Amyloid Structure and Properties and Its Relation to Human Diseases

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Abstract

Amyloid – a fibrillar, cross β-sheet quaternary structure –was first discovered and associated with a great variety of human diseases (Alzheimer's, Parkinson's, prion, diabetes, cataracts, etc.). It is believed that the misfolding and aggregation of amyloid proteins are responsible for the appearance and progression of these diseases. Protein aggregation is a highly complex process resulting in a variety of aggregates with different structures and morphologies. Oligomeric protein aggregates (amyloid oligomers) are formed as both intermediates and final products of the aggregation process. They are believed to play an important role in many protein aggregation-related diseases, and many of them are highly cytotoxic. Due to their instability and structural heterogeneity, information about structure, mechanism of formation, and physiological effects of amyloid oligomers is sparse. Here we review the molecular properties of amyloid proteins and relate them to the pathological conditions and the appearance of various diseases. We show how the structure of the amyloid protein at different hierarchical levels (from backbone to fibrills) is representative to the pathological changes that appear at the disease and how it can be potentially be employed to monitor the disease progression. We also review the cytotoxicity of the amyloid proteins and discuss how it might be related to the structure. In conclusion, we delineate the intervention strategies that prevent amyloid formation.

Introduction

Misfolding and aggregation of polypeptides and proteins is a central pathological and biochemical event shared by many neurodegenerative disorders, s such as Alzheimer's, Parkinson's, Huntington's diseases, as well as other human diseases, such as prion diseases, type II diabetes and systemic amyloidosis[1-3]. In addition, protein aggregates are believed to be involved in several physiological processes such as hormone storage[4] and memory formation[5-7]. Furthermore, many biologically important proteins act in a form of specific homo- or hetero-oligomers.

Protein aggregates are usually either classified as amyloid fibrils (structures in which the polypeptides are organized into cross-β sheets), amorphous aggregates, or soluble aggregates often generically described as amyloid oligomers. Amyloid oligomers are defined as soluble non-monomeric structures that appear as intermediates or final products in the process of protein aggregation lacking one or more of the hallmarks of fibrillar structure. They are highly heterogeneous in size, structure, stability and morphology. Here we will describe amyloid oligomers based on their major properties.

Structure of Amyloid Fibers.

I. Structure of Amyloid Spine

Amyloid is a fibrous quaternary structure formed by the assembly of protein or peptide monomers into intermolecularly hydrogen bonded β-sheets that display the cross-β fiber diffraction pattern as shown in Figure 1A[1,8]. In amyloid state, the elongated fibers are formed, with spines consisting of many-stranded β sheets; amyloid fibers share a common ''cross-β'' spine.

Determining the atomic details of the cross-β spine is complicated since the limited order of fibrils presents challenges to crystallographic, nuclear magnetic resonance (NMR), and electron microscopy (EM) methods. But important features have gradually emerged from studies by solid-state NMR[9,10], model-building constrained by X-ray fiber and powder diffraction[11,12], site directed spin labeling[13-16], cryo-EM, scanning mutagenesis[17] , and single-crystal X-ray diffraction[18]. The most general points to emerge are that

1. In all amyloid fibers, the strongest repeating feature is a set of β sheets that are parallel to the fibril axis, with their extended strands near perpendicular to the axis.

2. The β sheets can be either parallel or antiparallel, that is, adjacent hydrogen-bonded β strands within a sheet can run in the same direction or in opposite directions.

3. The sheets are usual ''in register,'' meaning that strands align with each other such that identical side chains are on top of one another along the fibril axis. In parallel sheets, identical side chains are separated by an interstrand distance of 4.8 Å (Figure 1), and in antiparallel sheets, they are separated by 2 × 4.8 Å= 9.6 Å.

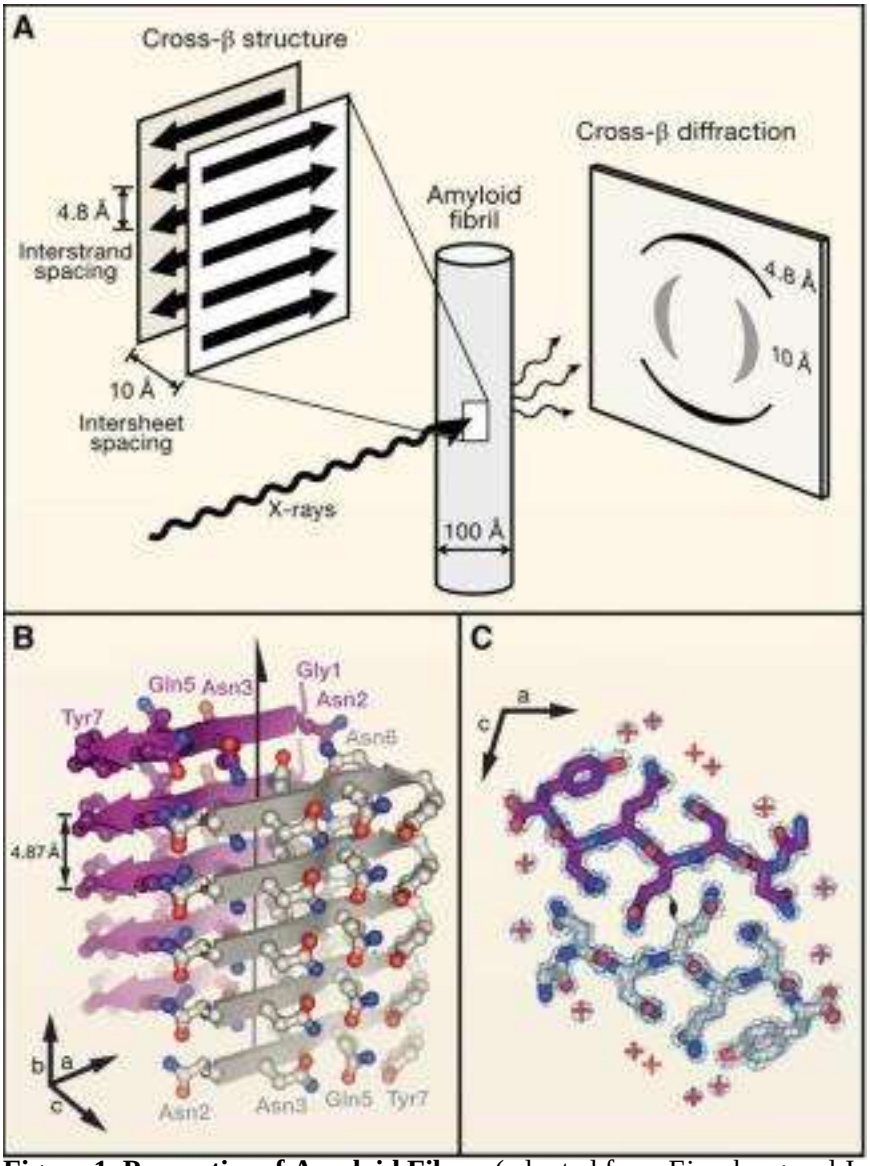

Figure 1. Properties of Amyloid Fibers (adopted from Eisenberg and Jucker, 2010; Nelson et al., 2005)
(A) The characteristic cross-β diffraction pattern observed when X-rays are directed on amyloid fibers. The diffuse reflection at 4.8 Å spacing along the meridian (vertical) shows extended protein chains running roughly perpendicular to the fibril and spaced 4.8 Å apart. The even more diffuse reflection at ~10 Å spacing along the equator (horizontal) shows that the extended chains are organized into sheets spaced ~10 Å apart. For less well-oriented fibrils, both reflections blur into circular rings.

(B) The steric-zipper structure of the sequence segment GNNQQNY from the yeast prion Sup35. Five layers of b strands are shown of the tens of thousands in a typical fibril or microcrystal. The front sheet shows the protein backbones of the strands as gray arrows; the back sheet is in purple. Protruding from each sheet are the sidechains. The arrow marks the fibril axis.

(C) The two interdigitating b sheets are viewed down the axis. Water molecules, shown by red + signs are excluded from the tight interface between the sheets. Red carbonyl groups and blue amine groups form hydrogen bonds up and down between the layers of the sheet (Nelson et al., 2005).

The architecture of at least the simplest cross-β amyloid spines has been clarified by determining short segments of amyloid-forming proteins[19-23]. The segments examined are those that seem to be the adhesive parts of amyloid proteins. In isolation from the rest of the protein, they form microcrystals and related fibers with morphological similarity to fibers of the entire parent proteins[24]. The atomic structures of the microcrystals reveal that the motif of the amyloid protofilament consists of a pair of β sheets that run the length of the fiber-like crystals (Figure 1B). Each sheet is a standard Pauling- Corey β sheet, in which each strand is hydrogen bonded to the strand above and below it through its backbone amide groups. When the side chains contain amides (glutamine and asparagine), those amides also form hydrogen bonds to the identical residue in the strands above and below. This creates parallel arrays of hydrogen bonds running along the fiber axis. The electrostatic interactions of all of these aligned hydrogen bonds mutually polarize one another, producing hydrogen bonds even stronger than those in ice[25]. The stability of such interdigitated β sheets explains the persistence of amyloid fibers and prions. Within the protofilament, the side chains emanating from the two sheets are tightly interdigitated, as shown in Figure 1C, like the teeth of a zipper. The interface between the sheets is devoid of water, and hence this motif has been termed the ‘‘dry steric zipper.’’ Dozens of atomic structures of dry steric zippers have been determined by X-ray crystallography and share the following properties:

1. Steric zippers form from self-complementary amino acid sequences, in which their sidechains can mutually interdigitate. The sequences can be polar or non-polar, with large side chains or small, but they fit together in complementary fashion.

2. Steric zippers have dry interfaces between the two sheets. Thus, the hydrophobic effect contributes to amyloid stability, as does the strong hydrogen bonding.

3. The β strands are most often in register, maximizing interstrand hydrogen bonding and permitting stacking of glutamine (Gln), asparagine (Asn), and tyrosine (Tyr) residues. Although all steric zippers are expected to be formed from complementary sequences, the sequences do not need to be self-complementary. There is strong evidence from solid-state NMR studies[26,27] that in Aβ, some close interactions are between b strands that differ in sequence (Figure 2). Such ‘‘heterosteric zippers’’ have not yet been observed in X-ray crystal structures.

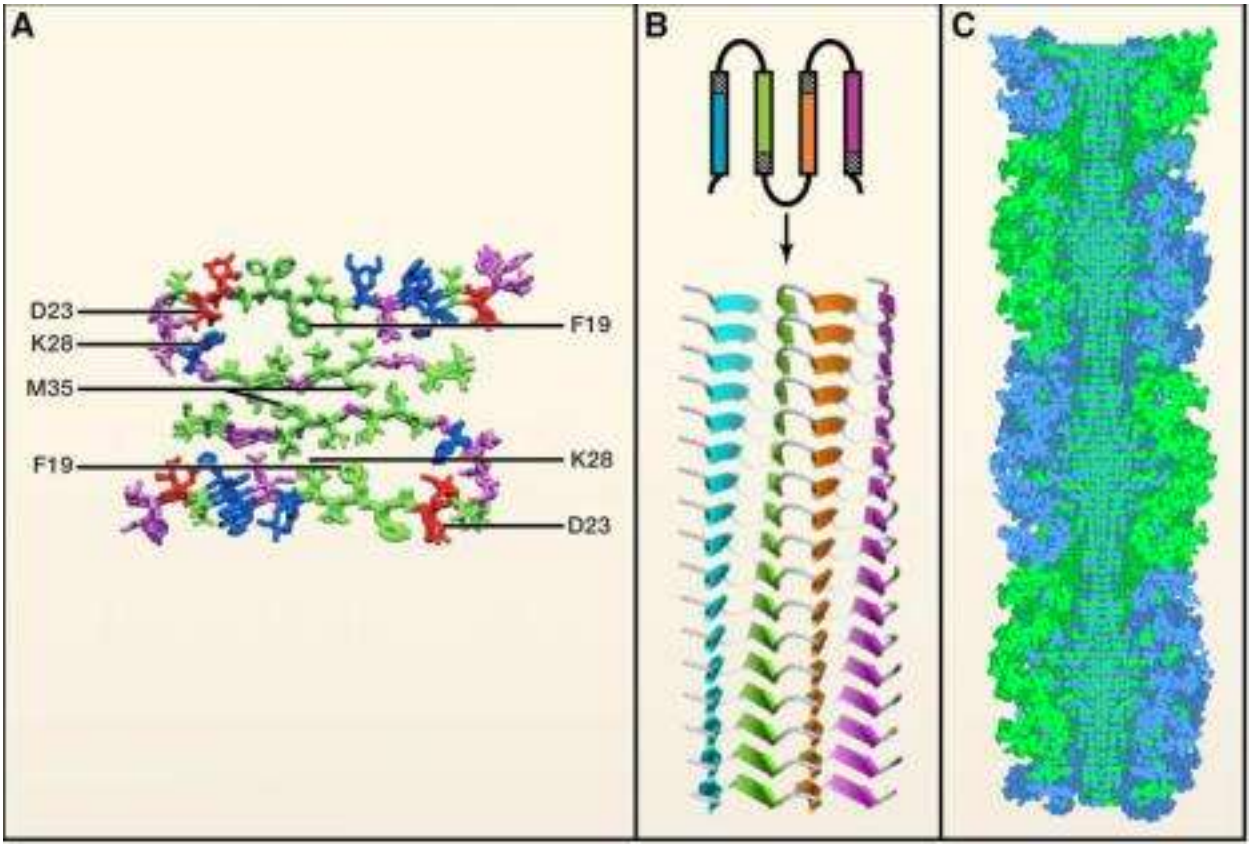

Figure 2. Models for Amyloid Fibrils Larger than a Single Steric-Zipper Spine (adopted from Eisenberg and Jucker, 2010)
(A) Model for Aβ1-40 based on solid-state NMR data with additional constrains from electron microscopy (Tycko, 2011). The view is down the fibril axis, showing two molecules of Aβ, each with a U turn or ‘‘β arch.’’ Where the green segments of the two molecules abut, they appear to form a homosteric zipper, and a heterozipper could exist between the two arms of each U. Both types of steric zipper need to be confirmed by higher-resolution structures.

(B) A proposed structure for longer amyloid proteins is a ‘‘superpleated b structure’’ (Kajava et al., 2010), in which the protein chain forms several U turns/β arches. The view of the upper diagram is down the fibril axis; the view of the lower is perpendicular to the fibril axis. In the lower diagram, each protein chain is hydrogen bonded to the ones above and below. Heterozippers may exist between pairs of differently colored β strands. This type of structure has been proposed for several proteins in the amyloid state including Ure2p, Sup35p, and α-synuclein.

(C) A model for a designed amyloid of ribonuclease A with ten glutamine residue inserted between the core and C-terminal domains (Sambashivan et al., 2005) based on X-ray and electron microscopy data and steric constraints. The view is perpendicular to a cut-away of the fibril. The twisting steric zipper can be seen at the center. Globular subunits of ribonuclease A, which are essentially in their native conformation, are at the periphery. The amyloid-like fibrils of this designed amyloid show enzymatic activity, confirming that ribonuclease molecules retain native-like structure.

The most common sheet-to-sheet arrangement for steric zippers is face to face (Figure 1B), but other arrangements occur (Figure 3). In these other arrangements, the two sheets can be face to back (classes 2 and 4), pack with opposite edges up rather than

both edges up (class 4), or contain antiparallel strands (classes 5–8), rather than parallel strands (classes 1–4)[21]. To date, no examples in class 3 have been observed.

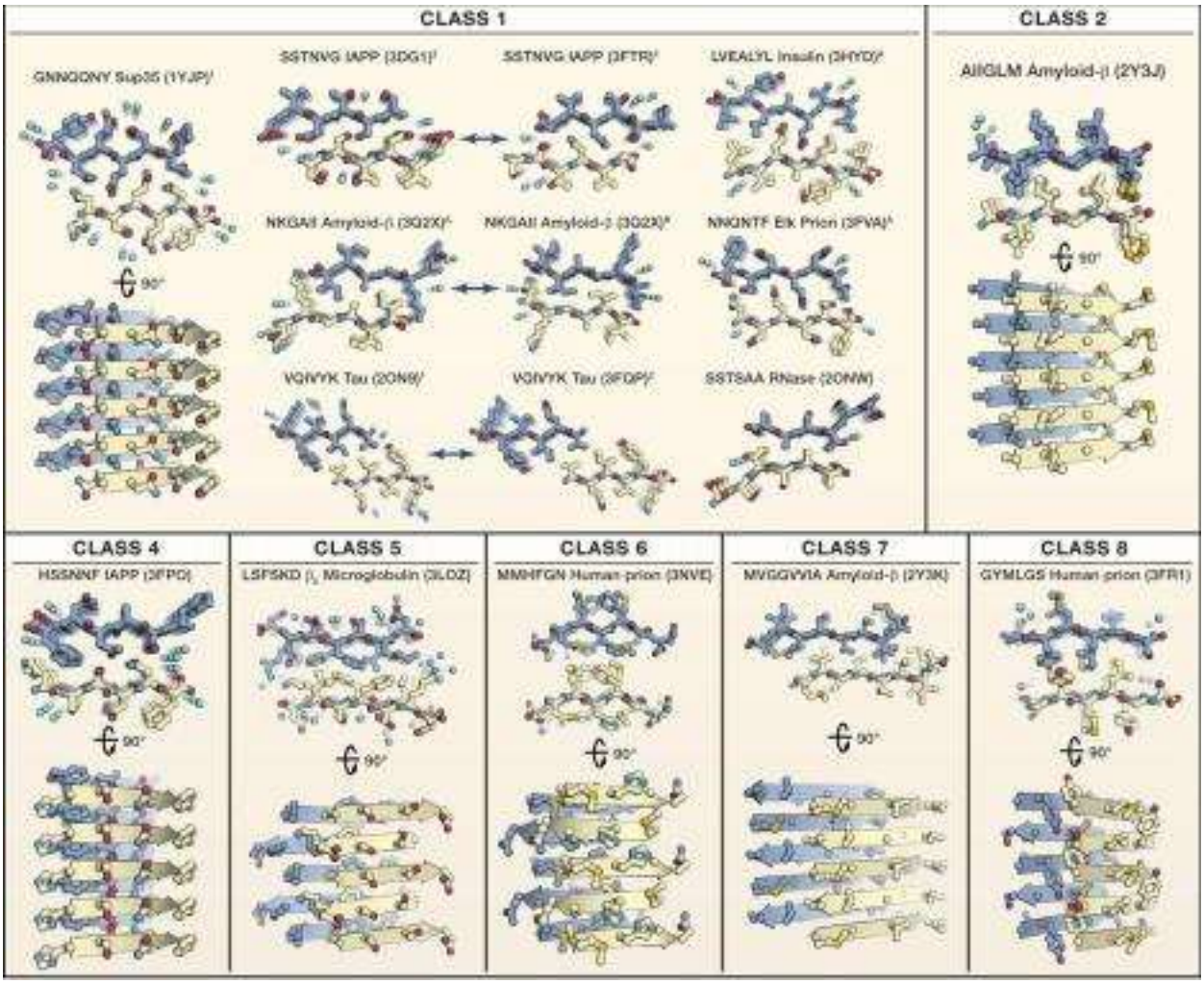

Figure 3. Steric-Zipper Protofilaments (adopted from Eisenberg and Jucker, 2010)
Twenty-eight atomic structures of steric-zipper protofilaments from amyloid-forming proteins, determined by X-ray diffraction. All are viewed projected down the protofilament axis, revealing the two sheets (one ivory and one blue) with their interdigitated sidechains. Selected zippers are also viewed perpendicular to the protofilament axis, with five layers of β strands shown with backbones as arrows. Water molecules are shown as aqua spheres; notice their absence from the interfaces between the paired sheets.

Some amyloid spines are more complex than single steric zippers. For instance, several different steric zippers all formed by the same protein can occur in the spine. In fact some 13 different steric zippers have been found for the 42-residue sequence of Aβ[28]. Many proteins, including have several potential steric zipper-forming segments within their sequences. Thus, Lewandowski et al.[29] provide solid-state NMR evidence that fibers of the yeast prion Sup25 contain three distinct steric zippers (one is shown in Figure 1B).

A second source of increased complexity is the likelihood of hetero-zippers formed from cross-complementary sheets. Heterozippers have been found by solid-state NMR[30]

(Figure 4). This structure, termed a solenoid by its discoverers, consists of a stack of two-layer protein loops. Each loop contains two extended strands with their side chains interdigitating in a similar manner as those in a steric zipper. Each molecule of contributes two such loops that stack on top of each other. This pair of loops then stacks on top of, and beneath, pairs of loops from its adjacent molecules in the fiber. The entire structure is amyloid like. Hetero-zippers probably are also found in spontaneous aggregates of proteins, such as those of Aβ, but they have not yet been fully defined at high resolution.

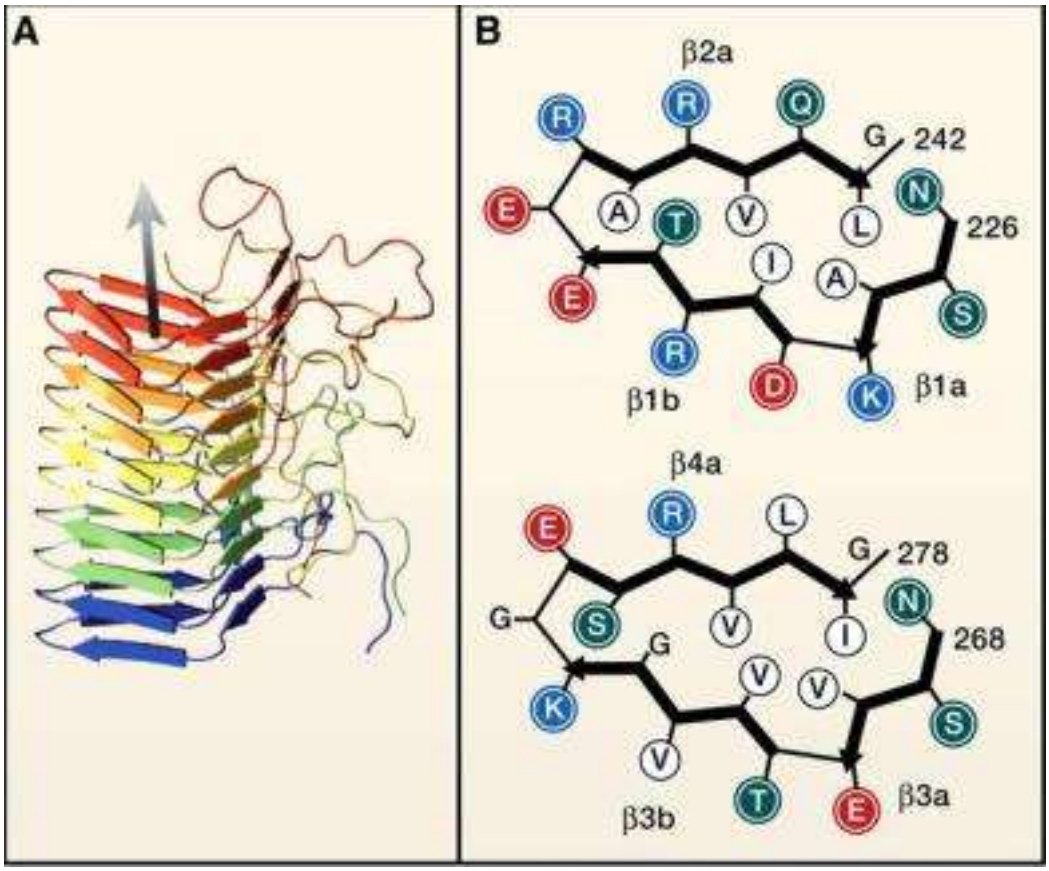

Figure 4. Structure of a Heterozipper (adopted from Eisenberg and Jucker, 2010; Wasmer et al., 2008)
The solid-state NMR-derived structure of Het-s shows heterozippers.
(A) The protein chain of each molecule (in a single color) contains six β strands, organized in double loops. The double loops of adjacent molecules sit on top of one another, hydrogen bonded up and down.

(B) The two layers are shown schematically with sidechains represented as circles. Each layer may be regarded as a hetero-zipper, in which the sidechains of opposing strands interdigitate.

II. Structure of Amyloid Fibrils

Full amyloid fibrils are more complicated than the simple spine structures; some fibrils appear to contain numerous protofilaments that are complex[29,31].

The findings about amyloid spines place severe constraints on fibril models. Given that proteins stack in register with strands spaced 4.8 Å along the fibril axis, the rest of the protein must be flattened out so that each

layer is only 4.8 Å high or it must somehow sit at the periphery of the spine, where it may extend more than 4.8 Å to avoid overlap with identical domains. A flattened model for Aβ is shown in Figure 2A, based on solid state-NMR measurements[10]. Each Aβ molecule makes a U-turn, called a β arch[32]. For longer proteins, it has been proposed that U turns are linked into a serpentine structure, termed a super-pleated β structure[32] (Figure 2B). In a super-pleated β sheet, the entire protein chain is flattened to fit in one 4.8 Å layer of the fibril.

Flattening is not necessary for an amyloid-forming protein to retain globular domains. In the model of a designed amyloid of RNase A (Figure 2C), the domains on the periphery of the spine, find space to retain their globular structure[33]. For larger globular domains, a greater circumference of the fibril and a longer protein linker to the steric zipper is required. This means that fibers formed from larger proteins would be expected to have greater diameters. Although the spines of amyloid fibers appear similar, fibrils show a great variety of structural complexity.

The observation that amyloid fibrils have spines composed of steric zippers explains why different proteins, when they enter the amyloid state, give fibrils of similar appearance in electron micrographs. The fibrils are all elongated and unbranched, just as their steric-zipper spines. The diameters of the fibrils vary because the lengths of the proteins that form them differ as well as the number of protofibrils that twist around each other to form the fibril. Thus, we would expect that cross-seeding of amyloid fibril formation in which the seed is formed from another, but similar, amyloid fibril is possible. All steric zippers formed from parallel

β strands have the same repeat – 4.8 Å in the fibril axis direction; similarly, all antiparallel zippers have repeat of 9.6 Å in the fibril axis (Figure 1). If the seeding steric zipper is complementary in shape to a segment of the seeded protein in solution, we could expect a hetero-steric zipper to form and to serve as a nucleus, as has been shown *in vitro* for Aβ[34].

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In human neurodegenerative diseases, the co-existence of more than one amyloid deposit is a common observation. For example, in Parkinson-related diseases, α-synuclein and τ-inclusions can occur in the same cell and form common inclusion bodies[35]. Although cross-seeding provides an attractive explanation for these observations[35,36], definitive proof is lacking, and other explanations are possible. For example, two amyloid deposits may simply develop independently of each other or there may be saturable cellular fractions for the removal of misfolded proteins and one aggregated protein may indirectly stimulate aggregation of the others by monopolizing clearance mechanisms. Also, colocalization of two amyloids is only apparent at the light-microscopic level and reflects common cellular niches prone to protein aggregation, while at the ultrastructural level, true co-aggreagtion of the two amyloids may not occur. Other observations indicate that the interaction of amyloidogenic proteins in human brain can impede, rather than promote, aggregation.For example, cystatin C co-localizes with Aβ plaques in

Alzheimer's disease, but the finding that cystatin C reduces Aβ plaque formation suggests a mechanism of cross-inhibition rather than cross-seeding[37].

III. Conformational Structure of Amyloid Strains

Three models for the molecular basis of prion strains and amyloid polymorphs have been proposed on the basis of atomic structures of amyloid-like fibers (Figure 3). The models suggest that strains are based in distinct steric-zipper spines of the associated amyloid fibers. The first model is termed packing polymorphism and is illustrated in Figure 3 by the pairs of zippers connected by double-headed arrows. In packing polymorphism, an amyloid segment packs in two or more distinct ways, producing fibrils with different structures and distinctive properties. The simplest form of packing polymorphism is a registration shift in which the two sheets forming the steric zipper in the second polymorph shift their inter-digitation from that in the zipper of the first polymorph. Because the nature and position of the side chains, the outer surface of the fibers differ in the two polymorphs, the properties of the fibers must be different[23,38]. Thus, in packing polymorphism, one sequence forms two or more ''conformations.''

The second structural model for strains is termed segmental polymorphism. In segmental polymorphism, two or more different segments of an amyloid protein are capable of forming steric-zipper spines. Figure 3 shows two segments from Aβ that form different steric zippers. Fibrils formed with different steric zipper spines will each have distinctive properties. Proteins particularly rich in different segments able to form steric zippers include Aβ[28], IAPP[23] and PrP[21].

In a third type of amyloid polymorphism, heterosteric zippers, the zipper is formed from the inter-digitation of non-identical β sheets. Though not yet seen in X-ray structures at the atomic level, heterotypic interactions between sheets are observed in the constrained models derived from solid-state NMR and cyro-EM[10,16,38]. The existence of such hetero-amyloid spines, in addition to self-complementary spines, greatly increases the number of potential amyloid polymorphs and prion strains. The hypothesis that distinct steric-zipper structures are at the basis of amyloid fiber polymorphism and prion strains is consistent with other observations about steric zippers. Steric zippers can be extremely stable[24]. Thus, steric zippers share with prion strains robust ''conformations'' that can conceivably be transmitted from cell to cell or organism to organism. Another similarity between steric zippers and prion strains is that environmental conditions seem to affect the formation of both[39]. Similarly, the differing steric zippers formed from the same protein segment in Figure 3 were created by incubating the segments under different solution conditions.

IV. Amyloid Morphology

In the brain, Aβ deposits are heterogeneous in histopathological appearance and biochemical composition, both within and among brain regions and patients[40-42]. Aβ aggregation can occur in association with the vasculature or in the brain parenchyma

as amyloid plaques. Point mutations within the Aβ sequence can lead to vascular amyloid, amyloid plaques, or both[43]. Vascular and parenchymal Aβ deposits differ in the ratio of deposited Aβ ending at amino acid 40 to Aβ ending at amino acid 42[43]. Plus, the Aβ40:Aβ42 ratio has been linked to different neurotoxicities and clinical Alzheimer's disease onset[44-46]. In addition, Aβ displays length variations due to truncations at the N terminus (e.g., Aβ starting at residue 3, 11, or 17) and variations in post-translational modifications (e.g., isomerization, pyroglutamyl formation, phosphorylation, or nitration). All these factors can profoundly influence Aβ aggregation and histopathological appearance of the amyloid[41,47-50]. A predominance of N-truncated and post-translationally modified Aβ distinguishes Aβ deposits in Alzheimer's disease compared to normal aging[51,52].

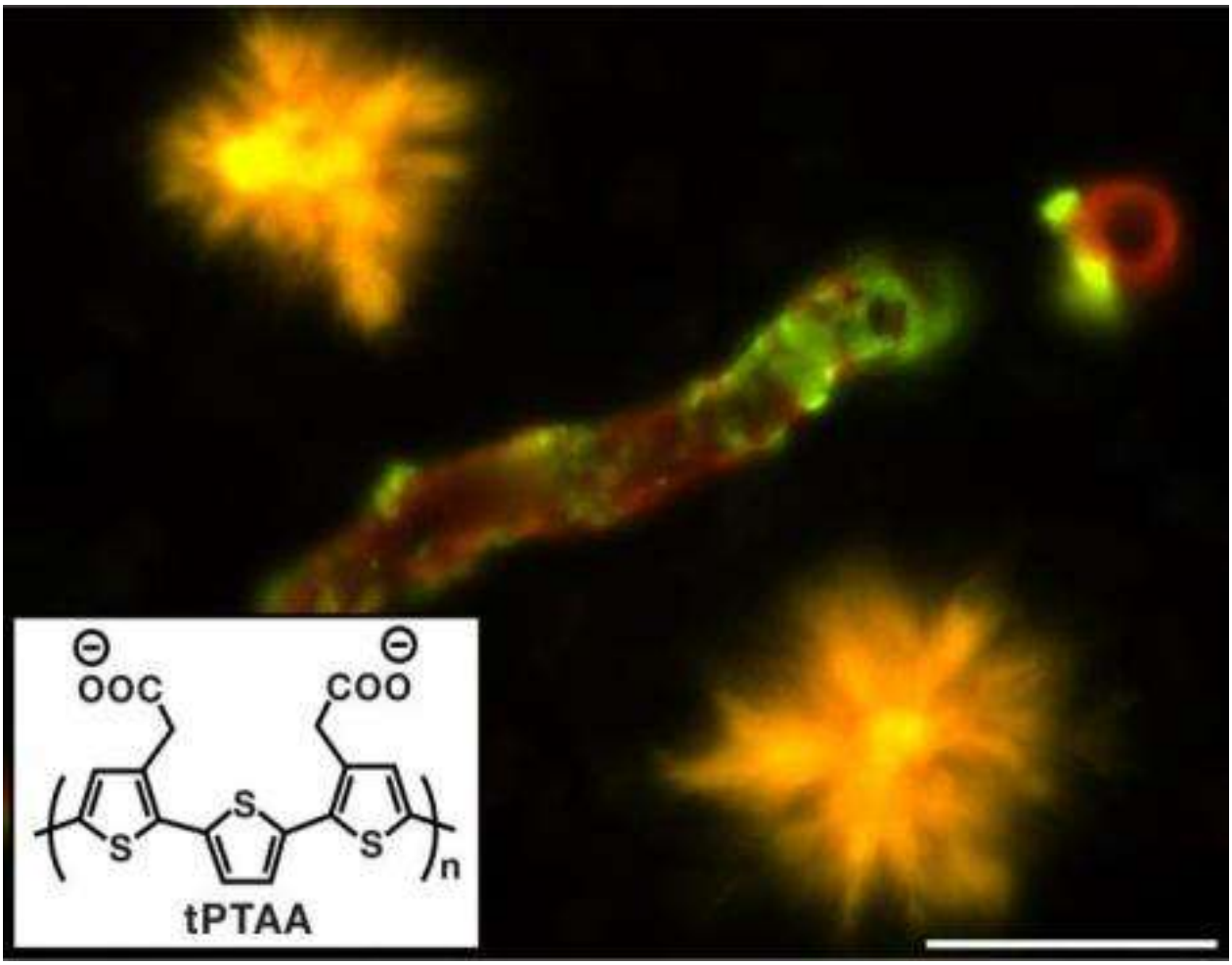

Figure 5. Rainbow Amyloid (adopted from Eisenberg and Jucker, 2010; Yamada et al., 1987)
Novel amyloid dyes can be used as surrogate probes of the supramolecular structure of protein aggregates. Shown are Aβ plaques (yellow) and Aβ amyloid angiopathy (green) in an AβPP-transgenic mouse (carrying the A β PP Swedish and AβPP Dutch mutation). Note the different spectral signatures upon staining with the luminescent conjugated polythiophene tPTAA (bottom left). The image was recorded using a combination of green and red filters. The scale bar represents 20 μm.

Although it remains difficult to study the conformational state of Aβ *in vivo*, indirect measures with luminescent conjugated polythiophene probes that detect particular amyloid conformations suggest the occurrence of conformationally distinct Aβ deposits in brain[53] (Figure 5). Different Aβ morphotypes in the brain may indicate that local factors influence the Aβ aggregates. They may also represent various stages in the disease[42] or reflect the templated propagation of conformationally distinct seeds[54]. Although these possibilities are not mutually exclusive, they suggest that the characteristics of prion strains may also apply to multimeric Aβ. However, the link between Aβ conformational variants and distinct clinical subtypes of β amyloidoses is still lacking.

Hetereogeneous amyloid morphotypes are also observed in other amyloidoses. Strikingly, amyloid heterogeneity is associated with the organ tropism (i.e., that the amyloid preferentially deposits in particular organs) and clinical manifestation of amyloidoses[55]. Similarly, length variants of the AA protein characterize two different histopathological AA amyloid patterns in the kidney with distinct clinical phenotypes[56]. In the brain, τ and α-synuclein inclusions reveal histopathological heterogeneity that is diagnostic of the various tauopathies and α-synucleinopathies, respectively[57]. Consistently, α-synuclein and τ-fibrils *in vitro* exhibit conformational diversity [58,59]. Although recent studies have reported the remarkable transmission of disparate proteopathic lesions, solid evidence for the hypothesis that the heterogeneous disease phenotypes are the result of the prion-like templated conversion of conformationally distinct TTR, AA, τ, and α-synuclein seeds has not been found as yet.

V. Amyloid Toxicity (cited from Eisenberg and Jucker, 2012)

Not all amyloids are toxic. First described in bacteria, fungi, and yeast, and more recently in mammals, amyloids can function in the formation of biofilms, the binding and storage of peptide hormones, the formation of melanin formation, or the launch of an antiviral innate immune response[4,60-62]. The type of amyloid and the controlled growth conditions may account for the lack of toxicity of so-called functional amyloids[38,63]. However, most amyloid formation in mammals occurs with aging and is associated with diseases commonly referred to as protein misfolding diseases, aggregation diseases, proteopathies, or, more specifically, amyloid diseases or amyloidoses[1,64]. An association of a given amyloid with a disease does not necessarily denote causality. However, a causal relationship between the amyloid formation and amyloid toxicity is suggested from familial cases in which a pathogenic mutation leads to an overproduction of the amyloidogenic protein or enhances the propensity of the protein to aggregate. It remains unclear which step of the amyloid formation cascade is toxic, and this step may be different for the various amyloid diseases.

Amyloid toxicity can result from losing the function of a protein or from the sequestration or mislocation of other proteins[65]. For most amyloid diseases, a gain of toxic function remains a favored hypothesis. Despite the longstanding knowledge that amyloids are associated with disease[66], we still lack a clear understanding of how amyloids lead to dysfunction, aside from the instances in which amyloids disrupt tissue structure and organ function via simple mass action[67,68]. This mass action mode of toxicity may well be the most important one for systemic amyloidoses and for the amyloid associated with the cerebral vessels (cerebral amyloid angiopathy, CAA) (Figure 6). CAA of various types (Aβ, ADan, British amyloid [ABri], and Cystatin C amyloid

[ACys]) all result in a thickening of the vascular basal lamina, loss of smooth muscle cells, perivascular inflammation, and, eventually, vessel wall rupture and hemorrhages[69]. Similar appearances and toxicities of CAA (independent of the amyloid type) are also seen in Aβ- and ADan-species[70,71]. Moreover, correlations between CAA severity and hemorrhage frequency was found in humans and mouse models, suggesting that the mass of amyloid fibrils may be the most important parameter mediating vascular toxicity[72-74].

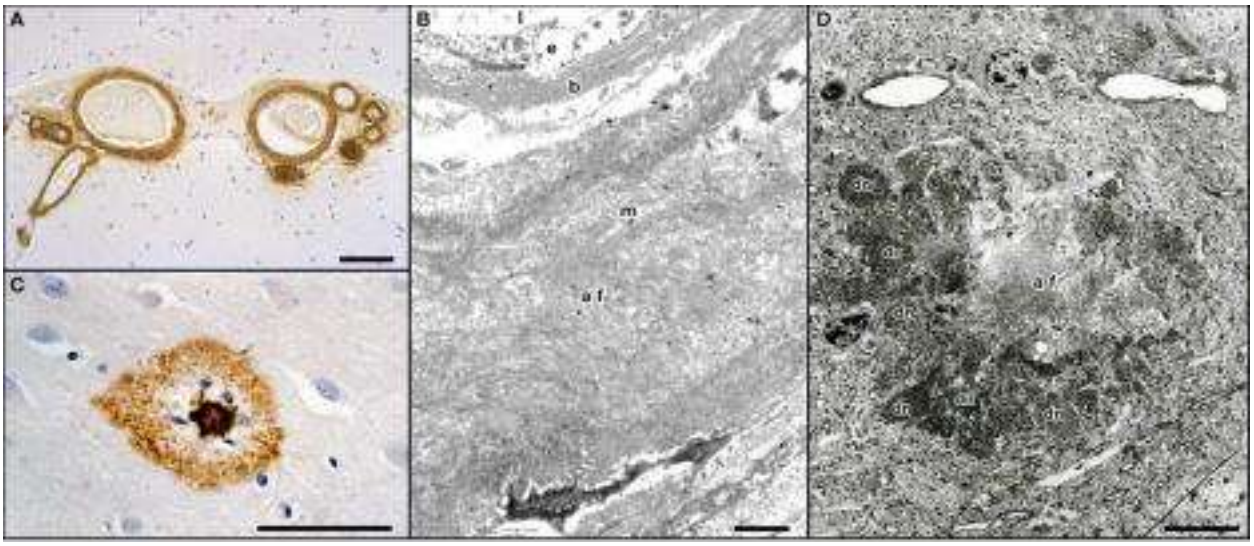

Figure 6. Histopathology of Cerebral β Amyloidosis (adopted from Eisenberg and Jucker, 2010)
(A) Aβ immunostaining (brown) reveals severe cerebral amyloid angiopathy (CAA) in superficial cortical vessels in a human case.

(B) Ultrastructural analysis of Aβ fibrils (af) in the vessel wall of an arteriole with CAA. Note that the amyloid has displaced nearly the entire vascular wall, disrupting normal vessel-neuron communications (b, basal lamina; e, endothelial cells; l, lumen; m, media).

(C) Aβ immunostaining (brown) of an amyloid plaque in a human Alzheimer's disease case. Note the dense amyloid core and glial nuclei (blue) surrounded by a halo of diffuse Aβ immunostaining.

(D) Ultrastructure of an Aβ plaque. Note the dense amyloid core with the amyloid fibrils (af) surrounded by numerous dystrophic neuritis (some are labeled with ''dn''). The Aβ plaque is from an AβPP-transgenic mouse brain due to better tissue preservation compared to postmortem human tissue. Scale bars represent 100 mm (A), 1 mm (B), 50 mm (C), and 5 mm (D).

Other amyloid deposits may not be the predominant toxic entity. In Alzheimer's disease autopsy material, the soluble Aβ species correlate more strongly with the degree of dementia than does the mass of Aβ plaques[75]. Indeed, a variety of soluble Aβ multimeric species (e.g., dimers, timers, dodecamers, and larger oligomers) have been isolated from the Alzheimer's disease brain, and they induce synaptic toxicity and dysfunction, both in cell culture and when injected into the rodent brain[76,77]. Similarly, synthetic, multimeric Aβ appears to be more toxic than Aβ monomers or fibrils[75,78,79], but it is often unclear how the synthetic Aβ species relate to the *in vivo* counterparts[80,81]. Also, for τ and α-synuclein, soluble oligomeric species appear to be more toxic than the corresponding amyloid fibrils[75,82,83].

The physicochemical properties of the toxic oligomeric species are not well understood, and a consistent nomenclature is needed [84]. It is generally assumed that the greater toxicities of oligomers are mediated by their unique structural features[85]. The higher relative toxicity of small soluble oligomeric species, however, may also mirror the greater diffusion capability of such small aggregates through the tissue and into various compartments. Along the same lines, the relatively lower toxicity of amyloid fibrils may reflect the fact that many of the toxic structural entities of the fibril are buried in the amyloid mass[75,86].

For Aβ toxicity, both receptor-mediated interactions and nonreceptor-mediated membrane interactions have been described[87,88]. The most significant toxicity of Aβ is toward the synapse. This is consistent with the profound loss of synapses in the Alzheimer's disease and the observation that oligomeric Aβ species inhibit LTP, an electrophysiological correlate of memory formation[89]. Soluble Aβ species bind to post-synaptic structures and interact with various putative ligands, such as PrP, NMDA receptor, EphB2, or downstream signaling events[90-94], but their *in vivo* relevance for Alzheimer's disease pathogenesis is still unclear. Non-receptor membrane cytotoxicity for Aβ has been suggested through the insertion of Aβ oligomers into membranes, resulting in membrane disruption, possibly with the formation of cationsensitive ion channels and dysregulation of calcium homeostasis[87,95]. Similar observations have been made with other oligomeric amyloid intermediates, suggesting that membrane disruption may be a more general mechanism in which amyloidogenic proteins exert their toxicity[95-97]. For example, Aβ plaques are responsible for local neuritic dystrophy (Figure 6), gliosis, and can eventually lead to disturbed neural network activity[98,99]. Aβ plaques may also serve as a source of the more toxic and soluble Aβ assemblies, consistent with the view that a dynamic continuum of the various amyloid intermediates, not a given protein entity, elicits toxicity[100-103].

Mechanisms of Amyloid Fibril Formation

Elucidating the mechanisms of the amyloid fibril formation is fundamental to understand fibrillogenesis and to identify assembly steps that could be therapeutic targets. Influential early investigations promulgated the idea that Aβ assembly was a specific example of the general class of nucleation-dependent polymerization reactions (Figure 7A, B). These reactions comprise a slow nucleation step, producing a "lag phase" during assembly monitoring, followed by a rapid fibril elongation step. Operating within this paradigm, nucleation kn and elongation ke rate constants for Aβ fibril formation were determined[104]. However, continuing elucidation of this ostensibly classical polymerization process revealed unexpected complexity in the numbers and types ("on-pathway" or "off-pathway" for fibril formation) of assembly paths and the structures.

Protofibrils, Paranuclei, and Monomer Folds

Figure 7C illustrates one pathway of fibril assembly. The penultimate fibril intermediate, the protofibril, was first

identified more than a decade ago[105]. Protofibrils were described as beaded chains, each bead of which was ~ 5 nm in diameter. The length of these structures generally was < 150 nm. Kinetics and solution-phase AFM experiments showed that protofibrils matured into fibrils[105]. To understand how protofibrils forme, methods to determine quantitatively the oligomer size distribution in nascent Aβ preparations were developed[106]. In Aβ42 assembly, these experiments suggested that a pentamer or hexamer, the "paranucleus," was the basic unit of the protofibril and that the beaded chains comprising protofibrils formed by the self-association of paranuclei. To understand the oligomerization process in atomic detail, computer simulations have been done[107]. These studies yielded oligomer frequency distributions similar to those determined experimentally, but in addition provided high resolution conformational information. Aβ40 oligomers were more compact than Aβ42 oligomers due to increased conformational freedom of the Aβ42 N-termini. This suggested that intermolecular interactions among Aβ42 N-termini might facilitate the C-terminal interactions obligatory for fibril formation. The importance of the C-terminus of Aβ in controlling Aβ assembly has also been revealed in experiments involving amino acid substitutions[106]. Systematic alterations in residue 41 side chain hydrophobicity showed that Gly or Ala largely eliminated paranucleus formation, whereas amino acids with hydropathic characteristics had no effect. Elimination of the Ala42 side chain blocked paranucleus self-association, whereas insertion of larger apolar side chains facilitated the process. Similar studies examined Met35 polarity. Thus, oxidation of Met35 had no effect on Aβ40 oligomerization, whereas Aβ42 paranucleus formation was abolished. Interestingly, the modified Aβ42 peptides oligomerized identically to Aβ40. The relative importance of the C-terminus in controlling Aβ assembly was also apparent in studies of Aβ40 and Aβ42 peptides containing substitutions linked to familial forms of AD. These substitutions produced oligomers of higher order when substituted in Aβ40 but had little effect on Aβ42 oligomerization. Removal of N-terminal residues in Aβ42 had no effect on its oligomer size distribution, whereas truncation of either the N-terminal two or four residues of Aβ40 produced higher-order oligomers. This observation was consistent with the aforementioned simulation data that suggested that collapse of the N-terminus of Aβ40 on the oligomer surface might shield underlying hydrophobic regions of the oligomers that otherwise might interact to form higher-order assemblies[107]. In fact, this process was observed in studies of the folding and assembly of urea-denatured Aβ[108]. Aβ40 formed an unstable but largely collapsed monomeric species, whereas Aβ42 existed in a trimeric or tetrameric state[108]. The solvent inaccessibility of the Ala21–Ala30 region of Aβ likely results from the formation of a turn-like structure that nucleates monomer folding[109]. This decapeptide region initiallywas identified due to its resistance to proteolysis, a resistance that remained in the isolated deca-peptide itself and that allowed NMR and computational determinations of its structure and dynamics[109]. Most recently, thermodynamics studies showed that the turn is destabilized by amino acid substitutions that cause AD[110].

Destabilization correlates with accelerated Aβ oligomerization and higher-order assembly and thus provides a mechanistic explanation for these familial forms of AD.

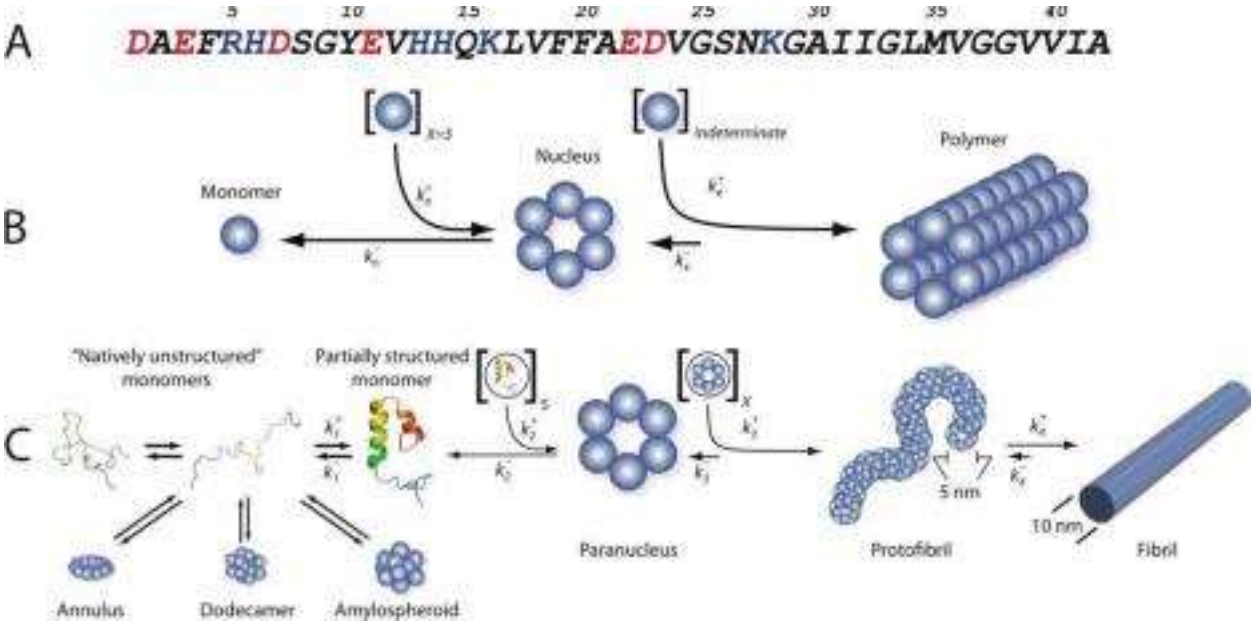

Figure 7. Aβ assembly (adopted from Roychaudhuri et al., 2009**)**
(A) The sequence of Aβ42 is shown in one-letter amino acid code. The side chain charge at neutral pH is color-coded (red, negative; blue, positive).

(B) Nucleation-dependent polymerization, reflecting the unfavorable self-association (rate constant $k_{n+} << k_{n-}$) of X natively folded monomers (in this case, six total) to form a fibril nucleus and the favorable addition ($k_{e+} >> k_{e-}$) of a large indeterminate number of monomers to the nucleus (nascent fibril) during fibril elongation.

(C) Aβ self-assembly. Aβ belongs to the class of "natively disordered" proteins, existing in the monomer state as an equilibrium mixture of many conformers. On-pathway assembly requires the formation of a partially folded monomer that self-associates to form a nucleus for fibril elongation, a paranucleus (in this case, containing six monomers). Nucleation of monomer folding is a process distinct from fibril nucleation. Fibril nucleation is unfavorable kinetically ($k_{2+} << k_{2-}$), which explains the lag phase of fibrillogenesis experiments, a period during which no fibril formation is apparent. Paranuclei self-associate readily ($k_{3+} >> k_{3-}$) to form protofibrils, which are relatively narrow (~5 nm), short (<150 nm), flexible structures. These protofibrils comprise a significant but finite number (*X*) of paranuclei. Maturation of protofibrils through a process that is kinetically favorable ($k_{4+} > k_{4-}$) yields classical amyloid-type fibrils (~10-nm diameter, indeterminate (but often >1 μm length). Other assembly pathways produce annular pore-like structures, globular dodecameric (and higher order) structures, and amylospheroids. Annuli and amylospheroids appear to be off-pathway assemblies.

Aβ Assembly and Disease

Thus far, we have discussed basic aspects of the physical biochemistry of Aβ assembly. However, the most fundamental biological question is, "what is the relationship between Aβ assemblies and AD?" Strong linkage exists between amyloid formation

per se and disease (for a comprehensive review, see Ref. 111), and this linkage formed, in part, the foundation for the "amyloid cascade hypothesis," which posited that amyloid fibril formation was the key pathogenetic process in AD[112]. As discussed above, elucidation of the mechanisms of fibril formation unexpectedly revealed a broad range of fibrillar and non-fibrillar structures. Aβ oligomers appear to be particularly important because they are potent neurotoxins and are isolable from AD patients, and their concentrations correlate positively with neuropathology *in vivo*. These facts have produced a fundamental paradigm shift resulting in a revised amyloid cascade hypothesis[75,113,114] one that posits the primacy of oligomeric forms of Aβ in AD causation.

A substantial experimental corpus exists demonstrating that Aβ is neurotoxic[88]. However, it recent advances in characterization of protofibrils allowed more structurally precise definition of Aβ that in turn enabled more precise structure-neurotoxicity correlations to be established[78,115]. An important goal of current research is to better define the mechanisms of this toxicity.

Human Diseases Associated with Protein Aggregation (cited from Chiti and Dobson, 2006)

Many diseases are associated with the formation of extracellular amyloid fibrils or intracellular inclusions with amyloid-like characteristics (for the complete review see Table 1 in Ref.1) along with the specific proteins that in each case are the predominant components of the deposits. The diseases can be broadly grouped into neurodegenerative conditions, in which aggregation occurs in the brain, non-neuropathic localized amyloidoses, in which aggregation occurs in a single type of tissue other than the brain, and non-neuropathic systemic amyloidoses, in which aggregation occurs in multiple tissues. Some of these conditions, such as Alzheimer's and Parkinson's diseases, are predominantly sporadic, although hereditary forms are well documented. Other conditions, such as the lysozyme and fibrinogen amyloidoses, arise from specific mutations and are hereditary. In addition to sporadic (85%) and hereditary (10%) forms, spongiform encephalopathies can also be transmissible (5%) in humans as well as in other mammals. It has also been found that intravenous injection or oral administration of preformed fibrils from different sources can result in accelerated AA amyloidosis in mice subjected to an inflammatory stimulus[116,117]. It has therefore been postulated that an environment enriched with fibrillar material could act as a risk factor for amyloid diseases[117]. Similarly, injection of the recombinant mouse prion protein in the form of amyloid-like fibrils has been reported to generate disease in mice that express the prion protein[118]. The extracellular proteinaceous deposits found in patients suffering from any of the amyloid diseases have a major protein component that forms the core and then additional associated species, including metal ions, glycol-amino-glycans, the serum amyloid P component, apolipoprotein E, collagen, and many others[119,120]. Ex vivo fibrils, representing the amyloid core structures, can be isolated from patients, and closely similar fibrils can also be produced

in vitro using natural or recombinant proteins; in this case, mildly denaturing conditions are generally required for their rapid formation, at least for proteins that normally adopt a well-defined folded structure.

The fibrils can be imaged in vitro using transmission electron microscopy (TEM) or atomic force microscopy (AFM). These experiments reveal that the fibrils usually consist of a number (typically 2–6) of protofilaments, each about 2–5 nm in diameter[121]. These protofilaments twist together to form ropelike fibrils that are typically 7–13 nm wide[12,121] or associate laterally to form long ribbons that are 2–5 nm thick and up to 30 nm wide[122-124]. X-ray fiber diffraction data have shown that in each individual protofilament the protein or peptide molecules are arranged so that the polypeptide chain forms β-strands that run perpendicular to the long axis of the fibril[12]. The fibrils have the ability to bind specific dyes such as thioflavin T (ThT) and Congo red (CR)[125], although the specificity of binding of CR to amyloid fibrils and the resulting green birefringence under cross-polarized light has recently been questioned[126,127].

The proteins found as intractable aggregates in pathological conditions do not share any obvious sequence identity or structural homology to each other. Considerable heterogeneity also exists as to secondary structure composition or chain length. Interestingly, some amyloid deposits in vivo and fibrils generated in vitro have both been found to include higher-order assemblies, including highly organized species known as spherulites, which can be identified from a characteristic Maltese cross pattern when observed under cross-polarized light [128,129]. Such species are also observed in preparations of synthetic polymers, such as polyethylene, a finding consistent with the idea that amyloid fibrils have features analogous to those of classical polymers.

Inhibition of Amyloid Formation (cited from Eisenberg and Jucker, 2012)

It can be accomplished in both chemical and biological interventions.

Chemical Interventions

There are four different approaches to perform chemical intervention

The first approach is to stabilize the structure of the protein soluble form diminishing the rate at which it undergoes conversion to the amyloid state. The pioneering demonstration of this strategy was on TTR[130]. TTR is a homotetramer that carries serum retinol binding protein and thyroid hormones, such as thyroxine. In several amyloid diseases, one of many mutations can destabilize TTR, leading to fibrous deposits in the heart and peripheral nerves. Using structure based design, several potent and specific binders to the TTR hormone pocket have been described that inhibit fibril formation[131]. The same strategy could be applied to other amyloid forming proteins that have a stable native structure.

The second approach is to screen for small molecules that disrupt fibril and oligomer formation. Thus, Necula et al.[132] list 16 screening studies for molecules that inhibit

fibrils of Aβ, and they go on to study molecules that inhibit formation of Aβ oligomers. In a recent study using small-molecule microarrays[133], 79 compounds were discovered that rescue cells from cytotoxicity. The authors suggested that the mechanism of the rescue is that a compound can accelerate Aβ aggregation past an early-forming toxic oligomer. Screening for compounds that inhibit fibrils of τ is also an active area[134]. Despite this huge effort, no compound for Alzheimer's disease treatment have been reported as yet.

The third approach uses the self-assembling property of amyloid fibers to poison the growth of amyloid fibers with peptides[135]. A biological system which apparently uses this strategy is Het-S, a native inhibitor of the HET-s prion[30]. Adoption of this principle for chemical design is based on the fact that β sheets are the fundamental structural unit of amyloid fibrils and the fibrils grow by addition of new strands to the β sheets. The fiber is poisoned or ''capped'' by adding a peptide that acts as a new strand via hydrogen bonding to the sheet at the fibril's growing edge but prevents the subsequent addition of another amyloid molecule. It was shown that the segment of Aβ with sequence KLVFF inhibits Aβ aggregation[136], however, this peptide itself forms steric-zipper fibrils[28]. More recent work emphasizes the modifications of the blocking peptide, to both inhibit fibrillation of the target protein prevent self-fibrillation of the blocker (for review, see Ref.135). Depending on the system, it has been found that blocking fiber formation could either increase or diminish the concentration of toxic oligomers[135]. The protein domain that has been found to inhibit fiber assembly of Aβ is the N-terminal domain of myelin basic protein[137].

The fourth approach is to inhibit fiber growth by the structure-based design of peptides targeted to block the ends of fibrils. This approach becomes possible by the determination of the atomic structures of steric zippers and has been shown to be effective for inhibition in vitro of two different amyloid fibers[22]. Based on the structure of the steric-zipper segment of the τ protein with sequence VQIVYK, an D-amino acid inhibitor was designed to cap the ends of VQIVYK fibrils. This 6 residue D peptide was found to inhibit fibrillation of both VQIVYK fibers and constructs of τ. This blocker designed to cap steric zippers also blocks fibrillation of the parent protein. This strengthens the hypothesis that steric zippers form the essential spine of amyloid fibrils.

Biological Interventions

Amyloid formation depends on the concentration of the amyloid-forming proteins. Thus, inhibition of the generation of amyloidogenic proteins or of their precursors is a primary therapeutic strategy. For example, suppression of the inflammatory process responsible for serum amyloid A protein (SAA) overproduction is a therapeutic option for AA amyloid and elimination of B cell clones that overproduce immunoglobulin light chains[138]. Likewise, genetic variability in the expression of amyloidogenic proteins at slightly higher levels than normal may contribute to the risk of amyloidoses[139]. However, because of the incomplete mechanistic understanding of such genetic

variability, no therapeutic strategies to reduce protein expression at the genetic levels have so far been developed.

Some amyloid-forming proteins are derived from longer precursor proteins that need cleavage to become amyloidogenic. The best-known example is AβPP that is sequentially cleaved by β-secretase and γ-secretase to release the Aβ peptide[47]. Secretase inhibitors are currently in clinical trials, but current inhibitors may need refinement to avoid unwanted side effects, i.e., blocking cleavage to other substrates[47]. Other amyloids (e.g., AA, AApoAII, and ACys) also consist of protein fragments of larger precursors; however, it is not always clear whether such fragmentation is necessary for the amyloidoses or whether truncation is a secondary event without physiological significance[68]. While the relationship between post-translational modification of amyloids and disease pathogenesis in general remains ill defined, inhibiting pyroglutamyl formation is pursued as a therapeutic target for Alzheimer's disease[140].

The finding that vaccination of AβPP-transgenic mice can prevent and reduce cerebral β amyloidosis has stimulated the development of antibody-based immune-therapeutics for Alzheimer's disease[141]. Although mechanistically still unclear, antibodies directed toward Aβ gain access to the brain where they bind to soluble and/or deposited Aβ species and promote their degradation. Phagocytosis of microglia as well as other mechanisms have been proposed for amyloid removal[141]. Subsequent human immune-therapy trials showed also a reduction of Aβ deposits in brains of Alzheimer's disease patients, as predicted from the preclinical mouse work[142]. However, unwanted side effects and lack of cognitive improvements in ''immunized'' Alzheimer's disease patients must be overcome in future trials by early preventative, rather than therapeutic, interventions[102,143]. Immunization against other amyloids, such as PrPsc, τ, and α-synuclein have also been reported in transgenic mouse models[144-146]. Along the same line, immunological depletion (in addition to pharmacological depletion) of serum amyloid P component (SAP) has been developed as a therapeutic strategy. SAP is claimed to stabilize amyloid fibrils and to be associated with most amyloids[147].

Conclusion

Although the research reviewed here, advances rapidly in knowledge of amyloid diseases, it is appropriate to define some of the critical questions for the future work. At the molecular level, we still lack high resolution knowledge of amyloid oligomers in all but the simplest fibers. Recent work has begun to reveal the structural basis of prion strains. Now we need to establish whether the amyloid strains play a physiologically significant role in other amyloid diseases, and, if so, we need the more comprehensive view of the amyloid polymorphism. Furthermore, we need better understanding of the molecular assembly mechanisms from functional proteins to amyloid oligomers and fibers as well the pathways of disassembly.

At the level of cellular biology, we need to learn which biological cofactors stabilize and destabilize amyloid structures, and to

elucidate the metabolic and signaling pathways that regulate degradation and disposal of amyloids. An urgent need is the further development of structural and physiochemical techniques that permit the analysis of aggregated proteins in cells and living tissues, as opposed to extracted or recombinant amyloid. A remaining mystery is the enormously greater potency of seeding by amyloid and prions extracted from tissues compared to recombinant amyloids. Is this greater potency due to undetected biological cofactors in the extracted material, or has the extracted protein been templated into some structure *in vivo* which the recombinant, apparently identical, material cannot achieve? Can biological factors be discovered which can convert recombinant proteins to forms that are as potent as extracted amyloid?

Another mystery involves the mechanisms and pathways for cellular toxicity of amyloid. Are there common mechanisms of the toxicities, or the mechanisms differ between systemic and cerebral amyloid diseases? What are the toxic structures? Are oligomers distinct from small fibers, and what accounts for their toxicity? Why can toxicity of PrP be recapitulated in animal models whereas the toxicity of Aβ is comparatively modest? What is different about functional amyloids that render them non-toxic? Finally, the implications for disease of the recently reported experimental transmission of non-prion amyloids need to be established. Are similar or different structures responsible for toxicity and transmission? Can amyloid in the environment seed human diseases, and, if so, what protective measures are necessary? As answers to these questions emerge, a class of diseases that afflict and kill millions will be understood and perhaps controlled by preventative and therapeutic interventions.

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