Published in final edited form as:

J Pept Sci. 2016 May ; 22(5): 368–373. doi:10.1002/psc.2879.

Peptide backbone modification in the bend region of amyloid- β inhibits fibrillogenesis but not oligomer formation

Erik C. B. Johnson^{a,c,*}, Jennifer D. Lanning^b, and Stephen C. Meredith^{a,b}

^aDepartment of Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, 60637, USA

^bDepartment of Pathology, The University of Chicago, Chicago, IL, 60637, USA

^cInstitute for Biophysical Dynamics, The University of Chicago, Chicago, IL, 60637, USA

Abstract

Current evidence suggests that oligomers of the amyloid- β (A β) peptide are involved in the cellular toxicity of Alzheimer's disease, yet their biophysical characterization remains difficult because of lack of experimental control over the aggregation process under relevant physiologic conditions. Here, we show that modification of the A β peptide backbone at Gly29 allows for the formation of oligomers but inhibits fibril formation at physiologic temperature and pH. Our results suggest that the putative bend region in A β is important for higher-order aggregate formation.

Keywords

Alzheimer's disease; amyloid- β ; oligomers; amyloid fibrils; 2-nitrobenzyl; peptide backbone

Introduction

Alzheimer's disease is the most prevalent neurodegenerative disease and is quickly becoming a public health crisis as the population of the world ages. Unfortunately, there are currently no therapies that affect the underlying disease process. A large body of evidence points to the amyloid- β (A β) peptide initiating the disease process that eventually leads to Alzheimer's dementia, with A β 1–42 identified as a particularly toxic A β species [1]. A β quickly aggregates into a variety of higher-order assemblies, including various types of oligomers and fibrils. One of the goals of research in this field is to understand and control A β aggregation in order to study the relative importance of each type of aggregate in the Alzheimer's disease process

The amino acid sequence of A β 1–42 is shown in Scheme 1A. Based largely on solution and solid-state NMR data, it is thought that during the aggregation process A β adopts a structure consisting of two β -strands connected by a bend region [2–5]. The details of the structural changes and intermediates are unknown, but this strand-bend-strand unit is ultimately able to

*Correspondence to: Erik C. B. Johnson, Gladstone Institute of Neurological Disease, 1650 Owens Street, San Francisco, CA 94158, USA. erik.johnson@gladstone.ucsf.edu.

assemble into the large polymeric amyloid fibrils found in amyloid plaques. Previous studies have suggested that the bend region plays an important role in the formation of higher-order A β aggregates [5–7]. We reasoned that modification of the peptide backbone in this region of the peptide would disrupt bend formation and, therefore, might disrupt amyloid fibril formation while allowing for the formation of amyloid oligomers that are either on-pathway or off-pathway to fibril formation.

Results and discussion

We chemically synthesized A β 1-42 to include a 2-nitrobenzyl (NO₂Bn) group attached to the amide nitrogen of glycine residues at position 29, 33, or 38 (Scheme 1). Gly29 is thought to be in or near the bend region, while Gly33 and Gly38 are proposed to be part of the C-terminal β -strand. We chose the NO₂Bn group because derivatives of this group attached to the peptide backbone have been shown to be labile to ultraviolet (UV) light, potentially offering a facile method to remove the chemical modification while leaving the native peptide backbone intact [8].

Peptides were synthesized stepwise on the solid phase, with the (*N*-2-nitrobenzyl)glycines introduced using the submonomer method [9]. The native A β 1-42 sequence eluted as a broad peak on reverse phase high performance liquid chromatography (HPLC), although mass spectrometry analysis showed one peptide species across the whole peak (Figure 1A). This suggested either conformational heterogeneity of A β 1-42 or tight-binding to the reversed-phase with consequent poor elution. While modification with NO₂Bn increased the apparent hydrophobicity of A β 1-42 and its retention time on HPLC, Gly^{33, 38} modification considerably sharpened the peaks (Figure 1C and D), suggesting that NO₂Bn modification disrupted conformation(s) adopted by the peptide that lead to modified interactions with the reversed-phase.

The purified peptides were then assayed for their abilities to form fibrillar structure using the thioflavin T (ThT) binding assay. Peptides were dissolved in neat dimethyl sulfoxide (DMSO) prior to dilution into phosphate buffer at pH 7.4 and 37 °C, a method that we have previously shown to be effective in reducing the number of potential fibril seeds that form during the synthesis and purification processes [6]. Fibril formation at a peptide concentration of 100 μ M was monitored by ThT fluorescence at regular intervals. Native A β 1-42 quickly formed fibrils, with maximum ThT fluorescence obtained within 16 h (Figure 2). A β 1-42 NO₂Bn^{33, 38} showed very similar kinetics of fibril formation to the native sequence. However, A β 1-42 peptides with the NO₂Bn modification at Gly29 (red and cyan traces, Figure 2) showed only minor ThT fluorescence, with a maximum reached by 6 h.

Further analysis by transmission electron microscopy (TEM) of these samples at 24 h showed that while A β 1-42 formed long fibrils (Figure 3A and B), peptide backbone modification at Gly29 with NO₂Bn completely inhibited fibril formation while allowing for the formation of numerous smaller oligomeric aggregates (Figure 3C and D. A β monomer cannot be visualized using this analytical approach). When the NO₂Bn group was added to Gly33 and Gly38 in addition to Gly29, aggregation of the oligomers was observed, possibly

through increased peptide hydrophobicity (Figure 3G and H). Although fibril formation appeared very similar between the native A β 1-42 sequence and the NO₂Bn-modified sequence at Gly33 and Gly38 by ThT fluorescence, TEM showed that the fibrils formed by the backbone modifications at Gly33 and Gly38 were shorter and narrower (Figure 3E and F). This finding suggests that although NO₂Bn backbone modification in this region of the peptide does not inhibit the formation of structure recognized by ThT, it does affect the ability of A β 1-42 to form the long-range ordered fibril structures characteristic of the native sequence. Although distinct in certain aspects of morphology, these shorter fibrils bear some resemblance to ‘protofibrils [10–14].’ However, they are unlikely to be precursors to longer fibrils given the structural constraints introduced by the two backbone NO₂Bn modifications. Analysis of these incubations beyond 24 h by TEM and HPLC showed stable aggregates without loss of the NO₂Bn group (data not shown). Of note, these oligomers were formed at neutral pH and room temperature, without special procedures required for their generation.

Our results suggest that the bend region in A β 1-42 is important for the formation of higher-order fibrillar assemblies and that modification of the peptide backbone in this region with the 2-nitrobenzyl group is sufficient to inhibit fibril formation. These results are in agreement with those from a prior study in which *N*-methyl (Nme) peptide backbone modification at position Gly29 was shown to inhibit fibril formation by A β 25-35 [15]. The precise effects of this modification on the structure of the bend region of aggregates are not yet clear. One possible effect is that introduction of NO₂Bn at Gly29 may remove a hydrogen bond that is critical for the stabilization of a bend structure conducive to fibril formation. Another possible effect is the addition of steric bulk by the NO₂Bn group that may preclude proper folding of the bend region and/or approximation of the N-terminal and C-terminal β -sheets that are likely required for fibril formation. Lastly, NO₂Bn would likely affect *cis/trans* isomerization of the modified glycine residue. *N*-alkyl modification has been shown to favor the *cis* conformation of the modified glycine [16], while *N*-aryl modification has been shown to favor the *trans* conformation [17]. Such alterations in stereochemistry at this site may have profound effects on the bend region and preclude structural rearrangements necessary for fibril formation while allowing for oligomerization through hydrophobic collapse of the hydrophobic amino acid sequences present in A β [14,18–21]. These potential effects of NO₂Bn modification are not mutually exclusive.

In contrast to Nme backbone modification at Gly33 in A β 25-35 [15], we did not find a strong inhibitory effect of NO₂Bn³³ on A β 1-42 fibril formation as assessed by ThT fluorescence and TEM. This could be due to the different chemical groups used for backbone modification or to the different length of peptides examined. In our previous work on Nme backbone modification in A β 1-40 [22], we found that Nme groups placed at positions 37 and 39 in the C-terminal β -strand were insufficient to inhibit fibril formation, similar to our findings reported here with NO₂Bn backbone modification at positions 33 and 38 in A β 1-42. The morphology of fibrils formed from each peptide is, however, notably different: long and twisted fibrils form from A β 1-40Nme^{37, 39}, whereas short straight fibrils form from A β 1-42NO₂Bn^{33, 38}. A β peptide length, the position of the backbone modifications, and the chemical nature of the modifications may all influence the types of fibrils that can be formed from these A β congeners.

Interestingly, a number of mutations in the amino acid sequence of A β 1-42 that lead to early-onset Alzheimer's disease appear to destabilize the bend region, whereas modifications in A β 1-42 that favor bend formation can lead to rapid amyloid formation [6,23]. We plan to characterize the biological activity of oligomers formed by A β 1-42NO₂Bn.²⁹ Because the 2-nitrobenzyl group used to modify the peptide backbone in this study is labile to UV light, this will provide experimental control over the A β aggregation process and could facilitate the structural and functional characterization of oligomers.

Experimental methods

Materials

2-(1H-Benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *p*-methylbenzhydrylamine resin, and Boc-amino acids were obtained from Peptides International, Kentucky. *O*-(7-Azabenzotriazol-1-yl)-*N,N,N',N'*-tetramethyluronium hexafluorophosphate (HATU) was obtained from GL Biochem, Shanghai. Boc-Val-OCH₂-Pam-resin was purchased from Rapp Polymere, Germany. Boc-Ala-OCH₂-Pam-resin and *N,N*-diisopropylethylamine were obtained from Applied Biosystems, Foster City. *N,N*-Dimethylformamide (DMF), dichloromethane, diethyl ether, and HPLC-grade acetonitrile were purchased from Fisher (Irvington, New York, USA). Trifluoroacetic acid (TFA) was obtained from Halocarbon Products, New Jersey. HF was purchased from Matheson. DMSO, *N,N*-diisopropylcarbodiimide (DIC), ThT, *p*-cresol, bromoacetic acid, and 2-nitrobenzylamine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Peptide Synthesis

All peptides were synthesized manually. Peptides were synthesized stepwise on the solid phase according to an optimized Boc *in situ* neutralization procedure [24] on a Boc-Ala-OCH₂-Pam resin. Side-chain protection for amino acids was as follows: Arg(Tos), Asn(Xan), Asp(cHex), Glu(cHex), His(Bom), Lys(2-Cl-Z), Ser(Bzl), and Tyr(Br-Z). The submonomer method of Zuckermann *et al.* [9] was used to prepare the 2-nitrobenzyl peptides with slight modification. Briefly, bromoacetic acid was activated with *N,N*-diisopropylcarbodiimide in dichloromethane (DCM) for 10 min, after which the DCM was removed under a stream of nitrogen and the activated acid redissolved in DMF and coupled in fivefold molar excess for 15 min. After washing the resin with DMF, 2-nitrobenzylamine (tenfold molar excess) was dissolved in *N,N*-diisopropylethylamine (1.1-fold molar excess), and a minimal amount of DMF, and then added to the resin and allowed to react for 9 h. The next amino acid was activated with HATU according to standard procedure and reacted for 12 h at fivefold molar excess. After chain assembly was complete, peptides were deprotected and simultaneously cleaved from the resin by treatment with liquid anhydrous hydrogen fluoride (HF) containing *p*-cresol (90 : 10, v/v) for 1 h at 0 °C. After evaporation of the HF under reduced pressure, the crude products were precipitated and washed with chilled diethyl ether, and the peptide products were dissolved in 1 : 1 CH₃CN:H₂O + 0.1% TFA. Final peptide composition was determined by liquid chromatography–mass spectrometry.

Peptide Purification

Preparative HPLC was performed with a Vydac C-4 (22 × 250 mm) silica column at a flow rate of 10 ml/min⁻¹. Peptides were eluted at 60 °C using a gradient of 20–25% solvent B over 3 min, then 25–45% solvent B over 40 min *versus* solvent A where A = H₂O + 0.1% TFA and B = CH₃CN + 0.08% TFA. UV detection was at 214 nm. Fractions containing the desired purified peptide product were identified by analytical liquid chromatography–mass spectrometry and then combined and lyophilized.

Liquid Chromatography–Mass Spectrometry Analysis

Analytical reverse-phase HPLC was performed on an Agilent 1100 system with a Vydac C-4 (5 µm 2.1 × 50 mm) silica column at a flow rate of 0.5 ml/min. Peptides were eluted using a 5–65% gradient of solvent B *versus* solvent A where A = H₂O + 0.1% TFA and B = CH₃CN + 0.08% TFA. UV detection was at 214 nm. Peptide masses were obtained using on-line electrospray MS detection with an Agilent 1100 LC/MSD ion trap.

ThT Fibril Assays

Aβ peptides were disaggregated by neat DMSO as described by Sciarretta *et al.* [6]. Briefly, approximately 1.5 mg of lyophilized peptide was weighed into 1.5 ml siliconized tubes and treated with 60 µl neat DMSO. Five microliters were removed and diluted with phosphate buffer (10 mM, pH 7.4) for concentration determination. Peptide concentration was assessed via absorbance at 274.6 nm, using a tyrosine extinction coefficient of 1420 M⁻¹ cm⁻¹. Fibril reactions were started by diluting peptide stock solution (in DMSO) to 1 ml with phosphate buffer (10 mM, pH 7.4, 0.005% sodium azide), for a final peptide concentration of 100 µM. Fibrillogenesis assays were performed under quiescent conditions in triplicate. Final DMSO concentration was <2% for all assays. Fibrillogenesis was followed by ThT fluorescence at 37 °C. At various time points, 10 µl aliquots were removed and diluted into 1 ml of 10 µM ThT solution in 10 mM phosphate buffer at pH 7.4. After vigorous mixing, fluorescence was measured on a Hitachi F2000 fluorescence spectrophotometer (Chiyoda, Tokyo, Japan) (λ_{EX} = 446 nm, λ_{EM} = 490 nm) with a 3 s delay. Measurements were averaged for 10 s, using a bandwidth of 10 nm and a photomultiplier voltage of 700.

Electron Microscopy

Five microliter aliquots of the Aβ peptide fibril reactions were applied to a glow-discharged, 1–400-mesh, carbon-coated support film, washed with water, and stained with 1% uranyl acetate for 30 s. Micrographs were recorded using an FEI Tecnai F30 electron microscope (Hillsboro, OR, USA) at magnifications of 15,000× and 98,000×, with additional magnification of 1.4× added by the charge-coupled device camera.

Acknowledgments

Electron microscopy was performed in the laboratory of Dr Robert Josephs at the University of Chicago. This work was supported by Department of Energy 'Genomes to Life' Genomics Program (grant DE-FG02-04ER63786). E. C. B. J. was supported by the MD/PhD Graduate Training in Growth and Development program at the University of Chicago (NIH T32 HD007009).

References

- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–356. [PubMed: 12130773]
- Hilbich C, Kisters-Woike B, Reed J, Masters CL, Beyreuther K. Aggregation and secondary structure of synthetic amyloid beta A4 peptides of Alzheimer's disease. *J Mol Biol*. 1991; 218:149–163. [PubMed: 2002499]
- Petkova AT, Ishii Y, Balbach JJ, Antzutkin ON, Leapman RD, Delaglio F, Tycko R. A structural model for Alzheimer's beta-amyloid fibrils based on experimental constraints from solid state NMR. *Proc Natl Acad Sci U S A*. 2002; 99:16742–16747. [PubMed: 12481027]
- Luhers T, Ritter C, Adrian M, Riek-Loher D, Bohrmann B, Dobeli H, Schubert D, Riek R. 3D structure of Alzheimer's amyloid-beta(1-42) fibrils. *Proc Natl Acad Sci U S A*. 2005; 102:17342–17347. [PubMed: 16293696]
- Lazo ND, Grant MA, Condrón MC, Rigby AC, Teplow DB. On the nucleation of amyloid beta-protein monomer folding. *Protein Sci*. 2005; 14:1581–1596. [PubMed: 15930005]
- Sciarretta KL, Gordon DJ, Petkova AT, Tycko R, Meredith SC. Abeta40-Lactam(D23/K28) models a conformation highly favorable for nucleation of amyloid. *Biochemistry*. 2005; 44:6003–6014. [PubMed: 15835889]
- Rajadas J, Liu CW, Novick P, Kelley NW, Inayathullah M, Lemieux MC, Pande VS. Rationally designed turn promoting mutation in the amyloid-beta peptide sequence stabilizes oligomers in solution. *PLoS One*. 2011; 6:e21776. [PubMed: 21799748]
- Johnson EC, Kent SB. Synthesis, stability and optimized photolytic cleavage of 4-methoxy-2-nitrobenzyl backbone-protected peptides. *Chem Commun (Camb)*. 2006:1557–1559. [PubMed: 16575459]
- Zuckermann RN, Kerr JM, Kent SBH, Moos WH. Efficient method for the preparation of peptoids [oligo(N-substituted glycines)] by submonomer solid-phase synthesis. *J Am Chem Soc*. 1992; 114:10646–10647.
- Walsh DM, Hartley DM, Kusumoto Y, Fezoui Y, Condrón MM, Lomakin A, Benedek GB, Selkoe DJ, Teplow DB. Amyloid beta-protein fibrillogenesis. Structure and biological activity of protofibrillar intermediates. *J Biol Chem*. 1999; 274:25945–25952. [PubMed: 10464339]
- Harper JD, Wong SS, Lieber CM, Lansbury PT Jr. Assembly of A beta amyloid protofibrils: an *in vitro* model for a possible early event in Alzheimer's disease. *Biochemistry*. 1999; 38:8972–8980. [PubMed: 10413470]
- Nilsberth C, Westlind-Danielsson A, Eckman CB, Condrón MM, Axelman K, Forsell C, Stenh C, Luthman J, Teplow DB, Younkin SG, Naslund J, Lannfelt L. The 'Arctic' APP mutation (E693G) causes Alzheimer's disease by enhanced Abeta protofibril formation. *Nat Neurosci*. 2001; 4:887–893. [PubMed: 11528419]
- Johansson AS, Berglind-Dehlin F, Karlsson G, Edwards K, Gellerfors P, Lannfelt L. Physicochemical characterization of the Alzheimer's disease-related peptides A beta 1-42Arctic and A beta 1-42wt. *Febs J*. 2006; 273:2618–2630. [PubMed: 16817891]
- Fandrich M. Oligomeric intermediates in amyloid formation: structure determination and mechanisms of toxicity. *J Mol Biol*. 2012; 421:427–440. [PubMed: 22248587]
- Hughes E, Burke RM, Doig AJ. Inhibition of toxicity in the beta-amyloid peptide fragment beta-(25-35) using *N*-methylated derivatives: a general strategy to prevent amyloid formation. *J Biol Chem*. 2000; 275:25109–25115. [PubMed: 10825171]
- Wu CW, Kirshenbaum K, Sanborn TJ, Patch JA, Huang K, Dill KA, Zuckermann RN, Barron AE. Structural and spectroscopic studies of peptoid oligomers with alpha-chiral aliphatic side chains. *J Am Chem Soc*. 2003; 125:13525–13530. [PubMed: 14583049]
- Shah NH, Butterfoss GL, Nguyen K, Yoo B, Bonneau R, Rabenstein DL, Kirshenbaum K. Oligo(*N*-aryl glycines): a new twist on structured peptoids. *J Am Chem Soc*. 2008; 130:16622–16632. [PubMed: 19049458]
- Cheon M, Chang I, Mohanty S, Luheshi LM, Dobson CM, Vendruscolo M, Favrin G. Structural reorganisation and potential toxicity of oligomeric species formed during the assembly of amyloid fibrils. *PLoS Comput Biol*. 2007; 3:1727–1738. [PubMed: 17941703]

19. Dobson CM. Protein misfolding, evolution and disease. Trends Biochem Sci. 1999; 24:329–332. [PubMed: 10470028]
20. Hoyer W, Gronwall C, Jonsson A, Stahl S, Hard T. Stabilization of a beta-hairpin in monomeric Alzheimer's amyloid-beta peptide inhibits amyloid formation. Proc Natl Acad Sci U S A. 2008; 105:5099–5104. [PubMed: 18375754]
21. Morgado I, Wieligmann K, Bereza M, Ronicke R, Meinhardt K, Annamalai K, Baumann M, Wacker J, Hortschansky P, Malesevic M, Parthier C, Mawrin C, Schiene-Fischer C, Reymann KG, Stubbs MT, Balbach J, Gorlach M, Horn U, Fandrich M. Molecular basis of beta-amyloid oligomer recognition with a conformational antibody fragment. Proc Natl Acad Sci U S A. 2012; 109:12503–12508. [PubMed: 22814377]
22. Sciarretta KL, Boire A, Gordon DJ, Meredith SC. Spatial separation of beta-sheet domains of beta-amyloid: disruption of each beta-sheet by *N*-methyl amino acids. Biochemistry. 2006; 45:9485–9495. [PubMed: 16878983]
23. Grant MA, Lazo ND, Lomakin A, Condron MM, Arai H, Yamin G, Rigby AC, Teplow DB. Familial Alzheimer's disease mutations alter the stability of the amyloid beta-protein monomer folding nucleus. Proc Natl Acad Sci U S A. 2007; 104:16522–16527. [PubMed: 17940047]
24. Schnolzer M, Alewood P, Jones A, Alewood D, Kent SB. *In situ* neutralization in Boc-chemistry solid phase peptide synthesis. Rapid, high yield assembly of difficult sequences. Int J Pept Protein Res. 1992; 40:180–193. [PubMed: 1478777]

Figure 1.

HPLC and mass spectrometry of A β 1-42 peptides. (A) A β 1-42 (expected mass 4514.0 Da, observed mass 4513.5 ± 0.6 Da). (B) A β 1-42 where the backbone nitrogen of Gly²⁹ is substituted with a NO₂Bn group (A β 1-42 NO₂Bn²⁹) (expected mass 4649.1 Da, observed mass 4648.5 ± 0.6 Da). (C) A β 1-42 where NO₂Bn is placed at Gly³³ and Gly³⁸ (A β 1-42 NO₂Bn^{33,38}) (expected mass 4784.3 Da, observed mass 4784.7 ± 0.6 Da). (D) A β 1-42 where NO₂Bn is placed at Gly²⁹, Gly³³, and Gly³⁸ (A β 1-42 NO₂Bn^{29,33,38}) (expected mass 4919.4 Da, observed mass 4919.1 ± 0.6 Da). Purified peptides were analyzed by analytical reversed-phase HPLC with online mass detection, using a 5–65% gradient of solvent B *versus* solvent A where A = H₂O + 0.1% TFA and B = CH₃CN + 0.08% TFA. UV detection was at 214 nm. Masses shown are a sum of all UV-active species. Each principal mass peak corresponds to the +3 molecular ion of the target peptide. The minor peaks on UV and mass spectra in (C) and (D) represent an unknown peptide product produced during synthesis that could not be removed during purification.

Figure 2. Fibril formation. The kinetics of amyloid fibril formation of Aβ1-42 wild-type and 2-nitrobenzyl backbone-modified peptides were monitored by ThT fluorescence.

Figure 3.

Transmission Electron Microscopy of A β 1-42 peptides. Negative-stained electron micrographs were obtained on samples withdrawn at 24 hours from the experiment shown in Figure 1. A) A β 1-42 at 15000X magnification and B) 98000X magnification. C) A β 1-42 NO₂Bn29 at 15000X magnification and D) 98000X magnification. E) A β 1-42 NO₂Bn33, 38 at 15000X magnification and F) 98000X magnification. G) A β 1-42 NO₂Bn29, 33, 38 at 15000X magnification and H) 98000X magnification. Scale bars at 15000X magnification represent 200 nm. Scale bars at 98000X magnification represent 50 nm.

Scheme 1.

Aβ1-42 with 2-nitrobenzyl substitution. (A) Amino acid sequence of Aβ1-42. Colored indications of secondary structure are approximate and are derived largely from solid-state NMR studies of Aβ fibrils. Orange, β-strand; brown, bend region; and green, modified glycines. (B) (2-nitrobenzyl)glycine, a photolabile peptoid moiety.