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Review Article

The role of prefibrillar assemblies in the pathogenesis of amyloid diseases

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Abstract

The formation of amyloid fibrils is associated with a large number of major diseases, including Alzheimer's disease, type 2 diabetes, prion diseases, Parkinson's disease and various familial and systemic amyloidosis disorders. The formation of well-ordered fibrils of 7-10 nm in diameter with a distinct X-ray fiber diffraction pattern and a β-sheet secondary structure is observed in all these amyloid diseases. The formation of the fibrils is correlated with cellular death that is co-localized with the fibrillar deposits. Nevertheless, recent studies have indicated that early prefibrillar assemblies, rather than large amyloid fibrils, may mediate the cytotoxicty that is associated with the fibrillization process. It was clearly demonstrated that unrelated amyloid-forming polypeptides form remarkably similar nanometric annular structures at the early stages of assembly. Those structures are kinetically transient and show strong membrane-interacting and -permeating abilities. The ultrastructure of the transient assemblies and their membrane activity are consistent with a membrane pore formation mechanism of cytotoxicity. The identification of prefibrillar structures as the key cytotoxic agents in amyloid disease suggests that therapeutic approaches to the treatment of amyloid diseases should be directed toward the early stages of molecular recognition that facilitate the formation of the early assemblies.

Amyloid disease

The formation of amyloid fibrils in various tissues and organs is the hallmark of a variety of diseases of unrelated origin (1-5). A partial list includes Alzheimer's disease, type 2 diabetes, Parkinson's disease, prion diseases (such as bovine spongiform encephalopathy, or BSE), and various familial and systemic amyloidoses (Table I). In all cases, soluble cellular proteins undergo a self-assembly process that leads to the formation of large and well-ordered protein deposits (Fig. 1). The mechanism of amyloid fibril formation is assumed to be a nucleation-dependent process. According to common models, unfolding events are followed by a series of equilibration steps to form a prefibrillar nucleus of a critical size. This is followed by thermodynamically favorable growth steps with addition of monomers to the growing nucleus. New observations, as will be described here, suggest that off-pathway steps exist and actually may play a key role in the pathology of amyloid diseases.

When examined by electron microscopy or atomic forces microscopy, these polypeptide deposits reveal typical fibrillar structures with a diameter of 7-10 nm and a length that can reach several microns. The fibrils are well ordered, as reflected by X-ray fiber diffraction, which shows a clear 4.6-4.8 Å reflection on the meridian. Such reflection is correlated with the hydrogen bonding distance between stacked β-strands. This is consistent with the predominantly β-sheet structure of proteins in amyloid deposits, as determined by Fourier transform infrared and circular dichroism spectroscopy. The well-ordered nature of the fibrils and their specific assembly process has led to the description of the amyloid fibrils as "one-dimensional crystals" (6).

Increasing evidence supports the hypothesis that the assembly of amyloid fibrils is a central factor in the development of the clinical symptoms of amyloid diseases (1-6). The formation of amyloid fibrils is correlated with cellular death in the affected tissues, with a clear spatial co-localization of amyloid deposits and damaged cells. Moreover, assembled amyloid structures were shown to be cytotoxic to cultured cells and to permeate lipid membranes. Finally, mutations within the amyloidogenic

Table I: Pathologies associated with amyloid fibril formation and the detection of prefibrillar assemblies.

Clinical syndrome	Amyloid-forming polypeptide	Site of amyloid formation	Detection of prefibrils
Alzheimer's disease	β-Amyloid (Aβ)	Brain	+
Parkinson's disease	α-Synuclein	Brain	+
Type 2 diabetes	Islet amyloid polypeptide (IAPP)	Pancreas	+
Medullary carcinoma	Calcitonin	Thyroid	
Aortic medial amyloid	Lactadherin	Atrial media	
Prion diseases	Pr protein	Brain	
Insulin injection amyloidosis	Insulin	Site of injection	
Primary systemic amyloidosis	Immunoglobulin light chain	Systemic	
Chronic inflammatory amyloidosis	Serum amyloid A (SAA)	Systemic	
Huntington's disease	Huntingtin	Brain	
Senile systemic amyloidosis	Transthyretin	Systemic	+
Pituitary gland amyloidosis	Prolactin	Pituitary gland	
Hereditary renal amyloidosis	Fibrinogen	Renal	
Familial British dementia	BriL	Brain and systemic	+
Finnish hereditary amyloidosis	Gelsolin	Systemic	
Familial non-neuropathic amyloidosis	Lysozyme	Systemic	

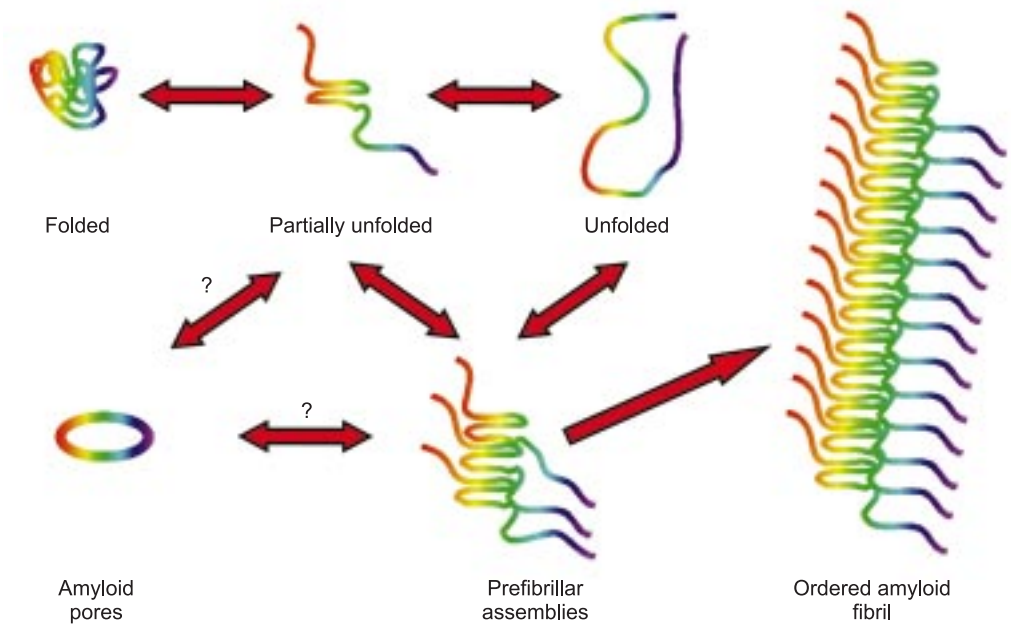

Fig. 1. A model for the assembly of amyloid fibrils. The model assumes equilibrium between the folded, partially unfolded and misfolded states (the thermodynamic equilibrium between the various forms varies between various proteins). The unfolded and partially unfolded states can to be sequentially added to the growing nucleus in a thermodynamically unfavorable manner. Assumed off-pathway trajectories, either from the partially unfolded and/or the prefibrillar state, result in assembly at the prefibrillar stage, which leads to the formation of amyloid pores (see Figs. 2 and 3). The second stage of the process occurs after the growing nucleus reaches a critical mass. After reaching the critical mass, there are further thermodynamically favorable additions of protein molecules to the nucleus. This eventually leads to the formation of a large stable aggregate.

peptides are associated with a high familial occurrence and early onset of some of these diseases.

As many amyloid-related diseases, including Alzheimer's disease and type 2 diabetes, are correlated with advanced age, they may become one of the major public health concerns of this century, due to the gradual increase in life expectancy. As an illustration, it is estimated by the U.S. National Institutes of Health (NIH) that only about 3% of men and women aged 65-74 have Alzheimer's disease, yet nearly half of those aged 85 and older may have the disease. A genuine understanding of the mechanisms that lead to the formation of amyloid fibrils, their pathological activity and means of inhibiting their formation is therefore of the utmost clinical importance.

Amyloid fibrils may represent a generic protein state

It has been previously shown that amyloid fibrils that are remarkably similar to those formed by the pathologically related proteins can also be formed *in vitro* by disease-unrelated proteins. Such proteins include an SH3 domain, an *Escherichia coli* cold shock protein and a fragment of *E. coli* HypF protein (7, 8). Moreover, the formation of amyloid fibrils was also observed in lower eukaryotes and prokaryotes. It was also demonstrated in prion-like diseases, in which amyloid proteins mediate the non-Mendelian inheritance of phenotypic traits in yeast (9, 10). More recently, it was demonstrated that the formation of biofilms by the *E. coli* bacterium is facilitated by the formation of amyloid fibril networks (11).

Taken together, it appears that the formation of amyloid fibrils is very common in nature and they may represent a generic form of assembled protein structures. However, in spite of the key importance of the amyloid fibrillization process in terms of both basic science and medical studies, this mechanism is not fully understood. A central feature in the process of amyloid assembly that has begun to unravel in the last few years is the identification of early intermediates in the process of amyloid assembly and their role in the pathogenesis of amyloid diseases. Here, we will present recent evidence for this notion and indicate directions for future research and drug development that are based on this concept.

Identification of prefibrillar assemblies

Early on in the study of amyloid diseases, scientific attention was mostly directed toward the characterization of the ordered fibrillar structures. Nevertheless, recent studies suggest that the prefibrillar species, rather than the full-length amyloid fibrils, may actually facilitate the pathological role of the amyloid assemblies. A central feature that supports the notion of the direct role of amyloid fibrils, rather than their side products, as pathological agents of amyloid disease is their cytotoxicity. According to this notion, the formation of toxic amyloid fibrils causes the apoptotic death of cerebral neural cells in the case of neurodegenerative amyloid diseases, or pancreatic cells in the case of type 2 diabetes.

The first prefibrillar species were identified during the study of the fibrillization process of the β-amyloid (Aβ) polypeptide, the major constituent of fibrillar plaques formed in the case of Alzheimer's disease (12, 13). Continuous monitoring of the aggregative assemblies using atomic force microscopy, electron microscopy, size exclusion chromatography and quasielastic light scattering clearly indicated the formation of distinct suparmolecular assemblies prior to the formation of the larger fibrils. These assemblies are metastable and therefore transient in nature, although they can be clearly identified using biophysical techniques.

These early assemblies were designated as protofibrils. While both the terms "prefibrillar" and "protofibrillar" can be used to characterize early assemblies of the amyloidogenic protein, in this review we will use the term "prefibrillar" as it is not clear whether the assemblies are early intermediates that lead to the formation of the fibrils or off-pathway assemblies (Fig. 1). Later studies have identified similar distinct molecular assemblies of transthyretin amyloidogenic protein, the Parkinson's disease-associated α-synuclein protein, the type 2 diabetes-linked islet amyloid polypeptide (IAPP) and the ABri peptide of familial British dementia (Table I) (14-18).

Cytotoxicity of the prefibrillar assemblies

Soon after the identification of prefibrillar assemblies, there was an indication that such structures may indeed play a role in the cytotoxicity associated with the amyloid formation process. An early observation indicated that the prefibrillar Aβ assemblies, and not the mature fibrils, induce acute electrophysiological changes in cortical neurons and have progressive neurotoxic activity (19, 20). Another study used a unique density-gradient separation technique to identify toxic activity towards neuronal cells by both fibrillar and prefibrillar species, but not by the monomeric Aβ (21). A reasonable interpretation for the partially conflicting results is that the metastability of the prefibrillar assemblies hampers the ability to fully identify the cytotoxic agents in the process of amyloid assembly. This is mainly due to the relatively short half-life of the prefibrillar assemblies as compared to the time scale of toxicity measurements.

Key support for the role of prefibrillar assemblies in the cytoxicity of the amyloid process emerged from studies on the Aβ "Arctic" mutant protein rather than the wild-type protein. The "Arctic" mutation within the Aβ polypeptide was identified to span 4 generations in a family in northern Sweden with an autosomal dominant pattern of inheritance. The mutation has a clear genetic link to early onset of Alzheimer's disease (mean age of onset 57 ± 2.9 years for carriers of the mutation). When the mutant protein was synthesized and studied *in vitro*, it was demonstrated that this mutation results in the preferential formation of stable prefibrillar assemblies compared to the wild-type Aβ protein. Both the rate of formation and the total level of prefibrillar assemblies were much higher with the mutant protein (22).

Ultrastructure of prefibrillar assemblies: are they membrane "pores"?

The relative stability of the prefibrillar assemblies of Aβ "Arctic" mutant polypeptide facilitated its high-resolution analysis using electron microscopy, which clearly demonstrated the existence of annular pore-like structures (22). Similar structures were further observed using mutant α-synuclein polypeptides (Fig. 2) (23, 24). In the case of both Aβ and α-synuclein polypeptides, the existence of annular structures was observed at significantly

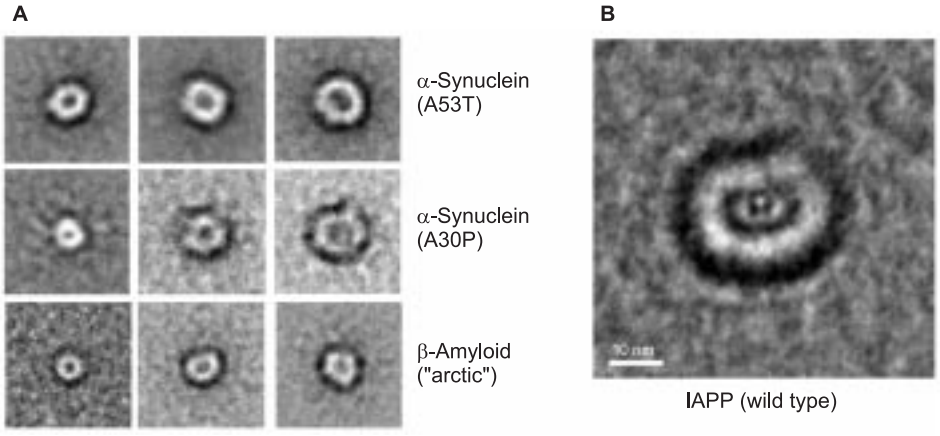

Fig. 2. Common annular structures formed by amyloidogenic polypeptides of unrelated origin. **A**. The formation of annular assemblies is observed with mutant amyloidogenic α-synuclein and β-amyloid polypeptides, which are associated with Parkinson's disease and Alzheimer's disease, respectively. **B.** Remarkably similar structures are formed by islet amyloid polypeptide (IAPP) associated with type 2 diabetes. Field emission gun transmission electron microscopy (FEG-TEM) allowed a clear visualization of the annular nature of the assemblies at high resolution. (Part **A** of the figure was reproduced from Ref. 24 with permission.)

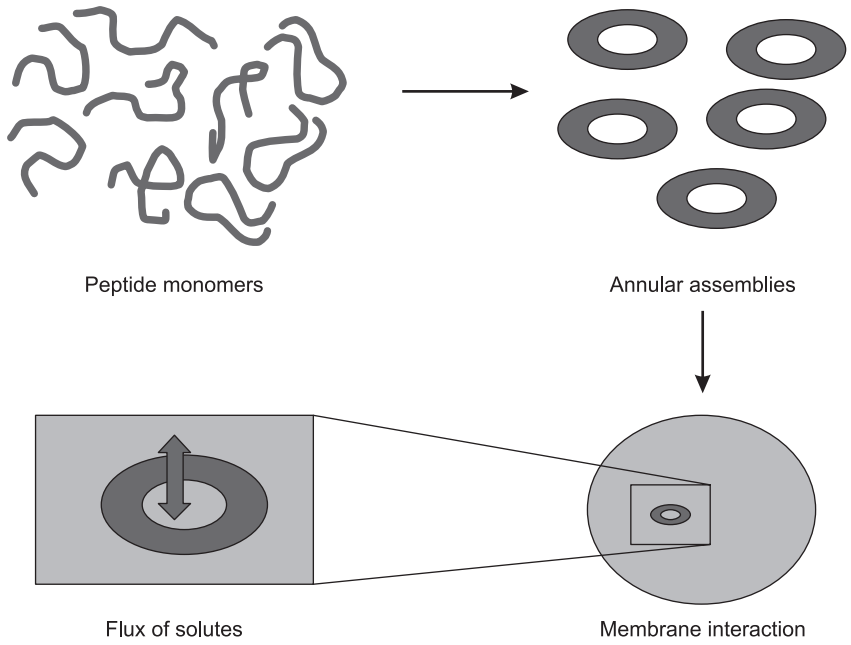

Fig. 3. A model for the cytotoxic activity of the annular prefibrillar structures. Transient annular prefibrillar assemblies, as described in Figure 2, are formed by the assembly of amyloid polypeptide (either from the partially unfolded state, another prefibrillar, or both). In the presence of lipid membranes, there is a partition of the assemblies into the lipid membrane due to high-affinity interactions. The interaction results in the permeation of the cellular membrane, with the final outcome of cellular death.

higher rates with the mutant as compared to the wild-type proteins. The ultrastructural properties of the assemblies and their prevalence preferentially in the mutant population led to the suggestion that the cytotoxic activity of prefibrillar structures, as presented above, is the result of unregulated pore formation upon interaction of the annular assemblies with the cellular membrane (Fig. 3) (22-26). It was therefore suggested that pore formation is similar to that observed with membrane-permeating bacterial toxins (27).

Support for this notion came from the study of the IAPP polypeptide (17). Very similar annular structures were observed with prefibrillar assemblies of the wild-type IAPP polypeptide (Fig. 2) (17). Those structures were transient in nature and were observed at early steps of peptide assembly. Field emission gun transmission

electron microscopy allowed clear visualization of the annular nature of the assemblies at high resolution. Indeed, clear pore-like planar structures were observed. Interestingly, the formation of these structures was clearly temporally correlated with the strong and transient membrane-interacting activity of the soluble fraction of the IAPP assemblies (17, 28), further supporting the membrane-permeating activity of the assemblies. The kinetic properties of membrane activity were concentration-dependent (17), further supporting an assembly process for the formation of the structures. Similar membrane-interacting activity was previously observed with the α-synuclein polypeptide, consistent with a common cytotoxic mechanism (28).

The remarkable similarity between the various planar ring-shaped assemblies is especially intriguing in light of a recent study that utilized an immunological approach to demonstrate a common structure for soluble amyloid assemblies (29). In this study, antibodies were raised against an oligomeric Aβ assembly that mimics the prefibrillar state. The antibodies were able to interact specifically with the prefibrillar form of 6 structurally unrelated amyloidogenic proteins, including the α-synuclein and IAPP polypeptides mentioned above. Furthermore, the antibodies were able to inhibit the toxic activity of the various prefibrillar assemblies.

Further work will be required to characterize the biophysical properties of the annular prefibrillar structure. Information regarding the structural properties of the polypeptide in terms of secondary structure, molecular arrangement of the molecules and exact kinetic properties of association and dissociation is still missing, mainly due to the metastable nature of the systems. Advanced techniques such as near-field scanning probe optical microscopy and high-resolution environmental scanning microscopy may provide new insight into this dynamic process. Such information will be of great value both from the basic scientific point of view and from the practical point of view for the development of future therapeutic agents.

Consequences for therapeutic approaches

In the past, much of the therapeutic attention in the context of amyloid diseases has been directed toward amyloid fibrils. Both drug-based and immunological approaches were aimed at inhibition of the formation of the fibrillar structures, or even their elimination. The current knowledge regarding the cytotoxicity of the early assemblies clearly suggests that attention should rather be directed toward the very early molecular recognition stages that lead to formation of the prefibrillar structures. Furthermore, the disintegration of established fibrils may lead to more damage than cure, as the availability of dissolved monomers can direct the formation of new early intermediates. It has even been argued that the fibrils themselves may actually be protective, as fibrillization would be an efficient way for the cell to sequester potentially toxic protofibrils (30). This model is highly relevant for the design of novel therapeutic agents for amyloid-related diseases due to the fact that compounds that inhibit fibril formation may actually result in accumulation of toxic prefibrillar assemblies (30).

We previously suggested that the stacking of aromatic residues may play a role in accelerating the process of amyloid fibril formation (31-35). As amyloid fibril formation is a process of molecular recognition and self-assembly, stacking interactions between aromatic residues may provide both an energetic contribution and directionality and orientation, which is conveyed by the restricted geometry of planar aromatic ring stacking. We further demonstrated that short aromatic dipeptides contain all the molecular information needed for self-assembly into well-ordered nanostructures which are structurally related to amyloid fibrils (36). Indeed, a number of studies have demonstrated the ability of remarkably simple unrelated aromatic molecules, such as nicotine (37), acetylsalicylic acid (38), tetracycline (39) and bis-1-anilinonaphthalene-8-sulfonate (40), to inhibit amyloid fibril formation. Further work should be done to determine the specific effect of these molecules on the early stages of recognition as compared to on elongation of full-length fibrils.

Conclusions

Transient prefibrillar assemblies are formed by amyloid-related polypeptides. The formation of such structures has been observed with the Alzheimer's disease-related β-amyloid polypeptide, the Parkinson's disease-related α-synuclein polypeptide and the type 2 diabetes-associated islet amyloid polypeptide. The assemblies are transient, have a remarkably similar ultrastructure and show potent membrane-interacting and -permeating properties. The morphology and biophysical properties of the assemblies are consistent with a membrane pore formation mechanism of toxicity. Together, these findings indicate that future therapeutic approaches should be directed towards the early stages of molecular recognition events.

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