Comparison of Alzheimer A β (1–40) and A β (1–42) amyloid fibrils reveals similar protofilament structures

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We performed mass-per-length (MPL) measurements and electron cryomicroscopy (cryo-EM) with 3D reconstruction on an A β (1–42) amyloid fibril morphology formed under physiological pH conditions. The data show that the examined A β (1–42) fibril morphology has only one protofilament, although two protofilaments were observed with a previously studied A β (1–40) fibril. The latter fibril was resolved at 8 Å resolution showing pairs of β -sheets at the cores of the two protofilaments making up a fibril. Detailed comparison of the A β (1–42) and A β (1–40) fibril structures reveals that they share an axial twofold symmetry and a similar protofilament structure. Furthermore, the MPL data indicate that the protofilaments of the examined A β (1–40) and A β (1–42) fibrils have the same number of A β molecules per cross- β repeat. Based on this data and the previously studied A β (1–40) fibril structure, we describe a model for the arrangement of peptides within the A β (1–42) fibril.

Alzheimer's disease | electron microscopy | prion | protein folding

Amyloid fibrils are fibrillar polypeptide aggregates that consist of a cross- β structure (1, 2). They accumulate inside the human body in the course of aging and are associated with several debilitating conditions such as Alzheimer disease (AD) (1, 2). AD amyloid fibrils are formed from A β peptide, which occurs in isoforms of different length. The 40-residue peptide A β (1–40) represents the most abundant A β isoform in the brain (3), while the 42-residue A β (1–42) shows a significant increase with certain forms of AD (4). A β amyloid fibrils have been analyzed with various biophysical and biochemical techniques, such as solid-state NMR (NMR) spectroscopy, solution state NMR spectroscopy coupled with hydrogen/deuterium exchange, or mutagenesis (5–8). These analyses have provided a wealth of information about specific structural details of the peptide in the fibril, for example dihedral torsional angles, protection factors or distances between specific atoms. Atomic models for A β peptides and their assembly in different amyloid fibrils have been constructed based on such data (6, 8–10) but these models have not been confirmed by more direct 3D imaging methods.

A hallmark of A β amyloid fibrils is their substantial polymorphism (11–14). We have recently shown by transmission electron cryomicroscopy (cryo-EM) and 3D reconstruction that A β (1–40) fibrils form a range of morphologies with almost continuously altering structural properties (13). Despite their different morphologies, the cross-sectional areas of the reconstructed fibrils were similar. The study suggested that the observed polymorphism may be the result of different packing of protofilaments that have the same basic structure. To obtain an image of a fibril at higher resolution, we established growth conditions that promote a specific A β (1–40) fibril morphology (15) and selected fibrils with this morphology from electron micrographs for further processing. Using newly developed image processing tools (16), we obtained a 3D image at 8 Å resolution from images of 188 fibrils (17). The image reconstruction revealed a fibril consisting of two protofilaments, and it resolved pairs of cross- β sheets at the core of each protofila-

ment. The observed cross- β sheet pairing resembled the recently proposed steric zipper model for amyloid spines (18) and suggested a protofilament core formed by two oppositely directed A β peptides. Mass-per-length (MPL) measurements further revealed that the fibril contains five peptides per cross- β repeat, or 2.5 peptides per repeat in one protofilament. The inconsistency between the 2.5 peptides per repeat indicated by the MPL measurements, and the two peptides per repeat implied by the paired β sheet model, was explained by additional peptide associated with the cross- β core of the fibril. The presence of additional peptide was also suggested by additional weak density visible at the periphery of the fibril core. The weak density is consistent with a more irregular peptide arrangement averaging 0.5 peptides per cross- β repeat, contrasting with the regular periodic structure of the fibril core.

In the present study, the structure of an A β (1–42) fibril morphology formed under physiologically relevant pH conditions is compared with two observed A β (1–40) fibril morphologies. Using scanning transmission electron microscopy (STEM), we show that all three morphologies have the same non-integer number of ≈ 2.5 peptides per cross- β repeat per protofilament. Furthermore, we use cryo-EM to generate a 3D reconstruction of the A β (1–42) fibril morphology and compare it to reconstructions of the A β (1–40) fibril morphologies. Although the reconstructed morphologies show significant differences, there is also evidence for common structural principles among different A β fibril morphologies.

Results

Amyloid characteristics of the analyzed A β (1–42) fibril. The analyzed A β (1–42) fibrils were obtained by in vitro incubation of 1 mg/mL A β (1–42) peptide in 50 mM Tris-HCl buffer, pH 7.4. After incubation for at least two days, the sample contained large quantities of fibrils, as confirmed by transmission electron microscopy (TEM) and standard biophysical methods (see *SI Materials and Methods*). Attenuated total reflectance Fourier-transform infrared (ATR-FITR) spectroscopy shows amide I and II maxima centered at 1,628 and 1,551 cm^{–1} (Fig. S1A). The recorded position of the amide I maximum is characteristic for the β -sheet structure of amyloid fibrils (19). Fibril samples also

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Data deposition: Density maps have been deposited in the Macromolecular Structure Database of the European Bioinformatics Institute [accession codes EMD-1649 [twofold symmetrized A β (1–42) fibrils] and EMD-1650 [13-nm A β (1–40) fibrils]].

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showed strong interactions with the amyloid-specific dyes Congo red or Thioflavin T (Fig. S1 B–D). Congo red bound fibrils produced apple-green birefringence when viewed in a polarizing microscope (Fig. S1 B and C). X-ray diffraction measurements of A β (1–42) fibrils gave rise to Bragg spacings at 4.67 ± 0.04 Å and 9.7 ± 0.3 Å (Fig. S2). Spacings at these positions represent typical characteristics of amyloid structures (20). The 4.7 Å reflection is termed “main chain” spacing and represents the distance of hydrogen bonded β -strands, while the 10 Å “side chain” spacing represents the packing distance of laminated β -sheet layers. Taken together, these data establish the amyloid-like characteristics of the A β (1–42) fibrils used in the present analysis.

Morphological Homogeneity of the Analyzed Fibril Sample. Cryo-EM shows that the analyzed sample contained large quantities of well-separated, unbranched fibrils of highly periodic structure. Almost all of the fibrils present regularly spaced cross-overs, indicative of a twisted fibril structure (Fig. 1A). Unidirectional platinum shadowing, coupled with TEM analysis, shows that the fibrils have a left-handed twist (Fig. S3). A left-handed helical twist is also found with many A β (1–40) fibrils (12, 13, 15). Measurement of the cross-over distances of 41 fibrils yields a relatively narrow distribution (Fig. 1B and Table 1). In addition, the fibrils are relatively similar in fibril width (Fig. 1C and Table 1). The low variation of these measurements indicates a high morphological homogeneity of the analyzed fibril sample. We compared this A β (1–42) fibril morphology with two A β (1–40) fibril morphologies present in a previously studied sample (15). These include the most frequently observed morphology with a fibril width between cross-overs of ≈ 20 nm (henceforth referred to as the 20-nm A β (1–40) morphology), and an infrequently observed morphology with a fibril width between cross-overs of ≈ 13 nm (referred to here as the 13-nm A β (1–40) morphology) (15). The standard deviations of the histogram distributions and errors are given in Table 1.

STEM Analysis. We performed STEM analysis combined with MPL measurements on all three fibril morphologies (Fig. 2). All MPL data were calibrated using Tobacco Mosaic Virus (TMV) and the standard deviations of the Gaussians fitted to the histograms closely match those of the MPL data for TMV (Fig. S4 A and B) (17). Therefore, the histogram distribution represents mainly an intrinsic error of the MPL measurements, and not a variability of fibril mass. The errors of the peak positions of the Gaussians fitted to the histograms are 0.4% or less in all cases (Table 1). For the A β (1–42) fibril, we obtained an average MPL value of 23.5 ± 0.1 kDa/nm. Using the theoretical molecular mass of an A β (1–42) molecule (4514 Da), the recorded MPL value translates into 2.44 A β (1–42) molecules for each 4.7 Å repeat of the fibril. Previous MPL analysis of the significantly wider 20-nm A β (1–40) fibril (17) yielded approximately twice this value, i.e., an average of 4.97 A β (1–40) molecules (4330 Da) or ≈ 2.5 A β molecules per cross- β repeat within one A β (1–40) protofibril. Virtually the same result of 4.91 molecules per cross- β repeat was found for the 13-nm A β (1–40) fibril. Hence, all analyzed A β (1–40) fibrils show a basic number of ≈ 5 peptide per cross- β repeat, while the presently reconstructed A β (1–42) fibril contains only half this number of peptides.

Three-Dimensional Reconstruction. We calculated a 3D reconstruction of the A β (1–42) fibril as described in ref. 17 using 14 fibril images from our cryo-EM dataset (Fig. 3 A and B). These were segmented into 572 overlapping segments for further processing (segment length: 158.4×158.4 nm). We selected fibrils with a minimal length of 300 nm and the smallest possible curvature. Furthermore, the selected fibrils included cross-over distances between 103 nm and 117 nm that were close to the distribution

Fig. 1. Fibril structure and structural homogeneity of the analyzed sample. (A) Cryo-EM images of A β (1–42), 20-nm A β (1–40) and 13-nm A β (1–40) fibrils. cross-overs and the widest points between cross-overs are indicated by black and white arrow heads, respectively. (B) Distribution of cross-over distances and (C) fibril widths (measured at the cross-overs and the widest points between cross-overs) for all three types of fibrils. The counts are plotted on a non-linear (square root) scale to make small counts more visible. Measurement results are provided in Table 1.

maximum of 108 nm (Fig. 1B). This represents $\approx 34\%$ of the imaged fibrils. The resolution of the reconstruction was ≈ 15 Å based on the 0.5 Fourier shell correlation (FSC) criterion (21) (Fig. S5A and Table 2). Projections of the 3D density map show good correspondence with the raw cryo-EM data (Fig. S5B). The observed match indicates that the obtained reconstruction is consistent with the raw data.

The reconstructed fibril is clearly polar. Fibril dimensions were measured using contour levels $1.5\times$ the estimated standard error of the map above background (bold lines in Fig. 3). The A β (1–42) fibril cross-section exhibits dimensions of $\approx 4 \times 11$ nm (Fig. 3B) and reflects the twofold symmetry imposed during reconstruction. The assumption of a twofold symmetry is based on a 3D reconstruction without imposed symmetry, which shows a similar cross-section and overall fibril topology (Fig. S6A). A twofold symmetry is consistent with most other amyloid fibril morphologies (13, 16, 22, 23). Hereafter, we will refer only to the twofold symmetrized reconstruction.

Fig. 2. STEM analysis of A β (1–40) and A β (1–42) fibrils. (A) STEM images of A β (1–42), 20-nm A β (1–40) and 13-nm A β (1–40) fibrils. (B) MPL measurements for all three fibril types. Measurement results are provided in Table 1. The standard deviation of the distribution of all histograms is the same standard deviation that is also found for TMV, which was used to calibrate the MPL measurements (Fig. S4). STEM data for the 20-nm A β (1–40) was taken from ref (17). The red and blue numbers above the histograms refer to number of A β molecules per 4.7 Å repeat, assuming A β (1–42) and A β (1–40) molecules, respectively.

the properties of P₄₀^b; they all display a diffuse density (Fig. 3B and C).

These comparisons provide further evidence that the analyzed A β (1–42) fibril consists of one protofilament, and that the C₄₂ region may harbor two juxtaposed β -sheets (Fig. 3B), similar to the core regions of the A β (1–40) protofilaments (Fig. 3C). The analogy to A β (1–40) further implies that the peptide N termini make up the P₄₂ regions, while the peptide C termini form the fibril core (C₄₂). Consistent with this proposal, we find that the C₄₂ substructure is comprised of two packed density cores that are separated from each other by a central lower density. However, a direct observation of this proposed packing arrangement will require higher resolution.

The MPL measurements indicating a non-integer number of ≈ 2.5 peptides per cross- β repeat in one protofilament are intriguing because they are incompatible with a fibril structure consisting only of peptides forming parallel in-register β -sheets.

Non-integer numbers of peptides per cross- β repeat were also obtained for other amyloid fibrils (25). This lends support to our MPL measurements. Furthermore, several other MPL measurements of A β fibrils have been published that report values with substantial deviations from those expected for an integer number of peptides per cross- β repeat (12, 26, 27). In these studies, the datasets were smaller than those used here, and the deviations were attributed to measurement error. In other cases, the fibril populations were too heterogeneous to yield MPL measurements that could be fitted by a single Gaussian (9, 12, 26, 28). The error analysis (Table 1) shows that in our study the deviation of the number of peptides per cross- β repeat from an integer is not due to a random measurement error. Furthermore, our fibril populations are sufficiently homogeneous to yield MPL data that can be fitted by single Gaussians with standard deviations comparable to that for TMV particles included with the amyloid fibrils.

It is possible that some A β molecules attach peripherally to the fibril with an average spacing of two cross- β repeats. Such additional molecules, decorating the actual fibril core, would be consistent with some low-density features seen with the 8 Å A β (1–40) fibril structure (17). Such disordered peptide may be difficult to detect by spectroscopic methods. Alternatively, instead of a loose peripheral association, peripheral A β peptides could swap domains with peptides from the fibril core with a frequency that averages to ≈ 0.5 peptides per cross- β repeat. Systematic errors can affect the observed MPL data. With the A β (1–42) fibrils, we regularly observe a systematic increase of the measured MPL values of $\approx 15\%$ at the positions of the cross-overs compared with measurements in between cross-overs. This leads to a 2% increase of the averaged MPL value for a typical fibril, compared with an average that excludes cross-overs. It is possible that the observed increase in mass is due to residual buffer that is not removed during freeze-drying, or due to denatured peptide that accumulates at the cross-overs. The average mass increase at the 13-nm and 20-nm A β (1–40) fibril cross-overs is $< 5\%$. The smaller value might be the result of the different morphologies or the different buffers used with the A β (1–42) (50 mM Tris/HCl at pH 7.4) and A β (1–40) fibrils [50 mM sodium borate at pH 8.7 (17)].

The 13-nm A β (1–40) fibril structure corresponds closely with one of the morphologies described previously (fibril 2 in ref. 13). However, due to the small size of the dataset, the resolution of the reconstruction is lower compared with the other two structures. Therefore, the interpretation of the density is less clear. Based on the similar cross-sectional area of the previously studied fibril morphologies (77 nm² for the 13-nm A β (1–40) fibril versus 90 nm² for the 20-nm A β (1–40) fibril), this fibril was also thought to consist of two protofilaments, each containing a pair of cross- β sheets (13). The difference of 13 nm² between the two cross-sectional areas may be due to the different quality of the two reconstructions, differences in the packing of the protofilaments, or different packing of the A β peptide within them. The MPL measurements further support the similarity between the 13-nm and 20-nm A β (1–40) fibrils.

The results obtained for all three fibril structures presented here suggest that they share the same fundamental protofilament structure, in agreement with earlier work using electron paramagnetic resonance (29). This would place the peptide N termini at the periphery of a protofilament, while the C termini would form its core. The different morphologies would, therefore, arise mainly from the different packing and number of protofilaments. This model does not exclude the possibility of variable side chain interactions between molecules within protofilaments, or differences in the structure and ordering of the N terminus. However, protofilaments would have an antiparallel arrangement of peptides in common that form pairs of parallel-stranded cross- β sheets.

Fibril Preparation. Synthetic A β (1–42) peptide was obtained from BACHEM (lot no. 0572194; the purity was at least 93%, measured by analytical HPLC/reverse phase chromatography). Fibrils were formed at 1 mg/mL concentration by incubation in 50 mM Tris/HCl (pH 7.4), at room temperature for a

Statistic	A β (1–42)	13 nm A β (1–40)
Pixel size on the specimen, Å	4.8	4.8
Resolution at FSC = 0.5, Å	15	23
Total length of non-overlapping segments, nm	4806	857
No. of fibrils	14	2
No. of segments	572	108
Segment size, nm	158.4	158.4
Size of 3D reconstruction, nm	77.7	77.7
Segment step size, nm	6.5	6.5
Crossover distance, nm	108	49
Repeat distance, nm	0.47	0.47
Fibril width, nm	10	13
Cross-sectional area, nm ²	44	77

minimum of 2 days. Other lots of A β (1–42) from BACHEM sometimes show a higher structural heterogeneity of fibril forms.

Electron Microscopy. CEM samples were prepared as described in ref. 15. Vitrified specimens were imaged in a Tecnai F30 microscope at 300 kV, with a magnification of 59,000 and low-dose conditions and a temperature of –180 °C. Micrographs were recorded at an underfocus of 1.75, 2.0 and 2.5 μ m on Kodak SO 163 film.

Scanning Transmission Electron Microscopy (STEM). Experiments were performed at the Brookhaven National Laboratory. The preparation and image acquisition conditions are described in ref. 35. Dark-field images with pixel sizes of 1 nm and 2 nm were analyzed using the computer program PCMASS29 (35) with the filament trace option and a mask of 40-nm length and appropriate width. The MPL measurements were calibrated with Tobacco Mosaic Virus (TMV) for each individual image as described in ref. 25, and a cross-repeat of 4.7 Å for all fibril morphologies was assumed.

Image Processing. Micrographs were selected for micrograph and fibril quality. Fibril quality was defined by length and straightness. Cross-over distances of appropriate A β (1–42) fibrils were limited to 110 nm $\pm$ 7 nm. The micrographs were digitized using a Zeiss SCAI flatbed scanner with a raster size of 7 μ m, resulting in a pixel size of 1.2 Å. A more detailed description is in ref. 16. The cross-over of 14 fibrils were boxed using EMAN's boxer program (36). Boxes along the fibril were added within 60 nm from each cross-over with 6.5-nm step size. The resulting image stack contained 572 squares of 158.4 $\times$ 158.4 nm. A pixel size of 4.8 Å was used for the reconstruction. For the image

processing we used SPIDER (37) and calculated a reconstruction of 77.7 $\times$ 77.7 $\times$ 77.7 nm in size. All studied fibrils appear polar and the image processing procedure (16) includes a test of polarity to ensure correct alignment. The parameters of out-of-plane tilt and helical axis angles were restrained during the alignment in addition to restraints applied to the x-shift and in-plane rotation angle, as described in refs. 16 and 17. The specimen out-of-plane tilt angle was measured by CTFTILT (38). A linear regression was performed based on the views, including cross-overs to predict the angles for the views between the cross-overs while obeying an ideal symmetry. Additional twofold symmetry was assumed by aligning each segment in two possible orientations differing by 180° rotation around the helical axis. For the reconstruction, we imposed helical symmetry with a rotation of 0.78° per repeat for the A β (1–42) fibril, and a rotation of 1.73° per repeat for the 13-nm A β (1–40) fibril. Table 2 contains details about the image processing. The standard error in the densities of the reconstructions was estimated from the difference between the two reconstructions used to estimate the Fourier shell correlation (FSC) curve as half the standard deviation. For the resolution estimation, the FSC was evaluated in a plane perpendicular to the fibril axis.

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