the search for a drug that will prevent Alzheimer's disease.[10] One way is to use small molecules that interfere with the aggregation process by disintegrating the formed oligomers or by remodeling the oligomers into nontoxic off-pathway aggregates.[11,12] Cyclodextrins belong to a well-studied group of molecules that are nontoxic and for many years have been used as additives in drugs, foods, and cosmetics.[13] They consist of a variable number of α-D-glucopyranoside units linked by α-1,4-glycosidic bonds that yield a conical cylinder (Figure 1A). The primary hydroxyl groups appear at the narrower end of the cylinder, while the secondary ones are at the wider opening. The three common forms of cyclodextrins are α-, β-, and γ-cyclodextrin, consisting of six, seven, and eight glucose units, respectively. In addition, the hydroxyl groups protruding from the molecules allow sites for modification, and there are more than 1500 known derivatives with varying characteristics.[13,14]

Received: March 13, 2012
Revised: May 2, 2012
Published: May 3, 2012

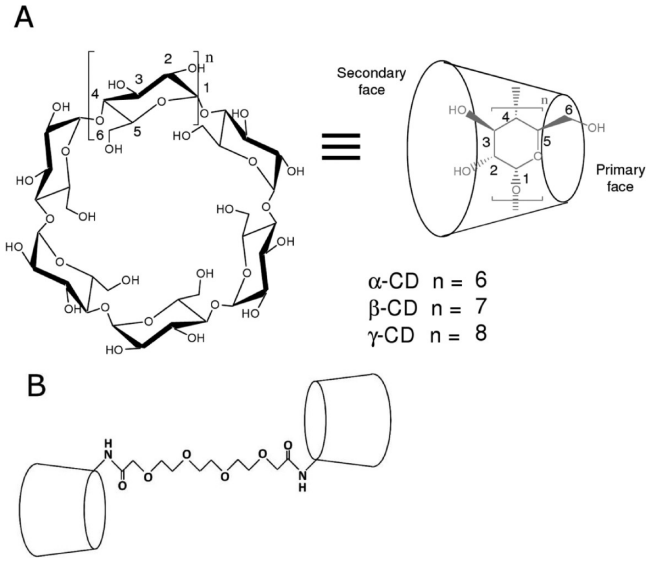


Figure 1. (A) Cyclodextrin molecule (left). α-, β-, and γ-cyclodextrin consist of six, seven, and eight α-D-glucopyranoside units, respectively. The units are linked by α-1,4-glycosidic bonds forming a cyclic molecule. At the right, the seven-unit cyclodextrin molecule is shown as a cone. (B) β-Cyclodextrin dimer. Two β-cyclodextrin monomers (cones) are connected by a flexible pentaethylene glycol-based linker.

The shape of the cyclodextrin molecule, combined with a hydrophobic interior and a hydrophilic exterior, allows host−guest interactions in water, where the cyclodextrin molecule acts like a host and surrounds typically a benzene group of the other molecule. It is also possible that the hydroxyl groups situated on the outside of the cyclodextrin interact with the other molecule, forming a noninclusion complex.[13]

The interaction among Aβ(1−40), various fragments thereof, and β-cyclodextrin has previously been characterized by NMR measurements.[15] The diffusion coefficient of Aβ(1−40) in the presence of an increasing concentration of β-cyclodextrin was carefully measured, and the resulting data were evaluated with one- and two-site binding models. Phenylalanine 19 and/or 20 and tyrosine 10 were found to interact with β-cyclodextrin with a dissociation constant of ∼4 mM.[15] The importance of the phenylalanines for binding was confirmed by using a variant of Aβ(12−28) in which the phenylalanines were replaced with glycines: in that case, no interaction with β-cyclodextrin was observed.[15]

In earlier studies, it was observed by mass spectrometry that β-cyclodextrin binds to Aβ(1−40) and that it has an antifibrillation effect based on Congo red binding experiments.[16] It has also been shown that β-cyclodextrin reduces the neurotoxic effect of Aβ(1−40) both in vitro and in vivo.[16,17] A circular dichroism (CD) and NMR study of Aβ(12−28) in the presence of α-, β-, and γ-cyclodextrin at pH 5.0 revealed that the peptide interacts with only β-cyclodextrin and that the interaction occurs via the aromatic groups of phenylalanines 19 and 20. The aromatic groups were proposed to be inserted into the β-cyclodextrin cavity and prevented Aβ(12−28) aggregation.[18] Besides this, β-cyclodextrin has also been reported to increase the level of aggregation of the peptide. In kinetic studies using thioflavin T (ThT) fluorescence, 10 mM β-cyclodextrin was found to accelerate induction of the ThT fluorescence of the Aβ aggregation in comparison to Aβ alone.[19] However, in a comparative study of many small molecules affecting Aβ oligomerization and fibrillation, the effects of β-cyclodextrin were found to be weak.[11]

β-Cyclodextrin in dimeric form has been observed to bind to polypeptide side chains[20] and to prevent association of the multimeric enzymes L-lactate dehydrogenase and citrate synthase.[21] β-Cyclodextrin dimers have also been seen to bind to peptides with hydrophobic amino acids and concomitantly induce α-helical structure.[22] In another study, β-cyclodextrin dimers and trimers were found by immunoassay and ThT methods to act as inhibitors of Aβ(1−40) aggregation.[23]

In this study, we investigated the effects of a β-cyclodextrin dimer, which consists of two β-cyclodextrin monomers connected by a flexible pentaethylene glycol-based linker (Figure 1B). The dimer was constructed as an attempt to improve the binding affinity for Aβ(1−40) in comparison to that of monomeric β-cyclodextrin and thereby improve its ability to disrupt the oligomer formation and/or aggregation process of the peptide. The interaction between Aβ(1−40) and the β-cyclodextrin dimer was investigated using NMR spectroscopy, and the aggregation kinetics was studied by a ThT fluorescence assay. We have also included an electron microscopy study of the aggregates formed by Aβ(1−40) in the presence of α- and β-cyclodextrin and the β-cyclodextrin dimer.

The aim of this study was to further explore the binding of a small molecule that may interact weakly with Aβ and despite the weak binding have a significant influence on the Aβ aggregation process. New information about the effects of such a weakly interacting binding partner may be important for further development of drugs against Alzheimer's disease.

EXPERIMENTAL PROCEDURES

Synthesis of the β-Cyclodextrin Dimer. 3,6,9,12-Tetraoxatetradecane-1,14-dioic acid and the corresponding disuccinimidyl ester were prepared according to the procedures reported by Wong and co-workers.[24] 6^A-Amino-6^A-deoxy-β-cyclodextrin was prepared according to the procedures described by Easton and co-workers.[25] Anhydrous DMF was purchased from Sigma-Aldrich. β-Cyclodextrin was supplied by Nihon Shokuhin Kako Co. and contained up to 10% water. It was dried under reduced pressure over phosphorus pentoxide, to a constant weight, before use. ^{1}H and ^{13}C NMR spectra were recorded on either a Varian Inova 300 or Varian Mercury 300 spectrometer. ^{1}H NMR chemical shifts are referenced to the external standard, 3-(trimethylsilyl)-3,3,2,2-tetradeuteropropionic acid sodium salt (TSPA-d_4). ^{13}C NMR chemical shifts are referenced to the solvent signal (DMSO-d_6, δ_C 39.5). Electrospray ionization (ESI) mass spectrometry was conducted with a Micromass VG Quattro II mass spectrometer. Thin-layer chromatography (TLC) was performed by using Kieselgel 60 F_{254} (Merck) on aluminum-backed plates, eluting with a 5:4:3:2 2-propanol/ethanol/water/acetic acid mixture, and visualized by wetting with a solution of 0.1% naphthalene-1,3-diol in a 200:157:43 (v/v/v) ethanol/water/H_2SO_4 mixture followed by heating. Cation-exchange chromatography was performed by using Toyopearl SP-650M (Tosoh). Anion-exchange chromatography was performed by using Toyopearl DEAE-650M (Tosoh). Low-pressure reverse-phase liquid chromatography was performed by using a Lobar prepacked column (310-25 LiChroprep RP-18).

A solution of the disuccinimidyl ester of 3,6,9,12-tetraoxatetradecane-1,14-dioic acid (0.3 mmol in 2.0 mL of CH_2Cl_2 and 1 mL of THF) was prepared according to the procedures reported by Wong and co-workers.[24] The solvent was removed

dx.doi.org/10.1021/bi300341j | Biochemistry 2012, 51, 4280−4289

by evaporation, and the residue was dissolved in anhydrous DMF (1 mL). The solution of the disuccinimidyl ester was then added to the cooled (0 °C) solution of the amino β-cyclodextrin (700 mg, 0.6 mmol) in anhydrous DMF (10 mL), followed by addition of Et_3N (85 μL, 0.6 mmol). The mixture was stirred for 10 min at 0 °C and for 12 h at room temperature (Figure S1 of the Supporting Information). The volume of the resulting solution was reduced by the rotary evaporator to approximately 3 mL. The concentrated solution was added dropwise to ice-cold acetone (75 mL). The precipitate that formed was collected by centrifugation and then resuspended in acetone. Repeating the resuspension−centrifugation process three times followed by removal of the solvent gave a crude product. The crude product was dissolved in water (10 mL) and passed through a cation-exchange column and then an anion-exchange column (3 cm × 5 cm for both). The obtained solution was concentrated to ca. 3 mL and then subjected to the low-pressure column (eluent, 25% methanol in water). Fractions containing the product were collected. Lyophilization of the product fractions gave the desired product (140 mg, 18.5%) as a white powder: TLC R_f = 0.45; ^{1}H NMR (300 MHz, D_2O) δ 5.16 (d, 1H, J = 3.3 Hz), 5.12−4.97 (m, 13H), 4.17−3.34 (m, 100H); ^{13}C NMR (75.43 MHz, DMSO-d_6) δ 169.3, 102.3, 102.0, 101.7, 84.0, 81.6, 73.1, 72.5, 72.0, 70.2, 69.8, 69.6, 59.9; ESIMS (+ve) calcd for $C_{94}H_{156}N_2O_{74}Na$ ($M + Na^+$) m/e 2519.8403, found 2519.8406.

NMR Measurements. The Aβ(1−40) sample was prepared as described previously[26] but in short included dissolution of the peptide (unlabeled, ^{15}N-labeled, or ^{13}C- and ^{15}N-labeled) (AlexoTech, Umeå, Sweden) in 10 mM NaOH, addition of cold destilled water followed by sodium phosphate buffer (pH 7.4), ending up at a peptide concentration of 50 μM and a buffer concentration of 10 mM. Between the additions, the sample was sonicated. The β-cyclodextrin dimer was dissolved in 10 mM sodium phosphate buffer to a stock solution of 12 mM, sonicated, and then added to the Aβ sample at concentrations ranging from 0.1 to 8 mM. In the diffusion experiments, titration of the dimer was performed only for the lower concentrations (up to 1 mM), and then a new sample with the peptide and dimer was prepared for every dimer concentration. This ensured that the peptide concentration was the same in every sample. For the ^{1}H−^{13}C HSQC measurements, titration was performed on the same Aβ sample because the purpose was to follow the chemical shift changes. For the diffusion and ^{1}H−^{13}C HSQC measurements, D_2O was used, and the samples for ^{1}H−^{15}N HSQC were based on a 90:10 H_2O/D_2O mixture.

The interaction between Aβ(1−40) and the β-cyclodextrin dimer was studied by translational diffusion, ^{1}H−^{15}N HSQC, and ^{1}H−^{13}C HSQC experiments at 25 °C on a 700 MHz Bruker NMR spectrometer equipped with a cryogenically cooled probe head. β-Cyclodextrin (Sigma-Aldrich), with a known diffusion coefficient,[27] dissolved in 10 mM sodium phosphate buffer in D_2O (pH 7.4) was used to calibrate the gradients. The diffusion studies were performed with a standard Bruker experiment based on the Stejskal−Tanner equation[28] with 16 gradient strengths, 256 scans, gradient pulses (δ) of 4 ms, and a diffusion delay (Δ) of 150 ms. The translational diffusion coefficients (D_t) were obtained by fitting the NMR signal attenuation of the methyl peaks from the diffusion experiments to the modified Stejskal−Tanner equation.[29] The error in the determined D_t was estimated by a Monte Carlo approach in which 10^4 fits were performed with signal intensity data randomly normal distributed, and the deviation in signal intensity was estimated from the signal-to-noise ratio. The dissociation constant (K_d) was obtained using a similar Monte Carlo approach and by fitting the calculated diffusion coefficients and the obtained deviations to a 1:1 stoichiometric binding model.[15] The viscosity change caused by an increased cyclodextrin concentration was corrected according to the method of ref 15. The ^{1}H−^{15}N HSQC spectra were recorded with 2048 × 256 complex points, a spectral width of 11.3 × 40.0 ppm, eight scans, and an acquisition time of 130 ms in the ^{1}H dimension. The ^{1}H−^{13}C HSQC spectra were recorded with 1750 × 512 complex points, a spectral width of 10.0 × 150 ppm, 16−64 scans, and an acquisition time of 125 ms in the ^{1}H dimension. The spectra were analyzed using NMRPipe and Sparky and referenced on the basis of the water signal.[30,31] The chemical shift changes between the ^{1}H−^{15}N HSQC spectra representing Aβ(1−40) in the absence and presence of the β-cyclodextrin dimer were calculated according to[32]

$$\Delta\delta_{avg}^{HN} = \{[(\Delta\delta^H)^2 + (\Delta\delta^N/5)^2]/2\}^{1/2} \quad (1)$$

Kinetic Aggregation Measurements. The influence of the α- or β-cyclodextrin monomer (Sigma-Aldrich) and the β-cyclodextrin dimer on Aβ(1−40) aggregation was studied by measuring ThT fluorescence in a 96-well plate reader (Fluostar Omega, BMG Labtech, Offenburg, Germany). The procedure of peptide expression, sample preparation, and kinetic aggregation is described in refs 33 and 34 and involves the following steps. Aβ(M1−40) was expressed in *Escherichia coli* with methionine before the first amino acid and purified from inclusion bodies using anion-exchange chromatography and centrifugal filtration. It was shown previously that the N-terminal methionine does not significantly perturb the aggregation process,[33] and the peptide used in this paper will be denoted as Aβ(1−40). This peptide with a methionine was used only in the experiments not involving NMR. The peptide was stored in 10 mM Tris-HCl buffer (pH 8.5) with 125 mM NaCl and 1 mM EDTA. It was kept at −20 °C and thawed before being used. To obtain the monomeric peptide and change it to 20 mM sodium phosphate buffer (pH 7.4) with 200 μM EDTA and 0.02% NaN_3, the thawed solution was applied to a Superdex 75 column for gel filtration. The concentration of the peptide was determined by measuring the absorbance at 280 nm with an ε of 1400 M^{-1} cm^{-1} for the single-tyrosine side chain absorbance. The sample was subsequently stored on ice.

Aβ(1−40) was used at concentrations of 3, 5, 8, and 15 μM; α- and β-cyclodextrin monomer concentrations were 0.1, 1, and 10 mM, and β-cyclodextrin dimer concentrations were 0.05, 0.5, 5, and 10 mM. The β-cyclodextrin dimer was added to Aβ(1−40), giving equivalent amounts of monomer units in the samples. ThT (13 μM) was added to the monomer solution after the gel filtration step, to the buffer, and to the cyclodextrin solutions. β-Cyclodextrin has been shown to weakly interact with ThT but does not, in contrast to γ-cyclodextrin, give rise to the typical increase in ThT fluorescence.[35] The samples with peptide and cyclodextrin were applied in triplicate or quadruplicate to a 96-well half-area plate of black polystyrene with a clear bottom and PEG coating (Corning 3881, Sigma-Aldrich) that was sealed with a plastic film (Corning 3095, Sigma-Aldrich). The plate reader recorded the ThT fluorescence (excitation at 440 nm and emission at 480 nm) at 37 °C every 6 min, and between the readings, the plate was shaken at 100 rpm. The resulting curves with a lag phase, a growth phase, and a final plateau, describing the

aggregation of Aβ(1−40) in the presence of the various cyclodextrins, were evaluated by applying the sigmoidal function:[34]

$$F(t) = F_0 + A/\{1 + \exp[-k(t - t_{1/2})]\} \quad (2)$$

where F_0 is the baseline before aggregation, A is the amplitude, k is the elongation rate constant, t is the time, and $t_{1/2}$ is the time at which the aggregation process is half-completed. The lag time, t_{lag}, is obtained from[34]

$$t_{lag} = t_{1/2} - 2/k \quad (3)$$

The Aβ(1−40) monomer concentration after incubation with the β-cyclodextrin monomer was investigated. One sample containing 10 μM Aβ(1−40) alone and one sample containing 10 μM Aβ(1−40) with 10 mM β-cyclodextrin monomer were incubated at 37 °C for 48 h. The supernatant, after centrifugation at 15000 rpm for 20 min, was injected into a Superdex 75 column, and the absorbance of the Aβ(1−40) monomer peak at 280 nm was monitored.

Electron Microscopy. Aβ aggregates were prepared for electron microscopy by dissolving monomeric Aβ (5 μM) into 10 mM Bis-Tris (pH 7.4) and 20 μM ThT in the absence and presence of 5 mM α-cyclodextrin, β-cyclodextrin, or β-cyclodextrin dimer. The aggregation process was monitored by ThT fluorescence as well as light scattering, and when the aggregation process was completed, carbon-coated 200 mesh copper grids were applied atop 20 μL droplets of the fibril preparation and blotted for 20 min. Negative staining was conducted with 1% (w/v) uranyl acetate. Images of 18500× magnification were recorded in a Technai G^2 Spirit BioTWIN microscope with a tungsten filament operating at 80 kV.

RESULTS

Sites of Interaction between Aβ(1−40) and the β-Cyclodextrin Dimer. ^{1}H−^{15}N HSQC spectra were recorded on 50 μM ^{15}N-labeled Aβ(1−40) in the absence and presence of 8 mM β-cyclodextrin dimer in 10 mM sodium phosphate buffer (pH 7.4) at 25 °C (Figure 2A). The assignment of Aβ(1−40) under these conditions was determined previously.[36] Not all cross-peaks of the peptide are seen in the HSQC spectrum, likely because of rapid chemical exchange of the amide protons with water molecules.[37]

For the ^{1}H−^{15}N HSQC cross-peaks, the largest chemical shift changes induced by the β-cyclodextrin dimer occur in the N-terminal part of the Aβ(1−40) peptide and in the central segment (residues 16−21) while the C-terminal part of the peptide is considerably less affected (Figure 2B). The amino acid residues that are influenced the most by dimer interaction are D7, Y10, L17, F19, and F20. This is in general agreement with earlier findings that Y10, F19, and F20 are binding sites for the β-cyclodextrin monomer.[15,18] Besides the cross-peaks belonging to Aβ(1−40), a few new peaks appeared in the ^{1}H−^{15}N HSQC spectrum (Figure 2A). These arise from the natural abundance of ^{15}N in the NH groups in the dimer linker.

The general intensity of the Aβ(1−40) cross-peaks did not significantly change upon addition of the β-cyclodextrin dimer, reflecting the fact that the peptide did not aggregate to any measurable extent during the HSQC experiment.

Induced chemical shift changes were further studied by ^{1}H−^{13}C HSQC measurements because not all amino acid residues are seen in the ^{1}H−^{15}N HSQC spectrum. Assignment of the cross-peaks in the aromatic region was performed by comparison with chemical shifts from M. Zagorski (personal communication) and previously published data,[38] but overlaps made it difficult to identify all cross-peaks unambiguously (Figure 3). The β-cyclodextrin dimer in steps of 0, 0.1, 0.2, 0.5, 0.9, 1.7, 4, and 6 mM was titrated into 50 μM ^{13}C- and ^{15}N-labeled Aβ(1−40), and the

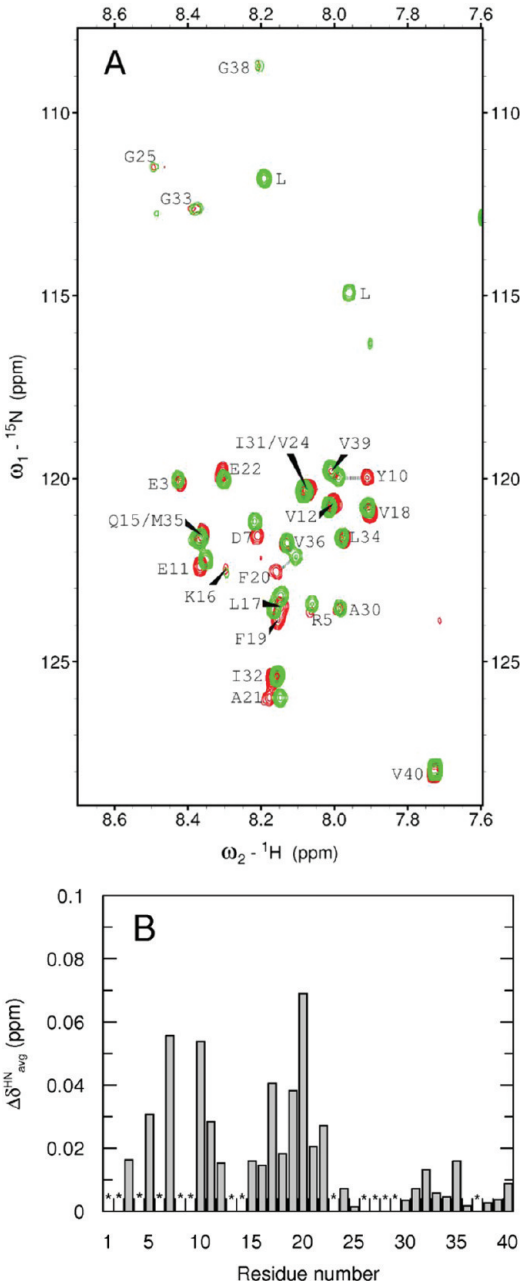


Figure 2. (A) ^{1}H−^{15}N HSQC spectra at 700 MHz of 50 μM [^{15}N]Aβ(1−40) in the absence (red) and presence (green) of 8 mM β-cyclodextrin dimer in 10 mM sodium phosphate buffer (pH 7.4) at 25 °C. The largest chemical shift changes are seen for D7, Y10, L17, F19, and F20. Suggestive chemical shift changes for Y10 and F20 are indicated by thin lines. Cross-peaks marked with an L belong to the linker in the dimer and arise because of the natural abundance of ^{15}N^{1}H groups. (B) Chemical shift changes, $\Delta\delta^{HN}_{avg} = \{[(\Delta\delta^H)^2 + (\Delta\delta^N/5)^2]/2\}^{1/2}$,[32] between [^{15}N]A$\beta$(1−40) in the absence and presence of 8 mM β-cyclodextrin dimer in 10 mM sodium phosphate buffer (pH 7.4) at 25 °C. Asterisks denote that the amino acid residue cross-peaks were absent in the spectra. Q15 and M35 overlap as well as V24 and I31 (panel A), and the same chemical shift changes are given. The N-terminus and the central part of the peptide are most influenced by the dimer as indicated by the largest induced chemical shift changes.

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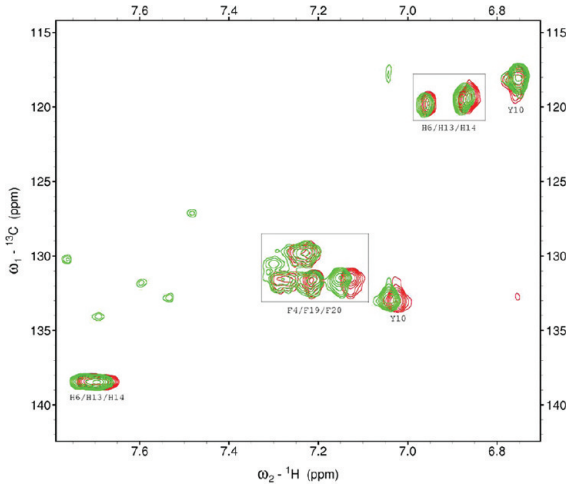

Figure 3. ^{1}H−^{13}C HSQC spectra at 700 MHz of 50 μM [^{13}C,^{15}N]Aβ(1−40) in the absence (red) and presence (green) of 6.6 mM β-cyclodextrin dimer in 10 mM sodium phosphate buffer (pH 7.4) at 25 °C. Weak and sharp new cross-peaks appearing in the ^{1}H−^{13}C HSQC spectra after addition of the β-cyclodextrin dimer may be due to natural abundance ^{1}H−^{13}C pairs from unassigned impurities.

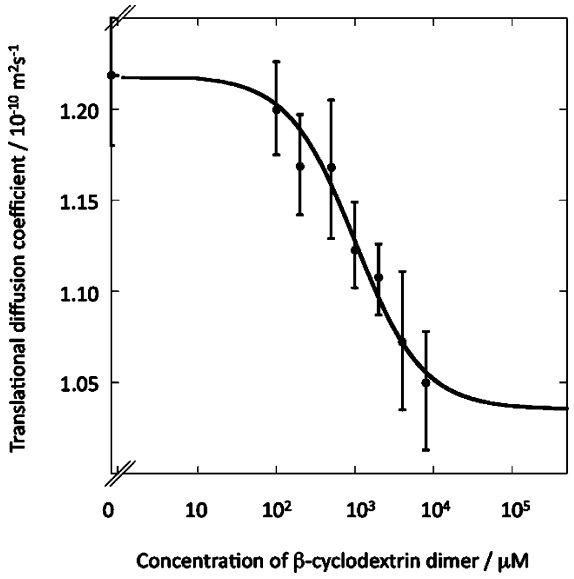

Figure 4. Translational diffusion measurements of 50 μM Aβ(1−40) in the presence of 0, 0.1, 0.2, 0.5, 1, 2, 4, and 8 mM β-cyclodextrin dimer in 10 mM sodium phosphate buffer (pH 7.4) at 25 °C. The diffusion coefficients are corrected for the viscosity increase caused by the dimer additions.[15] The dissociation constant between the peptide and the dimer is 1.1 ± 0.5 mM, found by fitting a one-site binding model to the data points.[15]

chemical shift changes that were induced were followed by recording ^{1}H−^{13}C HSQC spectra at each dimer concentration. Interestingly, all aromatic amino acid residues in the peptide, F4, H6, H13, H14, F19, and F20, exhibit significant chemical shift changes in the aromatic region (Figure 3). Induced chemical shift changes of the phenylalanines were also seen in the ^{1}H−^{15}N HSQC spectra, but the ^{1}H−^{13}C HSQC experiments gave complementary information about the histidines. Chemical shift changes for [^{13}C,^{15}N]Aβ(1−40) were also observed for the α- and β-protons (Figure S2A−C of the Supporting Information). The largest chemical shift changes are seen for amino acids D7, S8, and A21 and the aromatic residues. ^{1}H−^{13}C HSQC experiments were also performed with ^{13}C- and ^{15}N-labeled Aβ(1−40) in the presence of 6.6 mM β-cyclodextrin monomer. The results showed very similar chemical shift changes when compared with those of an Aβ(1−40) sample mixed with 3.4 mM β-cyclodextrin dimer (Figures S3 and S4A−C of the Supporting Information). The changes are seen foremost for the aromatic residues in Aβ.

The gradual shift of the aromatic cross-peaks upon titration of increasing concentrations of β-cyclodextrin dimer indicates that the Aβ(1−40) chemical exchange between its free and bound form is fast on the NMR time scale.[39]

Dissociation Constant of the β-Cyclodextrin Dimer Binding to Aβ(1−40). Translational diffusion measurements were used to quantify the interaction between Aβ(1−40) and the β-cyclodextrin dimer. Samples with 50 μM unlabeled Aβ(1−40) in the presence of increasing concentrations of dimer (0, 0.1, 0.2, 0.5, 1, 2, 4, and 8 mM) were prepared. The measured diffusion coefficients were corrected for the viscosity increase because of the dimer additions as described previously.[15] Without this correction, any added substance may be interpreted as a binding partner for Aβ because the diffusion coefficient is very sensitive to viscosity changes. The diffusion coefficient obtained for the peptide itself at 25 °C, 1.22×10^{-10} m^2/s (Figure 4), agrees with previously published data.[40] Addition of the dimer caused a gradual decrease in the diffusion coefficient of Aβ(1−40), and a value of 1.05×10^{-10} m^2/s was obtained in the presence of 8 mM dimer (Figure 4). Fitting of a one-site binding model[15] to the data revealed an apparent dissociation constant of 1.1 ± 0.5 mM. This suggests an increased affinity in comparison to that of the previously studied β-cyclodextrin monomer under the same conditions, 3.9 ± 2.0 mM.[15]

The diffusion coefficient of the pure β-cyclodextrin dimer was evaluated for several concentrations, and it varied less than 5% in a nonsystematic way (Figure S5 of the Supporting Information). This shows that although there are viscosity changes at high dimer concentrations, they are very small and in our concentration range negligible in the context of the kinetic aggregation experiments.

Kinetic Aggregation Studies. From the HSQC and diffusion experiments conducted by NMR, it is clear that Aβ(1−40) and the β-cyclodextrin dimer interact, but these measurements do not give any information about if and how the dimer influences the Aβ aggregation process. A common method for studying Aβ peptide aggregation is by ThT fluorescence. ThT is a positively charged aromatic compound that is considered to be a sensitive probe of the presence of amyloid fibrils and their soluble precursors. Its fluorescence is negligible in aqueous solution but increases significantly when it binds to amyloid structures.[41,42]

In this study, a well-developed procedure yielding reproducible aggregation kinetic data by ThT fluorescence[34] was applied to Aβ(1−40) in the absence and presence of α- and β-cyclodextrin monomers and the β-cyclodextrin dimer.

The lag time, elongation, and amplitude of the curve describing the Aβ(1−40) aggregation process in buffer are strongly dependent on peptide concentration, which has previously been shown for Aβ(M1−42).[34] The lag time for 15 μM Aβ(1−40) is 3.4 h, while it is considerably prolonged for 3 μM peptide, showing again this strong concentration dependence (Figure S6 and Table S1 of the Supporting Information). The amplitude behavior is more complex and difficult to evaluate.

The β-cyclodextrin monomer weakly affects the lag time of Aβ(1−40) (Figure 5B,C and Table S1 of the Supporting

dx.doi.org/10.1021/bi300341j | Biochemistry 2012, 51, 4280−4289

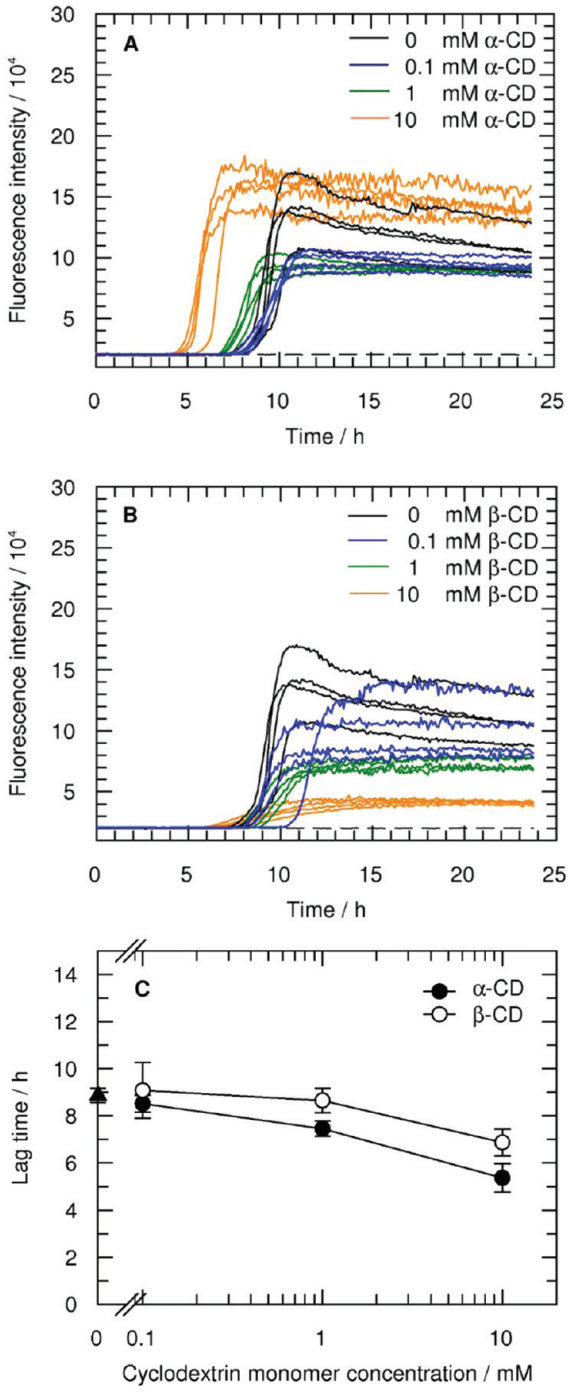

Figure 5. Kinetic aggregation experiments comparing effects of α- and β-cyclodextrin monomer. Aβ(1−40) (5 μM) in the presence of 0 (black), 0.1 (blue), 1 (green), and 10 mM (orange) α-cyclodextrin monomer (A) or β-cyclodextrin monomer (B) in 20 mM sodium phosphate buffer with 200 μM EDTA, 0.02% NaN_3, and 20 μM ThT (pH 7.4) at 37 °C and shaking (100 rpm). Dashed lines indicate background samples with 10 mM α-cyclodextrin (A) and 10 mM β-cyclodextrin (B) without the peptide under the same conditions that are described above. (C) Lag time as a function of α-cyclodextrin (●) and β-cyclodextrin (○) monomer concentration at 5 μM Aβ(1−40). The filled triangle shows the lag time for Aβ(1−40) in the absence of the cyclodextrin monomer. Each data point is an average of four replicates, and the standard deviation is represented by the error bars. The lines are guides for the eye.

Information), and the observed effect decreases with an increasing peptide concentration (Table S1 of the Supporting Information). However, as seen in Table S1 of the Supporting Information, which shows the statistical average of several data sets, the trend is that the β-cyclodextrin monomer at low concentrations (0.1 and 1 mM) seems to prolong the lag time of Aβ(1−40). When present at the higher concentration (10 mM), β-cyclodextrin shortens the lag time (Table S1 of the Supporting Information).

α-Cyclodextrin, like β-cyclodextrin in monomer form, has a limited effect on the lag time of the Aβ(1−40) aggregation process (Figure 5A,C and Table S1 of the Supporting Information). What actually differentiates between α- and β-cyclodextrin is their influence on the ThT fluorescence amplitude; while β-cyclodextrin reduces the amplitude in a concentration-dependent manner, α-cyclodextrin has no, or a slightly increasing, effect on the fluorescence intensity (Figure 5A,B). With an increase in the β-cyclodextrin monomer concentration, the ThT fluorescence amplitude is more strongly reduced. Neither α-cyclodextrin nor β-cyclodextrin monomer was found to give rise to any ThT fluorescence in the absence of peptide (Figure 5A,B).

The β-cyclodextrin dimer has a dual effect on the lag time. At concentrations of 0.05−5 mM, the dimer shortens the lag time for 3, 5, and 8 μM Aβ(1−40) (Figure 6 and Table S1 of the Supporting Information). Interestingly, a further increase in the dimer concentration to 10 mM reverses the effects on the aggregation process, and the lag time is instead prolonged for 5 and 8 μM Aβ(1−40) (Figure 6 and Table S1 of the Supporting Information). The ThT fluorescence amplitude reducing effect of the β-cyclodextrin monomer is less obvious for the dimer.

Monomer Concentration at Equilibrium. A possible explanation for the amplitude reducing effect of the β-cyclodextrin monomer could be that it keeps the peptide in monomeric form. This was investigated by measuring the Aβ(1−40) monomer concentration at equilibrium in samples incubated without and with 10 mM β-cyclodextrin monomer by liquid chromatography. The results showed no significant difference in peptide monomer peak area in the two samples (Figure S7 of the Supporting Information). This indicates that the modulating effect of the β-cyclodextrin monomer on the Aβ aggregation process does not cause the peptide to retain its monomeric form.

Electron Microscopy. Figure 7 shows TEM pictures of Aβ(1−40) aggregated either alone in buffer at pH 7.4 and 37 °C or in the presence of 5 mM α- or β-cyclodextrin or the β-cyclodextrin dimer. The results show the fibrillar structures of Aβ alone (Figure 7A). Similar structures are also seen in the presence of α-cyclodextrin (Figure 7B). Significant changes in the fibrillar morphology are seen in the aggregates formed in the presence of β-cyclodextrin (Figure 7C). Figure 7D shows that the presence of the β-cyclodextrin dimer gives rise to a profound change: a major fraction is present as amorphous aggregates, and only a minor fraction (<10%) is present as long fibrils.

■ DISCUSSION

In this work, we have used biophysical methods to characterize the interaction between Aβ(1−40) and a β-cyclodextrin dimer. NMR experiments describe the interaction on an atomic level and also reveal an estimated binding constant. Kinetic aggregation experiments visualize how the peptide aggregation is influenced in terms of ThT-positive assemblies.

Earlier studies reported that β-cyclodextrin, in contrast to α- and γ-cyclodextrin, binds to Aβ(12−28) and Aβ(1−40) and prevents aggregation.[15,16,18] The interacting sites on the peptide

dx.doi.org/10.1021/bi300341j | Biochemistry 2012, 51, 4280−4289

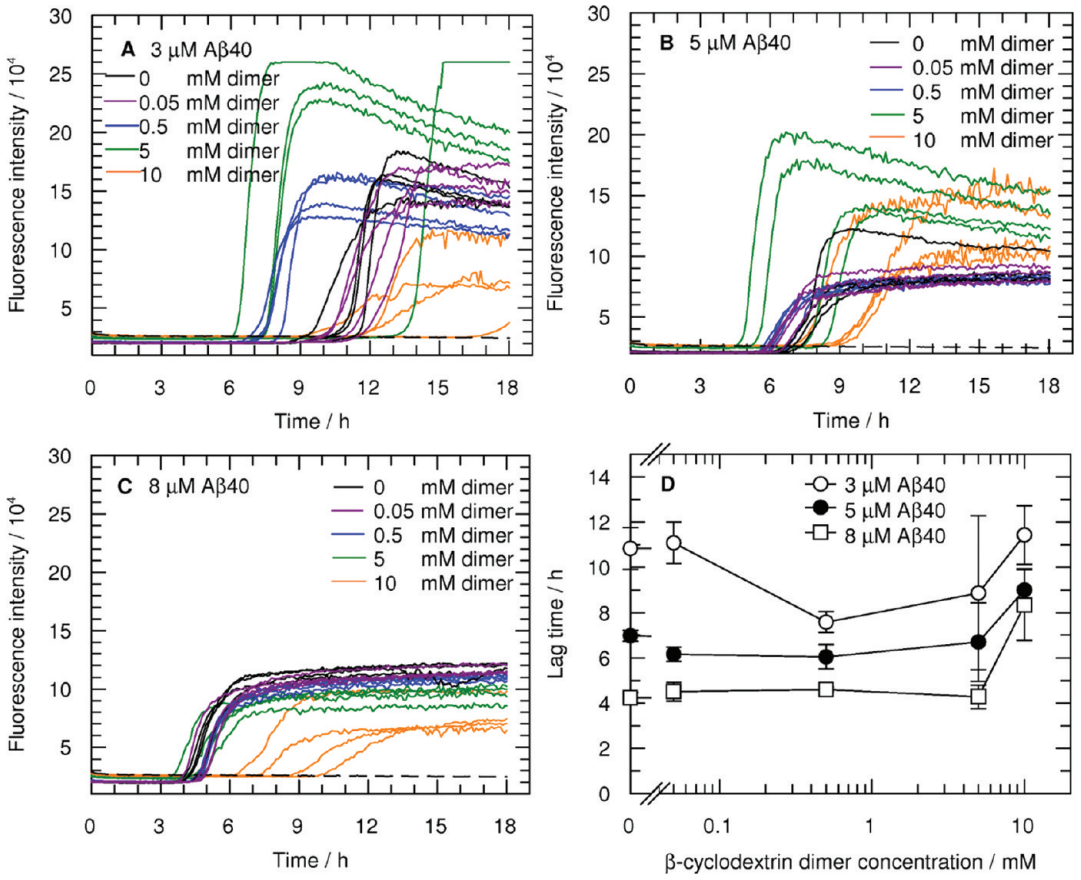

Figure 6. Kinetic aggregation experiments visualizing the effect of an increasing β-cyclodextrin dimer concentration. Aβ(1−40) at 3 (A), 5 (B), and 8 μM (C) in the presence of 0 (black), 0.05 (magenta), 0.5 (blue), 5 (green), and 10 mM β-cyclodextrin dimer (orange) in 20 mM sodium phosphate buffer with 200 μM EDTA, 0.02% NaN_3, and 20 μM ThT (pH 7.4) at 37 °C and shaking (100 rpm). Dashed lines indicate background samples with 10 mM dimer without peptide under the same conditions. (D) Lag time as a function of β-cyclodextrin dimer concentration at 3 (○), 5 (●), and 8 μM (□) Aβ(1−40). Each data point is an average of four replicates, and the standard deviation is represented by the error bars. The lines are guides for the eye.

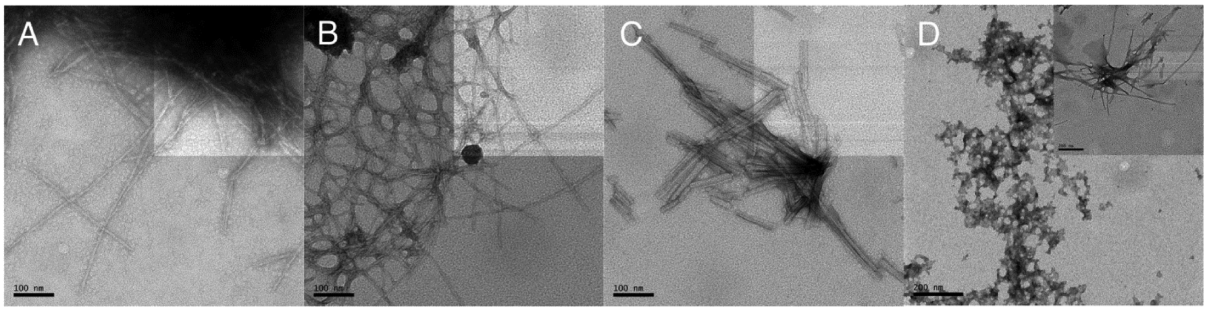

Figure 7. Electron micrographs of aggregated Aβ with and without cyclodextrin derivatives. Aβ(1−40) was agitated into ThT-positive aggregates under physiological conditions: 10 mM BisTris buffer (pH 7.4) at 37 °C. (A) Aβ alone forms long filamentous fibers typical for amyloid. (B) Aβ aggregated in the presence of α-cyclodextrin shows fibers indistinguishable from those formed by Aβ alone. (C) Aβ aggregated in a β-cyclodextrin solution forms ThT-positive fibers that are significantly shorter and wider than ordinary Aβ fibers, and the short fibers tend to pack into bundles. (D) Aβ aggregated in a solution with 5 mM β-cyclodextrin dimer mainly forms aggregates that seem to be amorphous. However, a small amount of long, typical Aβ fibers is found mixed with the nonfibrillar aggregates as shown in the inset of panel D.

were determined to involve aromatic amino acids Y10, F19, and/or F20, suggesting that the aromatic side chains interact with the cavity of β-cyclodextrin, which has a suitable geometry.[15,18] In accord with these results, the β-cyclodextrin dimer, consisting of two monomers linked by a flexible pentaethylene glycol-based linker, was also found to interact with Aβ(1−40). Chemical shift differences in the ^{1}H−^{15}N HSQC and ^{1}H−^{13}C HSQC spectra upon titration of the dimer with Aβ(1−40) were in good agreement with the previous one-dimensional NMR spectra showing the interaction between Aβ(1−40) and the β-cyclodextrin monomer,[15] but they also gave some additional information. In addition to Y10 and the phenylalanines, histidines H6, H13, and H14 were also seen to be affected by the presence of the dimer. In fact, using the same ^{1}H−^{13}C HSQC experiment for the interaction of Aβ with the monomeric β-cyclodextrin gave very similar results (Figure S3 of the Supporting Information). It is interesting that all

dx.doi.org/10.1021/bi300341j | Biochemistry 2012, 51, 4280−4289

the aromatic amino acid residues in the peptide display chemical shift changes. This is in agreement with the previously suggested hypothesis that the aromatic rings are inserted into the hydrophobic cyclodextrin interior.[16,18] In addition, because cyclodextrins have been observed to form water-soluble aggregates with surfactant-like effects,[43] Aβ(1−40) may also interact with these higher cyclodextrin assemblies. This less specific interaction could explain the chemical shift changes that are seen, not only for the amino acids mentioned above but also for the hydrophilic N-terminal part of the peptide in general (Figure 2A,B).

One rationale for constructing dimers of β-cyclodextrin monomers for Aβ interaction studies was to improve the binding affinity for the peptide, but we observed that connecting two monomers strengthened the binding to Aβ(1−40) only slightly: the dissociation constant decreased from 3.9 ± 2.0 mM for binding of the β-cyclodextrin monomer to Aβ(1−40) to 1.1 ± 0.5 mM for binding of the dimer to Aβ(1−40). The induced chemical shifts seen in the aromatic residues as well as in the histidines suggest a pseudospecific binding in which several sites on Aβ may serve as primary binding sites. Moreover, the dynamic range of the measured diffusion coefficients suggests that more than one cyclodextrin dimer binds to Aβ. The diffusion coefficient of Aβ bound to two cyclodextrin monomers was previously determined to be 1.17×10^{-10} m^2/s,[15] while the diffusion coefficient of Aβ with the bound cyclodextrin dimer is 1.05×10^{-10} m^2/s. This indicates more than one dimer per peptide. However, from the diffusion data, it is possible to estimate only the overall affinity in a simplified bound/free two-state model, which gives an indication of the general affinity of the dimer for Aβ. The mechanism of the stronger binding may be that after one of the monomers in the dimer has bound to a binding site in the peptide, the other monomer, restricted by the linker, probes the various remaining binding sites, resulting in a "constrained affinity".[44]

Despite a relatively low affinity, the dimer may still influence the formation of oligomers, larger aggregates, and the final state of the aggregation pathway. This was investigated by performing kinetic aggregation experiments with ThT. In contrast to the NMR measurements, which are performed while the peptide still mainly is in a monomeric form, the kinetic ThT experiments monitor the complete aggregation process.

Our study shows that both the α- and β-cyclodextrin monomer have only small effects on the lag time of Aβ(1−40) amyloid formation and that the effects depend on cyclodextrin concentration. This is in accord with earlier NMR measurements of Aβ(1−40) and β-cyclodextrin that showed weak binding and small induced chemical shift changes in the peptide.[15] However, the peptide concentration must also be considered when evaluating the cyclodextrin effect. With an increase in the peptide concentration, the aggregation is accelerated and the reproducibility of the kinetic traces is improved. Along with this effect, it seems that the aggregation pathway is harder to disturb, and accordingly, the effect of the cyclodextrins on the lag time is diminished (Table S1 of the Supporting Information).

In contrast to the weak influence on the lag time of Aβ(1−40) aggregation, the β-cyclodextrin monomer noticeably reduces the ThT fluorescence amplitude (Figure 5B). There are various possible mechanisms underlying this observed effect. One is that the β-cyclodextrin monomer reduces the number of ThT active aggregates of Aβ by keeping the peptide in monomeric form. This is, however, unlikely because the concentration of the monomeric peptide for samples incubated at 37 °C for 48 h was not affected by the presence of the β-cyclodextrin monomer (Figure S7 of the Supporting Information). Alternative explanations could be that a high concentration of cyclodextrin quenches the emission from the ThT bound to the fibrils or that the β-cyclodextrin monomer directs the peptide into formation of assemblies, or loosely associated aggregates, that do not bind ThT. This latter possibility is supported by earlier observations of the β-cyclodextrin monomer by AFM showing changed oligomeric and fibrillar structures when β-cyclodextrin was incubated with aggregating Aβ peptide samples.[19] Furthermore, the TEM picture (Figure 7C) indicating a changed morphology of the Aβ aggregates in the presence of the β-cyclodextrin provides another explanation for the altered ThT fluorescence intensity.

The β-cyclodextrin dimer has a more distinct influence on the lag time of Aβ(1−40) than the β-cyclodextrin monomer. The lower concentrations of dimer (0.05, 0.5, and 5 mM) considerably shorten the lag time of the Aβ(1−40) aggregation, while 10 mM dimer reverses the process and prolongs the lag time (Table S1 of the Supporting Information). That is, low dimer concentrations accelerate formation of the Aβ amyloid, while high concentrations slow the process. Instead of binding to an Aβ peptide and blocking its interaction with other peptides, the β-cyclodextrin dimer at low concentrations seems to act like a cross-linker, accelerating the association of peptides and the formation of ThT active aggregates. On the other hand, the high dimer concentration (10 mM) might prevent the number of events when peptides contact each other and/or may induce formation of amorphous ThT inactive aggregates that over time gradually transform into ThT fluorescence-inducing structures. This is supported by the large amounts of amorphous aggregates found in the TEM picture when aggregates are formed in the presence of the β-cyclodextrin dimer (Figure 7D). The turnover point at which the effect changes from acceleration to retardation thus occurs somewhere between 5 and 10 mM. With a dissociation constant of 1.1 mM, this corresponds to a bound fraction of Aβ(1−40) somewhere between 80 and 90%.

The weak binding of cyclodextrin to aromatic side chains of proteins has made the cyclodextrins useful as protein folding agents, or antiaggregators, in several systems because they block aggregation prone sites in folding intermediates but still allow varying intramolecular conformations and step by step let loose the protein upon folding.[45,46] In one structural study of β-cyclodextrin and insulin,[47] it was suggested that a probable mode of interaction is one in which the cavity of β-cyclodextrin interacts with a solvent-exposed aromatic side chain of the protein, in agreement with the interpretations made in this study, and in earlier studies on Aβ and monomers of cyclodextrin.[16−18] In another study, the refolding and aggregation of aminoacylase with the hydroxypropyl cyclodextrins, HP-α-, β-, and γ-cyclodextrin, was investigated.[48] It was observed that all HP-cyclodextrins affected the refolding in a concentration-dependent manner. Both the protein and the cyclodextrin concentration were important for the process, and it turned out that the cyclodextrin effects could both promote and inhibit aggregation depending on the ratio of cyclodextrin to protein. In general, HP-β-CD was the most effective in preventing aggregation of aminoacylase.[48] Our results are in accordance with this study, pointing out the importance of studying broad intervals of concentrations, for both protein and cyclodextrin, to reveal and clarify any effects on protein aggregation.

Obviously, the construction of a dimer changes the behavior of the β-cyclodextrin monomer, and the kinetic aggregation experiments suggest that they affect the Aβ aggregation by partially different mechanisms. These results are further emphasized by the results of the TEM study, which show the end points of the aggregation processes (Figure 7). As expected, Aβ alone forms straight nonbranched fibrils, similar to previous reports (ref 49 and references cited therein). The β-cyclodextrin monomer causes changes in the morphology of the fibrous aggregates, whereas the β-cyclodextrin dimer causes formation of a major fraction of very different amorphous peptide aggregates.

■ CONCLUSIONS

These results show that cyclodextrin interactions in general modulate the process of Aβ aggregation. The β-cyclodextrin monomer only weakly affects the lag time but instead reduces the ThT fluorescence amplitude. Via covalent dimerization of the β-cyclodextrin molecule, the affinity for Aβ(1−40) is increased by a factor of ∼3 relative to that of monomeric β-cyclodextrin. The aggregation pathway is modulated as evidenced by ThT binding kinetics for the aggregating peptide and the TEM analysis. A general pattern seems to be that lower cyclodextrin dimer concentrations decrease the aggregation lag time while a high concentration increases the lag time. Our results show that the weakly interacting β-cyclodextrin dimer has a significant influence on the structural outcome of the Aβ aggregation process, which is only partly reflected in its effects on the amyloid formation kinetics. The influence of the small cyclodextrin variants can obviously direct the peptide aggregation process toward different amyloid morphologies, with potential intermediates that may be very different both in their structures and in their neurotoxic activities.

■ ASSOCIATED CONTENT

Supporting Information

Schematic presentation of the synthetic route of the β-cyclodextrin dimer, $^{1}H-^{13}C$ HSQC spectra showing the α- and β-proton region of ^{13}C- and ^{15}N-labeled Aβ(1−40) in the absence and presence of 6 mM β-cyclodextrin dimer, $^{1}H-^{13}C$ HSQC spectra showing the aromatic region of 6.6 mM β-cyclodextrin monomer and 3.4 mM β-cyclodextrin dimer, $^{1}H-^{13}C$ HSQC spectra showing the α- and β-proton region of ^{13}C- and ^{15}N-labeled Aβ(1−40) in the absence and presence of 6.6 mM β-cyclodextrin monomer, translational diffusion coefficient of the β-cyclodextrin dimer at increasing concentrations, Aβ(1−40) kinetic aggregation traces at increasing peptide concentrations, an FPLC chromatogram of Aβ(1−40) incubated without and with β-cyclodextrin monomer, and a table with lag times from the kinetic aggregation experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +46 8 162450. Fax: +46 8 155597. E-mail: astrid@dbb.su.se.

Funding

This study was supported by grants from the Swedish Research Council, the Swedish Brain Foundation, the Knut and Alice Wallenberg Foundation, the Skaggs Institute, and the Estonian Ministry of Education and Research (Targeted Financing Theme SF SF0690034s09).

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Ms. Lisa Lang for valuable help with the TEM experiments and Mr. Torbjörn Astlind for valuable help with the NMR equipment.

■ ABBREVIATIONS

Aβ, amyloid β; NMR, nuclear magnetic resonance; HSQC, heteronuclear single-quantum correlation; ThT, thioflavin T; TEM, transmission electron microscopy.

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