Intrinsic Linear Heterogeneity of Amyloid β Protein Fibrils Revealed by Higher Resolution Mass-per-length Determinations*

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Amyloid β proteins spontaneously form fibrils in vitro that vary in their thermodynamic stability and in morphological characteristics such as length, width, shape, longitudinal twist, and the number of component filaments. It is vitally important to determine which variant best represents the type of fibril that accumulates in Alzheimer disease. In the present study, the nature of morphological variation was examined by dark-field and transmission electron microscopy in a preparation of seeded amyloid β protein fibrils that formed at relatively low protein concentrations and exhibited remarkably high thermodynamic stability. The number of filaments comprising these fibrils changed frequently from two to six along their length, and these changes only became apparent when mass-per-length (MPL) determinations are made with sufficient resolution. The MPL results could be reproduced by a simple stochastic model with a single adjustable parameter. The presence of more than two primary filaments could not be discerned by transmission electron microscopy, and they had no apparent relationship to the longitudinal twist of the fibrils. However, the pitch of the twist was strongly affected by the pH of the negative stain. We conclude that highly stable amyloid fibrils may form in which a surprising amount of intrinsic linear heterogeneity may be obscured by MPL measurements of insufficient resolution, and by the negative stains used for imaging fibrils by electron microscopy.

Amyloid fibrils are filamentous aggregates formed by many different proteins in various diseases both in and out of the central nervous system (1). Fibrils formed by 40-residue (Aβ40) and 42-residue (Aβ42) amyloid β (Aβ)2 proteins comprise the amyloid plaques of Alzheimer disease. Considerable effort has been expended to determine how Aβ proteins are folded within fibrils, yet the models emerging from these efforts differ significantly (1–8). Contributing to uncertainty about the internal structure of fibrils is a growing appreciation that Aβ fibrils are polymorphic when examined by transmission EM, cryo-EM, and AFM (7, 9–19).

Two morphological features have particularly important implications with regard to the internal structure of amyloid fibrils. One feature is a periodic narrowing observed in many transmission EM studies suggesting that fibrils twist around their long axis. The other feature is the fibril MPL, measured by dark field or tilted-beam EM. From this measure, the number of filaments comprising the fibril may be determined. We follow the terminology conventions defined by Kodali et al. (12) with the exception that “protofilament” is shortened simply to “filament.”

Fibrils with different morphologies can be created by varying fibril growth conditions such as temperature, buffer composition, agitation, and protein concentrations. Once formed, fibrils with a given morphology induce monomeric proteins to form “next generation” fibrils with the same morphology (10). Despite attempts to use identical conditions of formation, different laboratories often produce fibrils with distinctly different morphologies. These differences make it difficult to compare the physicochemical properties of fibrils from different laboratories. It is of interest, therefore, to understand how fibril morphology depends on the manner in which their morphologies are characterized.

In the present study amyloid fibrils were formed in relatively dilute aqueous solutions of Aβ40 (2.3–30 μM). The resulting fibrils exhibited a remarkably high thermal stability. They were examined using EM and a variety of different negative stains, as well as dark-field EM to determine their MPL. Negative stains are invariably used when examining amyloid fibrils by EM to provide contrast. Nearly a dozen different stains are in common use, with diverse chemical properties and patterns of interaction with biological materials. The pH of most stain solutions can be adjusted over a wide range, and will influence the pattern of interaction with biological materials. Yet, the pH of the negative stain is rarely mentioned when methods are described. MPL determinations, on the other hand, are performed without any stain, but with an analytical approach developed for homogeneous materials (20–24).

We have found that the morphology of these highly stable fibrils was strongly influenced by the pH of negative stains.
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used for visualizing amyloid fibrils. More importantly, however, the number of filaments in these fibrils changed frequently along the length of the fibril. Therefore, it is likely that the internal structure of the fibrils examined in this work differs from fibrils generated in other labs that exhibit lower thermodynamic stabilities (25–27), and those that yield narrow MPL distributions and narrow NMR spectra (7, 28).

EXPERIMENTAL PROCEDURES

Materials—Synthetic Aβ40 was custom synthesized and purified by the Keck Laboratory at Yale University. Protein mass was verified by MALDI-TOF mass spectrometry, which verified the protein cation mass \([M + 1] = 4330.9\), and minimal peaks at other masses. The concentration of protein in this material was assayed using bicinchoninic acid, tyrosine absorbance at 280 nm, and mass spectrometric determination of Ala, Val, and Leu in acid hydrolysates. All three methods agreed in suggesting that the powder was 87% Aβ protein, with the remainder most likely representing water or salts.

Preparation of Aβ40 Fibril Seeds—Samples of protein weighing 200–600 μg were weighed to the nearest microgram on a Cahn microbalance, dissolved at 1 μg/μl in HFIP, and lyophilized. Seeds were then prepared in two ways. Method 1: protein lyophilized from HFIP was redissolved at 100 μM in TBS buffer (50 mM Tris, 100 mM NaCl, 0.2% sodium azide, pH 7.4), briefly sonicated in a bath sonicator to solubilize the protein, and centrifuged at 20,000 × g at 4 °C for 30 min. The supernatant was injected onto a size exclusion chromatography column (TSKgel G3000SWXL, 7.8 mm ID × 30 cm, Tosoh Bioscience, King of Prussia, PA) with TBS buffer flowing at a rate of 400 μl/min. The size and elution time performance of the column was calibrated with albumin, cytochrome c, and aprotinin. Virtually all of the injected protein eluted at a time corresponding to a molecular weight range of 35–40 kDa (8–10 Aβ monomers). This fraction was collected and incubated for 7 days at 37 °C without agitation. The fibrils that formed were broken into short fragments by sonicating in a Branson 1510 water bath type sonicator (Danbury, CT) for 3 min. As illustrated in Fig. 1, seeds ranged in size from 10–150 nm in length. Method 2: protein lyophilized from HFIP was redissolved to 1% (w/v) AmMo in water was 5.4, and a second solution with pH 7.4 was made by adding concentrated ammonium hydroxide solution. The pH of 1% (w/v) NaMo in water was 5.4, and a second solution with pH 7.4 was made by adding concentrated ammonium hydroxide solution. Methylamine vanadate (MeaV) was obtained from Nanoprobes (Yaphank, NY) and used as a 2% (w/v) solution in water without pH adjustment (pH 8.0).

Approximately 20 ng of fibrillized Aβ40 in 1 μl of buffer was placed onto freshly glow-discharged carbon films on 300 mesh nickel grids for 2 min and then blotted with filter paper. A negative stain was then applied for 2 min, blotted, and air-dried. Images were recorded using a JEOL-1010 transmission electron microscope (Tokyo, Japan), operating at 80 kV, equipped with a side-mounted CCD digital camera.

Analytical HPLC—Samples containing 30 μM Aβ40 and 4% fibril seeds prepared by method 2 in 30 mM phosphate-buffered saline were incubated at 37 °C for 0–264 h without agitation. 550 μl aliquots were removed, centrifuged at 50,000 × g and 4 °C for 20 min, and the supernatant was injected onto a 4.6 × 250 mm reversed phase Vydac MS C4 column (Grace) and separated by the following gradient: Sol A: 1% ACN + 0.1% trifluoroacetic acid; Sol B: 100% ACN + 0.1% trifluoroacetic acid, 0–8 min; 0% B, 8–40 min linear increase to 60% B, with a constant flow rate of 0.7 ml/min. Protein was detected in a 10-mm flow cell with a Beckman model 168 diode array detector at 215 nm. A standard curve was prepared that correlated the integrated absorbances of monomer peaks to protein quantity determined by mass spectrometric amino acid analysis, and established linearity down to a concentration of 100 nm.

Electron Microscopy—Three heavy-metal negative stains were obtained from Sigma-Aldrich: uranyl acetate (UrAc), ammonium molybdate (AmMo), and sodium phosphotungstate (NaW). Prior to use, 2% w/v aqueous solution of UrAc at pH 4.2 was filtered through the cellulose acetate filter (0.2 μm) to remove small precipitates. The pH of 1% (w/v) AmMo in water was 5.4, and a second solution with pH 7.4 was made by adding concentrated ammonium hydroxide solution. The pH of 1% (w/v) NaW in water was 5.4, and a second solution with pH 7.4 was made by adding concentrated sodium hydroxide solution. Methylamine vanadate (MeaV) was obtained from Nanoprobes (Yaphank, NY) and used as a 2% (w/v) solution in water without pH adjustment (pH 8.0).

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Mass-per-length Measurements—Approximately 10 ng of seeded Aβ40 fibrils in 1 μl aliquots of buffer, prepared as described above, were applied to carbon films on 300 mesh nickel grids, and mixed with tobacco mosaic virus (TMV) at 5.0 ng/μl. After 2 min the grids were washed in distilled water, blotted, and air-dried. Dark-field images of unstained samples were obtained with a transmission electron microscope by shifting the objective aperture, but otherwise using the same electron optics as in bright-field TEM imaging.

The MPL of amyloid fibrils was determined using the method of Chen et al. (28) with modification. The signal intensity in a rectangular area of a digital image containing either Aβ40 fibril or TMV was integrated, and the signal intensity of a nearby background area was subtracted. The MPL of the Aβ40 fibril was determined by scaling a value of 131.4 kDa/nm for the MPL of TMV (32) according to the ratio of background-subtracted signal intensities for Aβ40 fibril and for TMV. The modification made to the published method was a decrease in the length of the fibril/virus covered by the
rectangular area from 80 nm to 40 nm. This modification enabled us to resolve linear heterogeneity in MPL along the length of a fibril. This approach was validated by comparing two TMV in the same image. Results for the second TMV took the form of a Gaussian distribution of MPL values with a mean of 130 kDa/nm and a half-width at half-maximum (HWHM) of 4 kDa/nm.

RESULTS

Bath Sonication and Seed Morphology—The effectiveness of amyloid fibrils at recruiting monomeric Aβ proteins into fibrils is likely to be greater with a greater number of fibril ends, so that many short segments would be more effective than many long segments for any given mass of Aβ protein. Therefore, seeds were prepared by subjecting fibrils in a capped polypropylene “bullet” tube to bath sonication for 3 min. As shown in Fig. 1, this treatment fragmented long fibrils into segments 10–150 nm in length bearing the same morphological appearance.

Equilibrium Monomer Concentration—Seeded solutions of Aβ40 were incubated for periods of 0 to 264 h without agitation, and centrifuged as described above to pellet fibrils. Monomer concentrations were determined in the supernatant by analytical HPLC. The detection system was able to determine protein monomer concentrations in the injected sample accurately down to 100 nM, with a limit of detection that was ~1/5 of that value. At 37 °C, the monomer concentrations declined rapidly from 30 μM to undetectable levels after ~15 h, and concentrations remained undetectable for 264 h (Fig. 2). The time course of the decline was somewhat slower in the presence of 10 μM Cu²⁺, and somewhat faster in the presence of a Cu²⁺ chelator, 10 μM DTPA. Nevertheless, at long times the concentrations were undetectable. At 4 °C monomer concentrations remained at nearly initial levels for up to 45 h.

Negative Stains and Fibril Morphology—Seeded fibrils were examined with six different stain/pH combinations, and representative images for each stain/pH combination are provided in Fig. 3. The images show that the distances between narrowings or nodes (the “internodal” distances) vary not only between fibrils, but also within single fibrils. Also, there is a distinct and consistent “handedness” to the nodes, as previously noted by AFM (33).

With each stain, some fibrils with relatively few nodes were observed, although close inspection revealed that at least one node could be identified in nearly all fibrils (Fig. 4). Nevertheless, in some cases, particularly with UrAc at pH 4.2, many fibrils had no discernable nodes. To quantify the distribution of internodal distances and determine whether there were subpopulations of fibrils with different morphologies, histograms of the observed internodal distances were generated for each stain/pH combination (Fig. 5). Results indicate that fibrils in each combination exhibited a near-continuous and
smooth distribution of distances (Fig. 5). Cryo-EM studies have confirmed that these nodes arise from a longitudinal twist of the fibrils, and that the pitch of the twist is subject to near-continuous variation (18). However, data in Fig. 5 also show that the pitch of the twist is sensitive to pH: only 1–3% of internodal distances were 50 nm with acidic stains, but 10–17% of internodal distances were 50 nm with alkaline stains. UrAc was distinctive in that most fibrils had 0 to 1 discernable nodes (Fig. 4E). Therefore, the counts in Fig. 5E are relatively small. Among fibrils with discernable nodes, the number of internodal distances 100 nm was greater for UrAc (78%) than any other stain (43–63%).

The minimum internodal distances observed were 30–32 nm. Assuming that these distances correspond to a 180-degree twist of the fibril, and that there is a 0.475-nm spacing between polypeptide strands in a fibril (34, 35), this minimum internodal distance implies a maximum pitch of 2.85°/chain. In comparison, the model illustrated in Fig. 8e of Petkova et al. (5) had a pitch of 4.0°/chain.

MPL Measurements—A typical dark-field image of unstained fibrils and TMV in the same field is shown in Fig. 6. Fibrils and TMV are both clearly seen in shades of lighter gray on a dark gray background. They are easily distinguished from each other, although morphological details are difficult to discern due to lack of contrast. The white rectangles in Fig. 6 represent the image windows over which digital image density was integrated for MPL determination. The windows were 80 nm wide perpendicular to the fibril/virus axis, and either 40 or 80 nm long parallel to the fibril/virus axis.

Histograms of MPL results are shown in Fig. 7. For 873 measurements derived from 40 × 80 nm windows, there are 5 distinct peaks corresponding to the MPL values expected for 2, 3, 4, 5, and 6 filaments per fibril. The peaks were statistically robust: the peaks remained prominent when any ran-
domly-selected half of the data were plotted. There were no
measurements suggesting that only one filament was present
in a fibril. For 613 measurements derived from the same
images, but using 80 × 80 nm windows, results were distinctly
different. The most common measurements corresponded to
non-integral numbers such as 2.5, 3.5, and 4.5 filaments per
fibril. Average MPL values were calculated according to
Equation 1,

\[ MPL_{\text{ave}} = \frac{\sum n_i M_i}{\sum n_i} \quad \text{(Eq. 1)} \]

where \( n_i \) is the number of results corresponding to mass \( M_i \).
For both the 40 and 80 nm windows, \( MPL_{\text{ave}} \) corresponds to
\( \sim 3.5 \) filaments per fibril. Because the same images were used
for both analyses, we conclude that fibrils have an intrinsic
heterogeneity with features that require windows <80 nm
long to resolve.

**Fibril Model**—To interpret the preceding results about the
number of filaments per fibril, a simple stochastic computational
model of amyloid fibril structure was developed. The
model assumes that two primary filaments are present at each
point along the fibril, but it does not make any assumptions
about the symmetry relationship between these filaments. A
distance of 4.75 Å is assumed between monomers along each
of the primary filaments.

Additional filaments are added to the lateral aspects of the
two primary filaments by nucleating a new lateral filament at
each monomer with a transition probability of \( P \). A lateral
filament is terminated with the same probability applied to
each monomer in the lateral filament. Denoting the original 2
filaments as \( aa \), and each of the lateral filaments added
through this procedure as \( b \), the fibril at the end of this proce-
dure will consist of two \( aa \), three \( baab \) or \( aab \), or four \( baab \)
filaments at various points because lateral filaments are al-
lowed on both sides of the original fibril. In the same manner,
c filaments are added to \( b \) filaments using the same value for
\( P \), yielding two additional ways to obtain four filaments per
fibril (\( aabc \) and \( cbaa \)). In the same manner, \( d \) filaments are
added to \( c \), and \( e \) are added to \( d \). Lateral filaments cease to
elongate when the filament to which they have been added
terminates. No upper limit is placed on the number of lateral
filaments, but the number of instances in which more than 6
filaments were generated along a 1,000,000-monomer fibril
was negligible.

Model fibrils generated in this manner in silico were ran-
domly sampled with 40- and 80-nm windows, and the num-
ber of filaments for the fibril segment in each window was
recorded. Values of \( P \) were adjusted so that histograms de-
derived from the model matched the histograms derived from
MPL measurements. Results for \( p = 0.005 \) are illustrated in
Fig. 8, and an illustration of the texture of the model is shown
in Fig. 9. Values of \( P \) ranging from 0.003 to 0.008 gave compa-
rable profiles and agreement with the experimental data of
Fig. 7. It was not possible to obtain a satisfactory distribution
from models in which additional filaments only formed along-
side only one of the primary filaments.

The model fibrils reproduce several key features of the ex-
perimental data. First, sampling with 40 nm windows yields
prominent peaks of roughly equivalent size in the histogram
for 2, 3, and 4 filaments per fibril, as well as non-integral val-
ues arising when filaments do not completely span the sam-
pling window. Second, values corresponding to 5 and 6 fila-
ments per fibril are found much less frequently, while >6
filaments per fibril are rare. Third, sampling with the longer
80-nm windows largely obscures the discrete peaks at integer
values observed with 40-nm windows. Fourth, the mean MPL
values for both window lengths correspond to 3.4 filaments
per fibril, similar to the experimental values of 3.5.
A statistical summary of filament lengths in the model is provided in Table 1 and Fig. 10. Sections with 3 and 4 filaments are each approximately twice as prevalent as sections with 2 or 5 filaments at shorter lengths (<60 nm). The average length of sections with the same number of filaments ranges from 24–48 nm, somewhat shorter than the internodal distances summarize in Fig. 5. However, Fig. 10 clearly indicates that sections with the same number of filaments are more common at shorter lengths in this model.

**DISCUSSION**

The first question to consider about these results is whether the proteins within an individual amyloid fibril are structurally heterogeneous, or whether they are structurally homogeneous and the samples consist of a mixed population of fibril types. Either of the two set of MPL data in Fig. 7, if considered alone, could be interpreted as indicating that mixed populations were present. However, the two panels were derived from the same set of EM images, and only the window size with which these images were analyzed was changed. Results using 40 and 80 nm windows on the same set of images should be similar for a mixed population of internally homogeneous fibrils. Instead, the use of smaller windows revealed fewer instances in which a non-integral number of filaments per fibril were observed. Therefore, we must conclude that there is structural heterogeneity within these fibrils.

The polymorphism of amyloid fibrils and their tolerance for different internal structures has been documented by other experimental techniques (12, 36). For example, amyloid fibrils have been created with fundamentally different and mutually exclusive disulfide cross-links (37, 38). Subtle interventions such as agitation during formation can also induce a change in the internal structure and morphology of fibrils (10, 13). The structures induced under different conditions are self-propagating, i.e. seeds derived from fibrils of a given morphology induce the same morphology in new fibrils. However, it is difficult to reconcile the self-propagation of a homogeneous morphology with the intrinsic heterogeneity we have now documented. It is possible that the nature of the interaction between fibrils and negative stains is self-propagated. Differences in experimental conditions may also have a role. Petkova et al. (10) observed self-propagation in seeded fibrils formed without agitation at 24 °C and at concentrations of 50–200 μM. Our fibrils were also formed without agitation, but at higher temperature (37 °C) and much lower concentration (2.3 μM), to more closely approximate conditions in brain tissue. It should be emphasized, however, that heterogeneity among the fibril seeds used in this work does not in any way explain the intrinsic linear heterogeneity within a fibril that is the principal finding of this report.

Larger windows may be expected to yield better results in MPL analysis because averaging over a larger area should yield a more precise measure of average image noise. In this case, the most popular window size, 80 × 80 nm, in our hands suggested that fibrils consisted of a non-integral number of filaments. However, reanalysis of the images with 40 × 80 nm windows resolved the MPL results into distinct peaks corresponding to integral numbers of filaments. The analyses in Fig. 7, A and B were performed on the same images, and yielded the same overall average MPL value. Therefore, the results imply that the number of filaments per fibril varies along the length of a fibril.

Narrower windows might be considered statistically weaker, possibly giving rise to random or fortuitous features in a histogram suggesting an integral numbers of filaments. However, we compensated for the statistical weakness of narrower windows by evaluating a much larger number of such windows, and the statistical validity of features in the histogram was tested by examining subsets of the data. In the case

**FIGURE 8.** Histogram of MPL results from a model fibril generated in silico. A, results obtained using 40 × 80 nm areas. B, results using 80 × 80 nm areas on the same data. The data were smoothed once with a 3-point smoothing scheme before plotting.

**FIGURE 9.** A small segment of the in silico model used to generate the results in Fig. 8. In this model, 2 filaments run the length of the fibril, and 1 or sometimes 2 additional filaments adhere to either side. The texture of this model should be compared with the texture of fibrils imaged without negative staining in the dark-field image of Fig. 6. Note, however, that the vertical scale is exaggerated — 20-fold compared with the horizontal scale.
of Fig. 7A, random subsets of half the available data yielded the same features when plotted as a histogram. Moreover, even considering only a random half of the available data, the number of integral values (the peaks in Fig. 7A) remained ~4-fold larger than the number of half-integral values (the troughs in Fig. 7A). Observer bias is also unlikely; background-subtracted signal intensities in each window were accumulated before the observer could know the ratios that were emerging from the analysis. Therefore, we conclude that both the vertical and the horizontal results depicted in Fig. 7A are statistically robust.

The results from 40-nm long windows we report in Fig. 7A are similar to those of Goldsberry et al. (11, 39) who performed MPL analysis with ~50-nm long windows and observed peaks corresponding to 2, 3, and 4 filaments per fibril. However, they interpreted these peaks as representing "subsets" of the fibril population. It is unclear whether subsets were present, or if their fibrils were also heterogeneous because, as we have shown, the difference cannot be discerned in negatively stained transmission EM images.

The results from 80-nm long windows we report in Fig. 7B are similar to results of Petkova et al. (Fig. 4C, right hand panel) who also examined seeded fibrils formed under quiescent conditions with 80 nm long windows (10). The average MPL in both sets of data appears to be ~3.5 filaments per fibril. They also reported internodal distances varying from 50–200 nm using uranyl acetate, which is qualitatively similar to the distribution we obtained with this stain in Fig. 5E. Chen et al. (28) obtained a slightly smaller average MPL with 80-nm long windows, but they used a different fibril formation procedure involving the use of DMSO. DMSO is an effective solvent for Aβ proteins, but its use raises concerns about methionine oxidation (40) and structural effects (41). Paravastu et al. (7) reported MPL data suggesting 3 filaments per fibril, and created a structural model with 3-fold symmetry to reconcile these results with their NMR data. Our results raise the possibility that there is a mixture of 2, 3, and 4 filaments per fibril in such cases. However, the MPL distributions of Chen and Paravastu were relatively narrow, and their methods of fibril preparation differed in many substantial ways from our methods. Therefore, it is also possible that these fibril preparations were linearly homogeneous with a single integral number of filaments per fibril.

MPL analysis has also been performed in conjunction with cryo-EM (16, 19), although the fibrils in these studies were formed at 230 μM without seeding, compared with our conditions of 2.3 μM with seeding. The MPL analyses were performed with an unspecified window size and suggested that 5 filaments were present in Aβ40 fibrils. Nevertheless, only 4 filaments could be clearly resolved in cryo-EM images. Studies of Aβ42 fibrils have yielded a non-integral MPL of 2.5 that is also unexplained by cryo-EM images (19). A very different cryo-EM structure was obtained when Aβ42 fibrils were formed from DMSO-treated protein incubated at 100 μM under strongly acidic conditions (17).

The distribution of 2–6 filaments per fibril observed experimentally (Fig. 7) was reproduced by a computer model (Fig. 8) with one adjustable parameter. The one parameter is the frequency with which additional filaments are nucleated or terminated along a fibril, and it determines whether MPL analysis with a given window size is able to resolve the additional filaments that are present. The degree of similarity between the histograms in Figs. 7 and 8 was the only fitting criterion applied for this parameter. Because nucleation and termination occur with equal frequencies in this model, one would expect additional filaments to form alongside 50% of each of the two primary filaments in a fibril irrespective of the value of the fitting parameter. When additional filaments form on 50% of both primary filaments, only 25% of a fibril is free of additional filaments (i.e. 25% of the fibril has only 2 filaments). This is indeed the case, as seen in Table 1.

The equilibrium monomer concentration observed in these experiments was substantially less than 100 nM, and lower than concentrations previously reported by several other laboratories (25–27). It is unlikely that small amounts of monomer are being lost in our system because a standard curve calibrated by amino acid analysis was linear down to 100 nM and had a zero intercept. The low concentration of monomers suggests that the protein samples are pure, or at least that they consist only of protein that fibrillizes. The low concentration at equilibrium also makes it unlikely that additional filaments are an artifact of sample drying, because there would be an insufficient amount of monomer present to form the additional filaments that are seen. Differences in equilib-
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Primary monomer concentration among laboratories most likely represent structural polymorphisms among the preparations studied by various laboratories.

Assuming that monomeric forms are in equilibrium with monomer binding sites on the lateral aspect (S\textsubscript{l}) and at the ends (S\textsubscript{e}) of a fibril, and that the number of S\textsubscript{l} sites is far greater than the number of S\textsubscript{e} sites, we can infer that S\textsubscript{l} \ll S\textsubscript{e} in terms of binding affinity. This inference has several implications. First, it suggests that changes in total protein concentration are unlikely to change the fraction of S\textsubscript{l} sites that are bound because an increase in overall protein concentration will cause the fibrils present to elongate and create more S\textsubscript{l} sites until the monomer concentration that is in equilibrium with S\textsubscript{e} sites is reestablished. Second, if proteins in S\textsubscript{e} sites have a relatively low affinity for each other compared with the affinity between proteins at S\textsubscript{l} sites, then the structure of proteins bound to S\textsubscript{l} sites must be different than the structure of proteins bound to S\textsubscript{e} sites. Given the likelihood that their structure is different, it is worth investigating whether S\textsubscript{l} sites are a repository of relatively toxic forms of \( \alpha \)B protein.

A third implication is that the smooth, albeit twisted appearance of fibrils in Figs. 3 and 4 may represent only the 2 principal filaments from which any proteins in additional filaments have dissociated. Given the quality of the electron micrographs available, it should be possible to identify portions of a fibril with 3–6 filaments and distinguish them from portions of a fibril with only 2 filaments. However, there is no evidence of additional filaments in the negatively stained images of Figs. 1, 3, and 4. With some imagination, the kind of texture illustrated in Fig. 9 may be seen in the unstained EM image of Fig. 6, but the contrast is insufficient to draw any firm conclusions. The negative stains used to enhance the contrast of fibrils in an electron micrograph are concentrated in the additional filaments. Negative stains used for EM can affect the pitch of the spiral twist, and may also displace the additional filaments to yield images of just the two primary filaments.

REFERENCES

1. Chiti, F., and Dobson, C. M. (2006) Annu. Rev. Biochem. 75, 333–366
2. Carter, D. B., and Chou, K. C. (1998) Neurobiol. Aging. 19, 37–40
3. Chaney, M. O., Webster, S. D., Kuo, Y. M., and Roher, A. E. (1998) Prot. Eng. 11, 761–767
4. Petkova, A. T., Ishii, Y., Balbach, J. J., Antzutkin, O. N., Leapman, R. D., Delaglio, F., and Tycko, R. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 16742–16747
5. Petkova, A. T., Yau, W. M., and Tycko, R. (2006) Biochemistry 45, 498–512
6. Lührs, T., Ritter, C., Adrian, M., Riek-Loher, D., Bohrmann, B., Döbeli, H., Schubert, D., and Riek, R. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 17342–17347
7. Paravastu, A. K., Leapman, R. D., Yau, W. M., and Tycko, R. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 18349–18354
8. Makin, O. S., and Serpell, L. C. (2005) FEBS J. 272, 5950–5961
9. Serpell, L. C. (2000) Biochim. Biophys. Acta. 1502, 16–30
10. Petkova, A. T., Leapman, R. D., Guo, Z., Yau, W. M., Mattson, M. P., and Tycko, R. (2005) Science 307, 262–265
11. Goldsbury, C., Frey, P., Olivier, V., Aebi, U., and Müller, S. A. (2005) J. Biol. Mol. 352, 282–298
12. Kodali, R., and Wetzel, R. (2007) Curr. Opin. Struct. Biol. 17, 48–57
13. Paravastu, A. K., Petkova, A. T., and Tycko, R. (2006) Biophys. J. 90, 4618–4629
14. Jiménez, I. L., Nettleton, E. J., Bouchard, M., Robinson, C. V., Dobson, C. M., and Saibil, H. R. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 9196–9201
15. Sachse, C., Xu, C., Wieligmann, K., Diekmann, S., Grigorieff, N., and Fändrich, M. (2006) J. Mol. Biol. 362, 347–354
16. Sachse, C., Fändrich, M., and Grigorieff, N. (2008) Proc. Natl. Acad. Sci. U.S.A. 105, 7462–7466
17. Zhang, R., Hu, X., Khant, H., Ludtke, S. J., Chiu, W., Schmid, M. F., Frieden, C., and Lee, J. M. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 4653–4658
18. Meinhardt, J., Sachse, C., Hortschansky, P., Grigorieff, N., and Fändrich, M. (2009) J. Mol. Biol. 386, 869–877
19. Schmidt, M., Sachse, C., Richter, W., Xu, C., Fändrich, M., and Grigorieff, N. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 19813–19818
20. Zeilier, E., and Bahr, G. F. (1962) J. Appl. Phys. 33, 847–853
21. Wall, J. S., and Hairfeld, J. F. (1986) Annu. Rev. Biophys. Biophys. Chem. 15, 355–376
22. Feja, B., Durrenberger, M., Muller, S., Reichelt, R., and Aebi, U. (1997) J. Struct. Biol. 119, 72–82
23. Feja, B., and Aebi, U. (1999) Micron 30, 299–307
24. Sousa, A. A., and Leapman, R. D. (2007) J. Microsc. 228, 25–33
25. Sengupta, P., Garti, K., Sahoo, B., Shi, Y., Callaway, D. J., and Maiti, S. (2003) Biochemistry 42, 10506–10513
26. Usui, K., Hulleman, J. D., Paulsson, J. F., Siegel, S. J., Powers, E. T., and Kelly, I. W. (2009) Proc. Natl. Acad. Sci. U.S.A. 106, 18563–18568
27. O’Nuallain, B., Shivaprasad, S., Khetarpal, I., and Wetzel, R. (2005) Biochemistry 44, 12709–12718
28. Chen, B., Thurber, K. R., Shewmaker, F., Wickner, R. B., and Tycko, R.
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(2009) Proc. Natl. Acad. Sci. U.S.A. 106, 14339–14344
29. Fezoua, Y., Hartley, D. M., Harper, J. D., Khurana, R., Walsh, D. M., Con- 
dron, M. M., Selkoe, D. J., Lansbury, P. T., Fink, A. L., and Teplow, D. B. 
(2000) Amyloid-Int. J. Exp. Clin. Investig. 7, 166–178
30. Liu, L., Komatsu, H., Murray, I. V., and Axelsen, P. H. (2008) J. Mol. Biol. 
377, 1236–1250
31. Jarrett, J. T., and Lansbury, P. T., Jr. (1992) Biochemistry 31, 
12345–12352
32. Namba, K., and Stubbs, G. (1986) Science 231, 1401–1406
33. Rubin, N., Perugia, E., Goldschmidt, M., Fridkin, M., and Addadi, L. 
(2008) J. Am. Chem. Soc. 130, 4602–4603
34. Eanes, E. D., and Glenner, G. G. (1968) J. Histochem. Cytochem. 16, 
673–677
35. Kirschner, D. A., Inouye, H., Duffy, L. K., Sinclair, A., Lind, M., and 
Selkoe, D. J. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 6953–6957
36. Kodali, R., Williams, A. D., Chemuru, S., and Wetzel, R. (2010) J. Mol. 
Biol. 401, 503–517
37. Shivprasad, S., and Wetzel, R. (2004) Biochemistry 43, 15310–15317
38. Wetzel, R., Shivaprasad, S., and Williams, A. D. (2007) Biochemistry 46, 
1–10
39. Goldsberry, C. S., Wirtz, S., Müller, S. A., Sunderji, S., Wicki, P., Aebi, U., 
and Frey, P. (2000) J. Struct. Biol. 130, 217–231
40. Shechter, Y. (1986) J. Biol. Chem. 261, 66–70
41. Jackson, M., and Mantsch, H. H. (1991) Biochim. Biophys. Acta. 1078, 
231–235
42. Benzinger, T. L., Gregory, D. M., Burkoth, T. S., Miller-Auer, H., Lynn, 
D. G., Botto, R. E., and Meredith, S. C. (2000) Biochemistry 39, 
3491–3499
43. Antzutkin, O. N., Leapman, R. D., Balbach, J. J., and Tycko, R. (2002) 
Biochemistry 41, 15436–15450
44. Selkoe, D. J., and Abraham, C. R. (1986) Methods Enzymol. 134, 
388–404
45. Mori, H., Takio, K., Ogawa, M., and Selkoe, D. J. (1992) J. Biol. Chem. 
267, 17082–17086
46. Roher, A. E., Chaney, M. O., Kuo, Y. M., Webster, S. D., Stine, W. B., 
Havrankova, L. J., Woods, A. S., Cotter, R. I., Tuohy, J. M., Krafft, G. A., 
Bonnell, B. S., and Emmerling, M. R. (1996) J. Biol. Chem. 271, 
20631–20635
47. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T. (1997) 
Chem. Biol. 4, 119–125
48. Hou, L., Kang, I., Marchant, R. E., and Zagorski, M. G. (2002) J. Biol. 
Chem. 277, 40173–40176
49. Wang, Z., Zhou, C., Wang, C., Wan, L., Fang, X., and Bai, C. (2003) Ul- 
tramicroscopy 97, 73–79
50. Karsai, A., Mártontalviz, Z., Nagy, A., Graña, L., Penke, B., and Keller- 
mayer, M. S. (2006) J. Struct. Biol. 155, 316–326
51. Bravo, R., Arimon, M., Valle-Delgado, J. I., García, R., Durany, N., Castel, 
S., Cruz, M., Ventura, S., and Fernández-Busquets, X. (2008) J. Biol. 
Chem. 283, 32471–32483
52. Harper, J. D., Lieber, C. M., and Lansbury, P. T., Jr. (1997) Chem. Biol. 4, 
951–959
53. Bose, P. P., Chatterjee, U., Xie, L., Johansson, J., Goethelid, E., and 
Arvidsson, P. I. (2010) ACS Chem. Neurosci. 1, 315–324
54. Arimon, M., Diez-Perez, L., Kogan, M. I., Durany, N., Giralt, E., Sanz, F., 
and Fernández-Busquets, X. (2005) FASEB J. 19, 1344–1346
55. Mastrangelo, I. A., Ahmed, M., Sato, T., Liu, W., Wang, C., Hough, P., 
and Smith, S. O. (2006) J. Mol. Biol. 358, 106–119
56. Harper, J. D., Wong, S. S., Lieber, C. M., and Lansbury, P. T., Jr. (1999) 
Biochemistry 38, 8972–8980