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Coalescence of Spherical Beads of Retro-HSP12.6 into Linear and Ring-Shaped Amyloid Nanofibers

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Abstract—The sequence-reversed form of a small heat shock protein, HSP12.6 (retro-HSP12.6), has been reported to fold and assemble into structured tetramers in aqueous solution. Upon raising the protein concentration to ~1.0-1.5 mg/ml, tetrameric retro-HSP12.6 is known to display a tendency to associate further into spherical beads of 18-20 nm in diameter containing folded protein subunits. Here we report that storage of this protein at low temperatures leads to further association of the beaded structures into linear and ring-shaped amyloid nanofibers of 18-20 nm in diameter. The electron micrographs presented in this communication provide the best visual evidence yet that amyloids can form through the association of smaller structured bead-like intermediates. The results also suggest that folded β-sheet-rich subunits can participate in amyloid formation.

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Two issues, in particular, intrigue investigators attempting to understand how and why proteins deposit into amyloid fibrils. One concerns the degree to which native structure is retained, or lost, by a polypeptide chain during deposition. The other concerns the issue of whether (i) individual chains deposit directly onto fibril surfaces, in a molecule-by-molecule fashion, or whether (ii) chains associate initially into small (not necessarily fibril-like) assemblies that then subsequently assemble into protofibrils and fibrils by mechanisms that are as yet unknown. Several subsidiary issues arise from these two fundamental issues. One could ask, for instance, why certain aggregates grow in a linear fashion to form fibrils whereas others extend in all directions to form apparently amorphous assemblies of no particular geometry; both forms are sometimes observed within the same sample of insoluble protein. Likewise, one could ask whether fibrils form exclusively through intermolecular β-sheet formation, while amorphous aggregates form also through other (e.g. hydrophobic) interactions. Similarly, there is the question of whether polypeptide chain organization tends to be similar or identical in all fibrils, irrespective of protein identity and amino acid sequence. Given that intermolecular β-sheets can theoretically form in a sequence-independent manner, it is difficult to anticipate that there would be any particular specificity of chain organization within fibrils. Even so, similar-looking structures of defined geometry are seen in fibrils derived from many different proteins. These and other related issues, e.g. regarding the involvement of hydrogen bonding and hydrophobic interactions in different types of amyloids and amorphous aggregates, have been discussed recently by Rousseau et al. [1] in a review that presents the current understanding of the subject.

In our laboratory we study the folding, stability, and aggregation of proteins from different viewpoints; one of these is to explore sequence–structure and structure–stability relationships in proteins through the study of naturally-occurring sequences subjected to protein engineering-based modifications such as backbone-reversal, substructure-swapping, and/or sequence truncation. We examined the folding behavior of the backbone-reversed (retro) forms of three globular all β-sheet proteins. Our interest was both to examine whether retro-proteins derived from such proteins can fold at all [2, 3] and also to examine whether retro-proteins that do not populate well-folded structures can efficiently utilize their intrinsic β-sheet-forming potential (unaltered by sequence reversal) to form intermolecular β-sheets. We found that one retro-protein, retro-CspA, forms poorly-soluble, poorly-

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folded structures that readily deposit upon increase of protein concentration into very well-formed amyloid fibrils, apparently without intermediates [4]. A second protein, retro-GroES, formed highly soluble (up to 3 mg/ml) well-folded tetrameric structures, with no tendency to aggregate (to be published). With a third protein, retro-HSP12.6 (the backbone-reversed form of an α-crystallin-like small heat shock protein from *C. elegans*), we obtained folding into soluble, small multimers [5]. At room temperature, retro-HSP12.6 showed a mild tendency to associate upon increase in protein concentration to form large spherical bead-like multimers, not unlike the large spherical multimers formed by the parent protein, the α-crystallin-like heat shock protein.

Here we describe additional findings with retro-HSP12.6 indicating that the structured beads associate with time upon storage into long fiber-shaped structures that very much resemble amyloid fibrils. To the best of our knowledge, the data presented here constitute the best (although not the first) evidence yet of bead-like intermediates associating into amyloid fibrils. We have previously shown that the beads have a high content of structure [5]. Therefore, the present data also provides evidence for structured forms associating to form fibrils, in a situation where chains—as an effect of backbone sequence reversal—may be anticipated to have some β-sheet forming tendencies that are not fulfilled through intramolecular structure formation.

MATERIALS AND METHODS

Approach. There are two ways of examining a transformational process that occurs very slowly and apparently irreversibly. One is to examine the population at different time points and see whether the bulk of the population presents itself in a different state at each time point (a kinetic study). The other is to examine in great detail a single population, or a set of comparable populations, to see whether components that have been transformed to different degrees are all present simultaneously in the population, in varying degrees of abundance. The second approach sometimes needs to be employed when the population does not progress in-step through different stages. The mechanism can then be worked out from the variety of states observed in the population.

Protein deposition conditions. HSP12.6 protein purified by methods described earlier [5], was incubated at 4°C following concentration by centrifugal ultrafiltration to above 1.5 mg/ml. Significant precipitation was observed upon 3-4 months of storage at 4°C; with no apparent change in polypeptide molecular weight (i.e. no degradation) as judged by SDS-PAGE.

Electron microscopy. The precipitates were examined by transmission electron microscopy (TEM) with negative staining using phosphotungstic acid, uranyl acetate, and nickel salts, to examine several tens of grids of retro-HSP12.6 prepared for electron microscopy after sonication for 5-10 min, or without sonication treatment, using the same methods used earlier to discover the bead-like structures formed by this protein [5].

RESULTS

Retro-HSP12.6 precipitates analyzed after months of storage were observed to exist in a number of different states. Figure 1 shows representative panels of these states. More than one panel has been used to show certain states, to provide an impression of the different ways in which some states present themselves on the grid. Besides the amorphous form seen in the background in most panels, the most common state observed was that of the structured bead-like forms earlier reported by us to have formed upon increase of protein concentration. Figure 1 (a and b) shows some representative depictions of these bead-like forms, for comparison with the other panels. Towards the bottom of Fig. 1b, one can see beads associating in twos and threes. In Fig. 1 (c-e), beads can be seen to be "lining-up" to different extents, with as many as seven beads lined-up in one "linear" structure associated with an amorphous mass that appears to contain other beads as well, as shown in Fig. 1e. In Fig. 1f, a representative mass is shown from which many different linear "maize-like" structures with roughly the same diameter as the beads appear to be emerging. The EM fields had many such amorphous masses from which the maize-like structures (of different lengths) can be seen to be emerging. One of the longer forms of such structures is shown in Fig. 1g, where the structure emerging can be seen to have assumed a filament-like shape. Numerous such long filaments could be seen. In all cases, the base of the filament near the amorphous mass could be seen to have a somewhat larger diameter (closer to the bead diameter), with the diameter narrowing down in the filament. The ends of such filaments appeared mostly to be irregular and broken, which could be explained by the fact that the samples were sonicated prior to electron microscopic analysis. As sonication could be expected to have caused breaking of filaments, samples that were not subjected to any sonication were also examined. A representative depiction of what was observed in such samples is presented in Fig. 1h, where one can see numerous filaments interconnecting regions with high densities of amorphous material. Close-ups of some filaments are shown in Fig. 1 (i and j). In Fig. 1i, the base of the filament near the attached amorphous mass can be clearly seen to consist of bead-like structures that appear to have not yet integrated with the filament. Similarly, in Fig. 1j also, bead-like structures are seen at one end of the filament. Figure 1k shows a magnified view of one filament with a tip perfectly rounded like a bead, rather than with the irregular broken appearance of the filament tip seen in

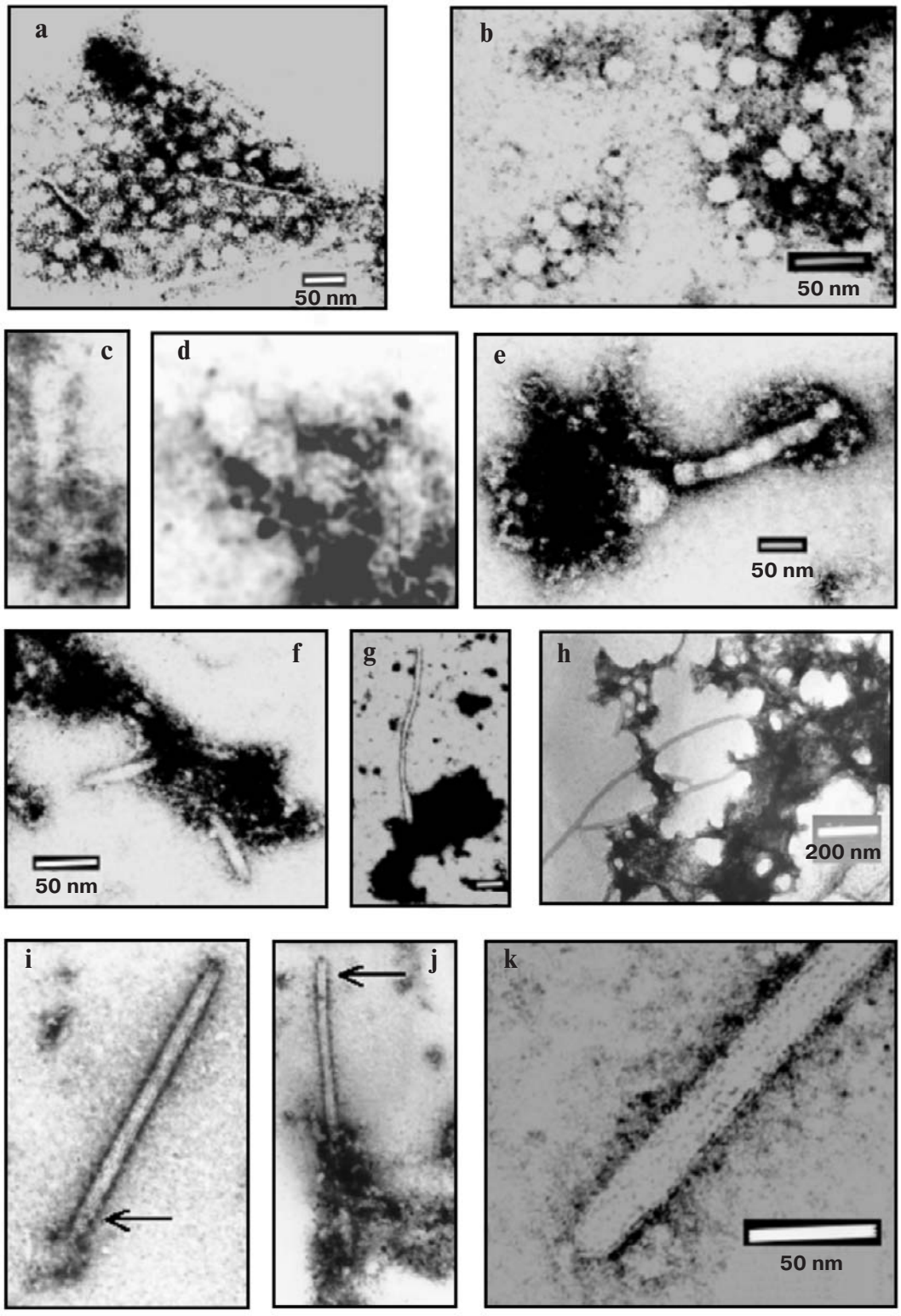

Fig. 1. Representative views of various types of structures seen in the same population of retro-HSP12.6 precipitated material visualized through electron microscopy after sonication (a-g) and without sonication (h-j). Panels (a) and (b) show bead-like forms. Panels (c)-(e) show beads "lining-up" into linear structures. Panel (f) shows a large aggregate associated with multiple "maize-like" linear structures as well as beads of comparable diameter. Panel (g) shows a linear structure with a filament-like appearance and a broken tip. Panel (h) shows filaments interconnecting dense amorphous aggregated material. Panels (i) and (j), respectively, show magnified views in which bead-like structures are visible at the base of a filament, or at the tip, as highlighted by arrows. Panel (k) shows a view of a filament with a hemispherical tip demonstrative of the origin from beaded structures.

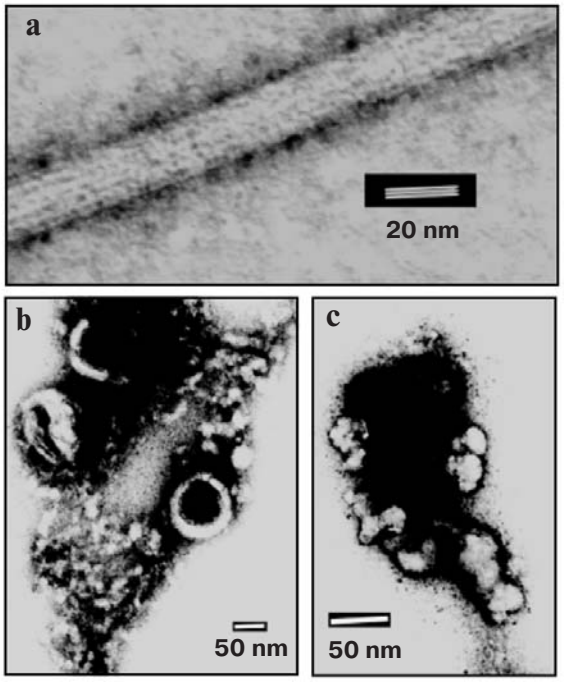

Fig. 2. a) A magnified view of the filament in Fig. 1k; b) a rare sub-population of fibrils associating head-to-tail to form ring-like amyloid structures; c) beads associated into irregular structures that are neither linear nor ring-shaped.

Fig. 1i. A further magnified view of the main filament in Fig. 1k is shown in Fig. 2a, where characteristic striations along the length of the linear structure (seen in many different amyloid fibrils) can be distinctly seen. Figure 2b shows fibrils associating head-to-tail to form ring-like amyloid structures. Notably such ring-like structures have also been recently reported to form in another amyloid-forming protein, equine lysozyme [6]. Sometimes, as Fig. 2c shows, the structures formed through associations of beads are neither linear nor ring-shaped and circular; instead, there is some irregular structure formed.

DISCUSSION

Our results suggest that 18-20 nm diameter beads are initially formed by structured, precipitated forms of retro-HSP12.6 within what appear to be amorphous masses of proteinaceous material. The beads appear to adhere to each other and coalesce into linear structures within this amorphous material. As this proceeds, the linear structure that has begun to form loses its beaded appearance and gives rise to a smooth striated cylinder-like form. That the origins of such cylinders can be traced back to the coalescence of 18-20 nm beads is suggested by the fact that the ends of cylinders can sometimes be seen to consist of material which has still not completely lost its bead-like appearance. Another interesting fact is that the ends of filaments always appear to be associated with some amorphous material. We examined many fields of filaments; in most of these, broken filaments had at least one end associated with an amorphous aggregate, while in some cases filaments also had both ends associated with such aggregates. Thus, the amorphous material associated with the filaments could probably be thought of as "nurseries" supporting the formation and coalescence of beaded proteinaceous material. Fibrils would appear to be "growing" from the end still associated with such a nursery through the addition of more beads supplied by the nursery. It may be noted that the preliminary material that associates to form filaments here was previously demonstrated to be structured [5]. This suggests that association of structured material into filaments can occur if molecules retaining unsatisfied potential for hydrogen bonding through edge strands assemble through intermolecular contacts. What remains unaddressed, of course, is the question of why the structured proteinaceous material appears to initially form beads and then associate into filaments. We have no answers to offer at this time.

It is clear that different protein systems support different fibril assembly models [7, 8]. Much evidence now exists to suggest that α-helical proteins can undergo profound conformational changes to adopt β-sheet structures during fibril formation. This change can occur spontaneously, in a mutation-induced fashion, or through a prion-like mechanism in which a preexisting fibril somehow induces the further deposition of protein from solution onto its surfaces [9], or even in a manner forced by the use of extreme physicochemical conditions [10]. In certain instances, the entire native structure of a polypeptide chain appears to be destroyed and replaced by a β-sheet-based structure in the amyloid [9]. In other instances, amyloids appear to form through more subtle conformational changes such as the swapping of a single β-strand between neighboring molecules [11-13]. This indicates that native-like structure can sometimes be retained within chains using only a part of their structure to participate in amyloid formation [14, 15].

In principle, therefore, chains that are naturally folded into β-sheets could potentially retain much of their native-like structural format while depositing into amyloids, utilizing only a few solvent-facing β-strands to undergo intermolecular associations [16]. Lower order multimers could eventually also associate into higher order multimers, and ultimately, into fibrils. Interestingly, there are reports of the association of bead-like intermediates into amyloid fibrils [17-22], just as there are reports of fibrils growing out of amorphous material, through the molecule-by-molecule bleeding of dissociational equilibria at the surfaces of amorphous protein aggregates [23]. Our results add to this growing body of literature.

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