Exposing Asymmetry between Monomers in Alzheimer's Amyloid Fibrils via Reductive Alkylation of Lysine Residues

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Alzheimer's disease amyloid fibrils are composed of the self-assembled 40−43 residue peptide Aβ[1] (Figure 1). Solving the atomic-level structure of these fibrils represents a key step in the study of biochemical processes related to Alzheimer's Disease. Unfortunately, progress toward this goal has been slow because conventional X-ray and NMR structural methods cannot be applied to fibrous protein samples. In developing any structural model of fibrils the space groups and symmetry operators for Aβ monomers are important considerations. In this communication, data is presented which demonstrates that when Aβ is free in solution, the amino terminus and the side chains of both lysine residues (16 and 28) are equally accessible for reductive alkylation. In contrast, when Aβ is self-assembled into fibrils, chemical access to lysines 16 and 28 can be differentiated. Furthermore, fractional alkylation of lysine 28 supports the argument that the "one-dimensional unit-cell" of an amyloid fibril contains at least two ***non***-equivalent Aβ molecules. Together these data offer powerful new constraints for future models of fibril architecture.

The global architecture of amyloid fibrils has been postulated to be in the range of ∼80−120 Å in diameter, assembled from four to five protofibrils, each containing two to three *sub*-protofibrils.[2] The peptide chains are proposed to run perpendicular to the fiber axis, with β-sheet hydrogen bonds lying parallel to the fiber axis (cross β-structure). *Sub*-protofibrils are thought to be laminates of multiple β-sheets. During the past several years, several very different structural models have been proposed for Aβ fibrils.[3] In each of these models there is a strong effort to comply with the proposed overall diameter of the fibrils. However, some of the models are not based upon a hierarchical assembly of *sub*-protofibrils into protofibrils, and finally into fibrils. In addition, there has been little experimental information on how symmetry should be treated during the refinement of these models, or as a basis for the development of new models. To specifically probe for asymmetry in Aβ fibrils, we have been evaluating

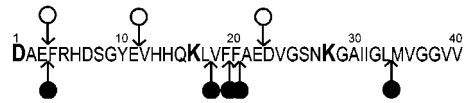

Figure 1. Aβ(1−40) with sites for reductive alkylation in **BOLD** (Asp 1, Lys 16, and Lys 28). *Open circles* (○) and *closed circles* (●) correspond to cleavage-sites for endoproteinase Glu-C, and pepsin (*respectively*).

different methods for modification of selected amino acids. The most desirable criteria for modification protocols include high efficiency, quantitative yields, and mild reaction conditions (to avoid altering the fibril structure). A suitable protocol was identified which relies upon the specific reaction of primary and secondary amines with formaldehyde to form the corresponding Schiff's base, followed by reduction with sodium cyanoborohydride [1° amine < 1° aldimine < 2° amine < 2° aldimine < 3° amine].[4] Reaction progress at specific sites can then be analyzed via limited-proteolysis and MALDI mass spectrometry. Under near physiological conditions this protocol can quantitatively alkylate the amino terminus as well as exposed ϵ-amino groups of lysine residues in soluble Aβ (Figures 1 and 2). Any hindrance of the alkylation in amyloid fibrils will expose site-specific nonequivalence within individual Aβ molecules and monomer asymmetry in fibrils.

Highly purified monomeric solutions[5] of Aβ(1−40) (**1**) were prepared. The samples were divided, a portion was subjected to "controlled-seeding" fibrilization, and the carefully assembled fibrils were gently isolated via centrifugation.[6] Monomeric solutions and resuspended fibrils were then subjected to reductive alkylation.[7] A solution of **1** was quantitatively alkylated to yield **3** (+6 methyl groups) (Figures 1 and 2). However, when **1** was pre-assembled into fibrils, the predominant products were roughly comparable amounts of (**2**) or (**3**) (Aβ(1−40) with +4 or +6 methyl groups added).

Other products were only seen transiently.[8] Hypothetically, alkylation might be equally incomplete at all sites. To observe roughly comparable amounts of Aβ(1−40) with 4 and 6 methyl groups, the overall product ratio for 2, 4, and 6 methyl groups should be 14.1%:42.2%:42.2% (or 1:3:3, *respectively, (for 75% overall yield)*). But it is difficult to clearly resolve a MALDI peak of sufficient intensity corresponding to just 2 added methyl groups after 48 h, raising the question of whether reductive alkylation in fibrils might be site specific.

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(1) (a) Glenner, G. G.; Wong, C. W. *Biochem. Biophys. Res. Commun.* **1984**, *122*, 1131−1135. (b) Kang, J.; Lemaire, H. G.; Unterbeck, A.; Salbaum, J. M.; Masters, C. L.; Grzeschik, K. H.; Multhaup, G.; Beyreuther, K.; Muller-Hill, B. *Nature* **1987**, *325*, 733−736. (c) Castano, E. M.; Ghiso, J.; Prelli, F.; Gorevic, P. D.; Migheli, A.; Frangione, B. *Biochem. Biophys. Res. Commun.* **1986**, *141*, 782−289. (d) Kirschner, D. A.; Inouye, H.; Duffy, L. K.; Sinclair, A.; Lind, M.; Selkoe, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 6953−6957.

(2) Teplow, D. *Amyloid: Int. J. Exp. Clin. Invest.* **1927**, *5*, 121−142.

(3) (a) Lansbury, Jr., P. T.; Costa, P. R.; Griffiths, J. M.; Simon, E. J.; Auger, M.; Halverson, K. J.; Kocisko, D. A.; Hendsch, Z. S.; Ashburn, T. T.; Spencer R. G. S.; Tidor, B.; Griffin R. G. *Nat. Struct. Biol.* **1995**, *2*, 990−998. (b) Chaney, M. O.; Webster, S. D.; Kuo, Y−M.; Roher, A. E. *Protein Eng.* **1998**, *11*, 761−767. (c) Li, L.; Darden, T. A.; Bartolotti, L.; Kominos, D.; Pedersen, L. G. *Biophys. J.* **1999**, *76*, 2871−2878. (d) Benzinger, T. L. S.; Gregory, D. M.; Burkoth, T. S.; Miller-Auer, H.; Lynn, D. G.; Botto, R. E.; Meredith, S. C. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 13407−13412. (e) Benzinger, T. L. S.; Gregory, D. M.; Burkoth, T. S.; Miller-Auer, H.; Lynn, D. G.; Botto, R. E.; Meredith, S. C. *Biochemistry*, **2000**, *39*, 3491−3499. (f) Antzutkin, O. N.; Balbach, J. J.; Leapman, R. D.; Rizzo, N. W.; Reed, J.; Tycko, R. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 13045−13050.

(4) Jentroft, N.; Dearborn D. G. *Methods Enzymol.* **1983**, *91*, 570−579.

(5) Peptides were synthesized and purified via HPLC according to (a) Lee, J. P.; Stimson, E.; Lu, Yi-An; Felix, A. M.; Maggio, J. *Biochemistry* **1995**, *34*, 5191−5200. (b) Esler, W. P.; Stimson, E. R.; Ghilardi, J. R.; Vinters, H. V.; Lee, J. P.; Mantyh, P. W.; Maggio, J. E. *Biochemistry* **1996**, *35*, 749−757. (c) Tseng, B. P.; Esler, W. P.; Clish, C.; Stimson, E. R.; Ghilardi, J. R.; Vinters, H. V.; Mantyh, P. W.; Lee, J.P.; Maggio, J. E. *Biochemistry* **1999**, *38*, 10424−10431. Stable monomeric solutions of Aβ were prepared according to (d) Zhang, S.; Iwata, K.; Lachenmann, M. J.; Peng, J. W.; Li, S.; Stimson, E. R.; Lu, Y.; Maggio, J. E.; Lee, J. P. *J. Struct. Biol.* **2000**, *130*, 130−141.

(6) Aggregates were prepared in two steps. (1) 100 μL of Aβ at ∼1 mM was aggregated using dissolution artifacts as "spontaneous seeds" for 1 week at 25 °C in saline at pH 7.4, with frequent sonication and sedimentation. The resulting "first generation seeds" consisting of fibrils and amorphous material are then used in a 1:10000 ratio with ∼10 μM monomeric solutions of Aβ. "Controlled-seeding" of fibrillization then occurs for 14−28 days at 37 °C *without* mixing. "Controlled-seeding" obeys first-order kinetics. Fibrils are obtained via mild centrifugation.

(7) Reductive-alkylation was performed on 100 μM samples of monomeric or fibrillar Aβ at pH 7.4 in the presence of 2 mM formaldehyde and sodium cyanoborohydride. For monomers the reaction was allowed to proceed for 2−3 h, while for fibrils the reaction was judged complete in 1−2 days. Soluble Aβ was isolated on a C18 SEP-PAK column, eluted with 50% acetonitrile-water (0.1% trifluoroactetic acid (TFA)), and lyophilized. Fibrils were sedimented and washed extensively with water, dried under vacuum, and dissolved in 50% acetonitrile−water (0.1% TFA). MALDI cocrystallization was performed using sinapinic acid or α-cyano-4-hydroxy-cinnamic acid.

(8) Only at <24 h were one, two, three, or five added methyl groups detected.

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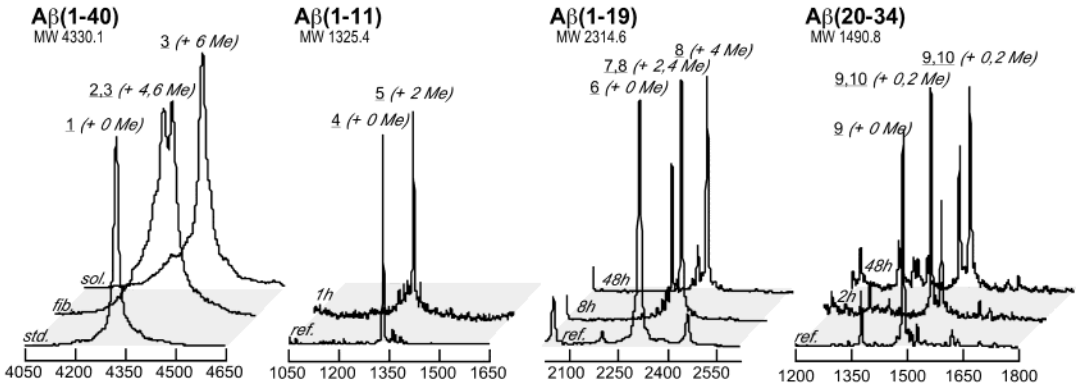

Figure 2. MALDI of Aβ(1−40) and its proteolysis products. The *x*-axis corresponds to the bottom spectrum, upper spectra are offset for display. *(Far-left)* are Aβ(1−40) STANDARD (**1**), and the FIBRIL (48h) (**2** and **3**) and SOLUBLE (2 h) (**3**) alkylation products. The remaining spectra are all derived from fibril alkylation reactions. *(Left-middle)* (**4** and **5**) are endoproteinase Glu-C products for residues Aβ(1−11) at 0 & 2h, *(right-middle)* (**6**, **7**, and **8**) are pepsin products for residues Aβ(1−19) at 0, 8 & 48 h, *(far-right)* and (**9** and **10**) are pepsin products for residues Aβ(20−34) at 0, 2, and 48 h.

To probe for specificity, alkylated fibrils of Aβ(1−40) (**2**, **3**) were dissolved in formic acid and digested with endoproteinase Glu-C[9] or pepsin[10] (Figures 1 and 2). The resulting proteolytic fragments were then analyzed by MALDI to determine the extent of alkylation at each site. A proteolytic fragment corresponding to Aβ(1−11) (**4**) was quantitatively transformed to (**5**) (+2 methyl groups) after 1 h, showing that the amino termini of *ALL* molecules are completely exposed when Aβ(1−40) is within fibrils. In contrast, after 8 h of alkylation, a fragment corresponding to Aβ(1−19) (**6**) was transformed to roughly comparable amounts of (**7**) and (**8**), (+2 and +4 methyl groups respectively), and after 48 h of alkylation, followed by proteolysis, the only product was (**8**) (+4 methyl groups). Hence, while all Aβ molecules in fibrils are exposed, the amino termini and lysine 16 can be kinetically differentiated. In addition, since the limited proteolysis shows that both the amino termini and lysine 16 are fully alkylated, the data strongly suggest that lysine 28 is the site of incomplete alkylation after 48 h. This suspicion was confirmed by monitoring another proteolytic fragment corresponding to Aβ-(20−34) (**9**). MALDI data shows that after only 2 h, there is appreciable alkylation of lysine 28 within Aβ(1−40) fibrils. However, between 8 and 48 h, alkylation of lysine 28 does not proceed to completion. Instead it appears to approach roughly comparable amounts of (**9**) and (**10**) (0 and +2 methyl groups).[11] Hence a fraction of the lysine 28 residues are either completely inaccessible, or the reaction is much slower for that fraction of the sites, compared to the amino terminus or lysine 16, when Aβ is within fibrils.

These data unravel new details on the architecture of amyloid fibrils. First, *all* Aβ(1−40) molecules are partially exposed to solvent. Second, residues 1, 16, and 28 are chemically indistinguishable in solution, but clearly distinguished in fibrils. Third, the data are consistent with nonequivalent Aβ molecules in fibrils. It follows that the structural heterogeneity between individual molecules requires further characterization, and ultimately it should be used to help define the "one-dimensional unit cell," within these "beta-crystallites."[12]

A critically important prerequisite for this work was the carefully preparation and isolation of Aβ(1−40) fibrils from monomeric solutions and defined seeds.[6] Initially aggregated Aβ was obtained via rapid spontaneous self-assembly, relying upon incomplete dissolution remnants as seeds. However, data was erratic and nonreproducible.[13] The clarity of the current data was only possible using more stringently defined fibrils. It is noteworthy that in the past, qualitative markers of fibril formation, such as electron microscopy or dye binding, have provided the benchmarks for the presence of fibrils. But one limitation of these methods is difficulty in calibrating the amount of amorphous or protofibrillar material that is present. It follows that the current results should also lead to a chemical "benchmark" of fibril preparations, and perhaps allow further subclassification of different possible fibril morphologies.

Finally, while this work should impact new structural models, it is also tempting to try to discriminate between existing models. *However Caution is Warranted*. Fibril preparations can be polymorphic, and data derived from different samples may detect mutually exclusive structural features. Because the current preparation methods are highly stringent, the relatively well-defined architecture in this work is likely to be a subset of those in other preparations.

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Supporting Information Available: Several full MALDI spectra of proteolytic digests taken at different time-points (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) Proteinase Glu-C E.C.3.4.21.19 (Boehringer 791 156), 0.5 mg/mL in pH 7.4 ammonium carbonate for 10 min.

(10) Pepsin E.C.3.4.23.1 (Sigma P6887), 0.5 mg/mL, sat. sinapinic acid (pH ~2.5) in 50% acetonitrile−water for 10 min.

(11) See Supporting Information.

(12) Inouye, H.; Fraser, P. E.; Kirschner D. A. *Biophys. J.* **1993**, *64*, 502−519.

(13) Presumably under conditions where amorphous aggregates or protofibrils are present. Isolated fibrils exhibited high structural stability.