

Molecular Mechanisms of Polypeptide Aggregation in Human Diseases

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Abstract: Protein aggregation is implicated in a plethora of neurodegenerative diseases. The proteins found to aggregate in these diseases are unrelated in their native structures and amino acid sequences, but form similar insoluble fibrils with characteristic cross- β sheet morphologies called amyloid in the aggregated state. While both the mechanism of aggregation and the structure of the aggregates are not fully understood at the molecular level, recent studies provide strong support for the idea that protein aggregation into highly stable, insoluble amyloid structures is a general property of the polypeptide chain. For proteins with a unique native state, it is known that aggregation occurs under conditions that promote native-state destabilization *in vitro* and *in vivo*. Taken together, the results of several important recent investigations suggest three broad molecular frameworks that may underlie the conversion of normally soluble peptides and proteins into insoluble amyloid fibrils: (1) edge-strand hydrogen bonding, (2) domain-swapping, and (3) self-association of amyloidogenic fragments. We argue that these underlying scenarios are not mutually exclusive and may be protein-dependent – i.e., a protein with a high content of hinge-regions may aggregate *via* a runaway domain-swap, whereas a protein with a high content of amyloidogenic fragments may aggregate primarily by the self-association of these fragments. These different scenarios provide frameworks to understand the molecular mechanism of polypeptide aggregation.

INTRODUCTION

Protein aggregation-related toxicity is implicated in a plethora of neurodegenerative diseases including Alzheimer's, Parkinson's, prion, and motor neuron diseases [1]. Although the symptoms in these diseases vary widely, they are characterized by the presence of proteinaceous deposits, first identified in the 19th century, and often referred to as amyloid [2,3]. The proteins found to form amyloid in diseases are unrelated in their native structures and amino acid sequences [4], but form morphologically similar, usually fibrillar, deposits in tissues where neurodegeneration is most severe [5]. Mutants of disease-associated proteins associated with familial versions of the diseases can be induced to form fibrillar deposits *in vitro* [6,7]. Analysis of these deposits shows that the principal structural feature of fibrillar aggregates is the presence of cross β -structures [8], as observed in disease. It has also been found that many proteins without any known connection to diseases, including proteins such as myoglobin, can give rise to fibrillar structures with all the characteristics of those found associated with the clinical amyloidoses [9]. The presence of a common structure in aggregates of proteins with unrelated sequences suggests that certain general properties of the polypeptide main chain govern the aggregation and, possibly, the toxicity observed in diseases [10].

While the existence of a conclusive causative link between aggregation and cytotoxicity in disease remains unestablished, several lines of evidence indicate that it is not the insoluble fibrillar aggregates but soluble oligomers of proteins that are responsible for cytotoxicity [11,12]. Be-

cause there is increasing evidence that protein aggregation may be the causative step in neurodegeneration, several recent biophysical studies have aimed at understanding the underlying molecular mechanisms of aggregation by uncovering the mechanism of aggregation of purified proteins *in vitro* or by studying the process of aggregation *in silico*. Here, we review the major insights obtained from some of these studies, and suggest a unified framework for understanding the underlying mechanisms of protein aggregation. A better understanding of the underlying biophysical principles of aggregation may lead to therapeutic intervention aimed at inhibiting aggregation and cytotoxicity. Indeed, mechanism-based inhibitors of aggregation are being pursued as leads for developing drugs [13,14].

Aggregation-related diseases are usually age-related, occurring in midlife or later, and it is not surprising that the *in vitro* aggregation of disease-associated protein solutions under physiological conditions is slow. It is an established paradigm that a protein in solution or in the cell explores various conformations – for a stable protein under physiological conditions, most of these conformations are folded, native conformations, but a small fraction of the conformations adopted by the protein may be less stable, partially folded or misfolded. Under destabilizing conditions, the fraction of protein chains in these non-native, misfolded conformations will be higher, and misfolded chains may interact with other chains to form soluble oligomers of various morphologies, and eventually self-associate into an insoluble amyloid (Fig. 1). Therefore, to understand the mechanism of aggregation it is common to study aggregation by perturbing the environmental conditions, lowering the pH, adding mild denaturants such as trifluoroethanol, using heat treatment, such that disease-like aggregates can be detected on experimental time scales [15,16]. Exposure to the appropriate de-

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stabilizing conditions lowers the energy barriers for the multi-step aggregation process (Fig. 1). Next, we review some proposals for the underlying forces causing self-association of protein chains as well as the structural evidence for the formation of various species indicated in Fig. 1. While several proteins known to aggregate in diseases are natively unfolded, i.e., do not have a unique native structure, this review primarily deals with proteins that are known to have a unique native structure. We expect that some of the underlying mechanistic ideas will be useful for understanding the aggregation of intrinsically disordered proteins as well. Finally, we briefly review suggestions for how oligomers may induce cytotoxicity.

STRUCTURAL BASIS OF AMYLOID-FORMATION

It is well known that large conformational transitions occur during the aggregation of proteins from their native states into amyloid fibrils, suggesting that partial unfolding and/or misfolding [17-24] are necessary for aggregation. The thermodynamic stability of a protein determines the propensity of the protein to adopt native-like structures. Thus, the formation of stable protein aggregates is linked to changes in the thermodynamic stability of aggregating proteins, and conditions which promote aggregation are expected to destabilize the native-state. Such conditions include mutations in the protein [25-28], changes in the local cell environment that affect processing of the polypeptide chain during localization/transport into organelles [29], post-translational modifications [30], interactions with reactive metabolite spe-

cies [31], and aberrant interactions with the proteolytic or chaperone machinery [32].

Many of the proteins involved in aggregation in diseases, and a significant fraction of soluble proteins in the cell are intrinsically disordered [33]. In contrast with natively folded proteins that aggregate, these proteins may have to gain structure (i.e., “fold”) for aggregation to occur [34]. While it is long established that destabilization or (at least partial) unfolding/folding (for intrinsically disordered proteins) is necessary for aggregation, the driving forces for formation of a specific aggregate topology, the cross- β amyloid fibril, are less well understood. We classify the proposed driving forces for amyloid formation from partially unfolded or misfolded states of polypeptides into three overlapping categories:

Edge-Strand Interactions

Protein folding *in vitro* is potentially a highly inefficient process, because of competing off-pathway reactions. However, in the non-diseased cell, a large fraction of proteins folds to its native state, avoiding off-pathway processes that might lead to aggregation. While the high fidelity of protein folding *in vivo* is due to a variety of factors including chaperones and the protein degradation machinery, it has been postulated that in the course of evolution, nature has selected sequences that not only fold into stable native states but also avoid non-native aggregation into amyloid fibrils. This is the so-called principle of negative design [35], which offers structural clues into protein aggregation. Upon a systematic

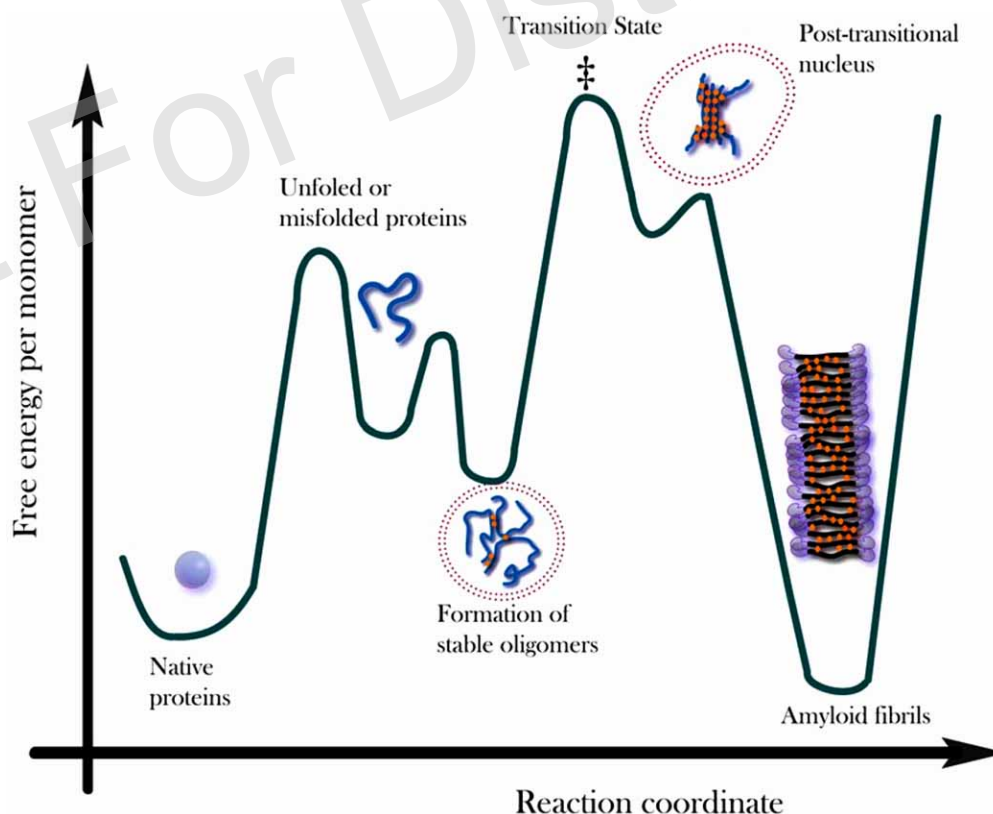


Fig. (1). A free energy schematic profile for a generic mechanism for protein aggregation into amyloid fibrils. This representation is a projection of a very high-dimensional space onto a one dimensional plot, for illustration. The reaction coordinate for aggregation is also not easily defined.

analysis of the database of protein structures, it was postulated that all β -sheet proteins can form edge-to-edge aggregates unless they employ some 'blocking' strategies to prevent aggregation. There are two such 'blocking' strategies: (i) minimization of unsatisfied hydrogen bonds (H-bonds), and (ii) inward-pointing charged residues that block aggregation [36].

In the folded states of naturally occurring proteins, the number of unsatisfied H-bonds is minimized, and the presence of a large number of unsatisfied H-bonds promotes aggregation. It was shown that for pathological monomeric conformations of the prion proteins from mouse and Syrian hamster the proportion of unsatisfied H-bonds (14%) is significantly larger than the average number of unsatisfied H-bonds in naturally occurring proteins (6%) [37]. Furthermore, the β -helix, which is a putative topology of amyloid fibrils, does not contain unsatisfied H-bond donors/acceptors. Thus, the formation of H-bonds may be the driving force for protein aggregation. In this scenario, formation of protein aggregates is governed by a competition between specific amino acid interactions within proteins, and non-specific hydrogen bond formation between the unprotected edge-strands of different proteins [27,38]. Interactions other than the hydrogen-bond – such as hydrophobic collapse, electrostatics – are involved in the aggregation process, too.

Another possibility for hydrogen-bond mediated assembly is the so-called "dehydron" mechanism for protein assembly [39]. Fernandez et al. have classified amide-carbonyl hydrogen bonds that are water-accessible as dehydrons [40]. These dehydrons promote the removal of surrounding water through protein-protein or protein-lipid association because these interactions will lead to burial of hydrogen bonds [41]. Buried hydrogen bonds are much stronger than those exposed to water [40]. Thus, dehydrons on different polypeptide chains may induce inter-polypeptide associations, and thereby promote aggregation.

The template hypothesis of aggregation states that pre-formed oligomers or aggregation nuclei provide edge-strands, and these interaction centers cause the conversion of a normally soluble protein into an aggregation-prone conformation [42]. The docking of the aggregation-prone conformation to the pre-formed nucleus results in growth of the oligomer. Consistent with the template hypothesis, computational studies have shown that pre-formed or spontaneously formed templates with hydrogen-bond donors/acceptors can cause the inter-conversion of α -helical peptides to β -strands that propagate the aggregate [43,44]. Conversely, it is expected that disruption of edge-strand mediated interactions will lead to the inhibition of assembly. In an elegant test of this hypothesis, Wang and Hecht first designed peptides containing amphiphilic β -strands (with alternating hydrophobic and hydrophilic sidechains) which were found to spontaneously assemble into amyloid-like fibrils, and showed that aggregation was inhibited if a charged residue was placed at the non-polar face of a terminal amphiphilic β -strand [45]. The charged residue is expected to stabilize the monomeric protein compared to the aggregate because of the large desolvation penalty associated with charge burial during aggregation. A similar observation of the inhibition of ag-

gregation by presence of charged residues in contiguous hydrophobic patches was made by Otzen and Oliveberg in their studies of the folding of the ribosomal protein S6 [46]. They found that the presence of charged residues in the key regions of the protein is required for the two-state folding of the protein – in the absence of these charged "gatekeepers" intermediate partially folded states of the protein are populated which leads to exposed edge-strands and aggregation. These results also suggest that key residues required for maintaining fold fidelity and preventing aggregation can be identified by studying the folding mechanism of proteins and uncovering kinetic aggregation-prone traps on the free energy landscape of the protein. Based on simplified models of protein folding, we identified a set of charged residues on the surface of the protein Cu, Zn superoxide dismutase (SOD1), a subset of which were later shown by Oliveberg and co-workers as being the "gatekeepers" required for avoiding aggregation of SOD1 [47,48]. Thus, removal of charged groups leads to aggregation mediated by exposed hydrophobic groups and hydrogen bond donors and acceptors.

Domain-Swapping

In this scenario, aggregates are formed from partially unfolded protein chains by a mechanism called "runaway domain swapping". Three-dimensional domain swapping is an event by which a monomer exchanges part of its chain with other identical monomers to form an oligomer or polymer in which each subunit has a similar structure to the monomer, albeit the subunit is now made of more than one chains. Domain swapping, initially proposed as a mechanism of functional regulation, has also been proposed to lead to misfolding and aggregation [49-51]. Although there is little direct evidence for domain swapping as a mechanism for aggregation and amyloid formation, several experimental (Ref. [50] and references therein) and computational [52-54] studies support the role of domain swapping in aggregation. For example, a correlation between domain swapping propensity of the protein p13suc1 was found to be correlated with its rate of aggregation [55]. Eisenberg and coworkers have designed both domain-swapped dimers and high-order oligomers from the same three-helix bundle structural motif but with different topologies [49,56,57]. Furthermore, domain-swapped forms of both the human prion protein and the amyloidogenic human cystatin C [58,59] have been crystallized.

Domain-swapping has been most clearly implicated in the aggregation of polyglutamine (polyQ)-containing model systems. The aggregation of polyQ-containing proteins is implicated in at least nine neurodegenerative diseases [60], and model systems in which polyQ stretches are inserted into small, well-characterized, non-disease-associated proteins have been used to understand the structural basis of aggregation [61-63]. It has been found that at small repeat lengths of polyQ (<35-40 glutamines), the destabilization induced by their insertion can be overcome by domain-swapped dimer formation by the chimeric protein [61]. For longer repeat lengths (>35-40 glutamines), the presence of polyQ stretches can destabilize the protein sufficiently to form higher-order oligomers and fibrils. A striking example of this phenomenon was demonstrated in the model system ribonuclease A by Eisenberg and co-workers. They had previously found

that ribonuclease A chains form domain-swapped dimers and trimers by exchanging identical structural elements on either side of a hinge region [49,57]. Upon a Q_{10} insertion in this hinge loop of ribonuclease A, polyQ self-association induces a “runaway” domain swap i.e. higher-order oligomerization and amyloid fibril formation [64].

Computational studies [52-54] using simplified native-structure based Gō-models [65,66] have shown that the monomeric protein topology alone is sufficient for predicting how a protein will form domain-swapped complexes, including higher-order oligomers. In computational domain-swapping studies of the SH3 domain [54,67], two types of topologies have been detected: “closed” domain-swapped dimers which are observed in X-ray crystal structures [68], and more “open” oligomers which can be propagated to form fibrils by a “runaway domain swap”. Structural features of the computationally obtained fibrils agree with the X-ray diffraction pattern of amyloid fibrils obtained in experiments [69]. Ding *et al.* further analyzed several proteins known to form domain-swapped structures and found that, in many cases, domain swapping occurs at positions around which the protein tends to unfold prior to complete unfolding. This, in turn, enabled prediction of protein structural elements that are responsible for domain swapping [70]. Thus, in the domain-swapping framework, the strong bias towards the native-state of the protein leads to “closed” domain swapped dimers or trimers upon small destabilization. As the degree of destabilization increases, lower-order oligomers can no longer stabilize the protein chain in a native-like conformation. Instead, a “runaway” domain-swap occurs, resulting in the formation of amyloid fibrils, in which elements of native structure may still be retained.

Self-Association of Amyloidogenic Fragments

In this scenario, unfolding may result in the exposure of amyloidogenic sequence fragments which, in turn, self-associate to induce oligomerization. This scenario is supported by the findings that several small (>5 residues long) sequence fragments of many aggregating proteins themselves form amyloid fibrils in vitro [71,72]. In some cases, these amyloidogenic sequence fragments have been found in the aggregated state to be arranged as parallel β -strands in a sheet in which the amino acid sequence is in exact register [73,74]. Thus, it has been argued that unfolding makes the self-association of amyloidogenic “hotspots” possible, and the self-association of amyloidogenic fragments nucleates the aggregation of the entire chain [75,76].

Mutational analyses of fragment aggregation in vitro and in silico suggest that hydrophobicity, net charge and β -sheet propensity modulate fragment self-association propensity [77-79]. Algorithms have been developed to predict the location of amyloidogenic fragments based on polypeptide sequence alone [77,80,81], and it has been found that in well folded globular protein sequences amyloidogenic fragments are surrounded by residues that have a very low aggregation propensity (“amyloid-breakers”) [77]. Analysis of protein structures also suggests that natural selection has led to amyloidogenic sequence fragments being protected in the native states of protein structures found in nature [35,82]. Therefore, the ability of amyloidogenic sequences to induce ag-

gregation is modulated by the global stability and the structure of proteins. Protein aggregation propensity is, then, the interplay between the stability of the native structure, which prevents protein aggregation, and the self-association of amyloidogenic sequence fragments from different polypeptide chains into in-register structures, which promotes protein aggregation. Consequently, mutations associated with familial forms of neurodegenerative diseases may promote aggregation by either destabilizing the native state and/or increasing the self association propensities of exposed sequence fragments under destabilizing conditions.

The relationship between aggregation and folding hotspots (nuclei) is also an important, but unsettled issue. Chiti *et al.* found that the residues important for mediating aggregation of AcP protein do not correspond with the folding nucleus of the protein [83]. However, in the human prion it is the folding nucleus whose modification triggers aggregation [84,85]. The folding and aggregation nuclei may or may not overlap, and an overlap between the two may also implicate domain swapping as a possible mechanism of aggregation: the success of the folding reaction requires that the nucleus residues make key contacts, if these contacts happen to be inter-chain, a domain-swapped structure may result which may be on the aggregation pathway. The balance between intra- and inter-chain interactions is dictated, apart from protein concentration, by the relative heights of the folding and aggregation barriers. Mutations in the folding nucleus residues may increase the intra-chain interaction barrier compared to the inter-chain one, which leads to aggregation instead of folding.

It should be noted that the three frameworks for aggregation outlined above are not mutually exclusive – for example, it is possible self-association-prone regions of the protein are in regions that also have a high propensity to domain-swap, i.e., in this case, domain swapping and self-association of amyloidogenic fragments occurs simultaneously during the oligomerization process, and this may lead to the formation of stabilizing hydrogen bonding networks in the oligomers. This is the putative scenario our studies on the aggregation of the protein Cu, Zn superoxide dismutase have revealed (Fig. 2). It is also possible that the underlying driving forces are protein-dependent – i.e., a protein with a high content of hinge-regions aggregates *via* a runaway domain-swap, whereas a protein with a high content of amyloidogenic fragments aggregates primarily by the self-association of these fragments. Using some of the insights described above, Shea and co-workers are using model misfolded conformations to design aggregation inhibitors [86].

STRUCTURAL FEATURES OF OLIGOMERS AND FIBRILS

Amyloid fibrils of different peptides share common structural features: they bind to dyes such as Congo Red, and Thioflavin T, and display a characteristic X-ray diffraction pattern called the cross- β pattern [74]. Because of the large size of amyloid fibrils, obtaining atomic level structures by X-ray crystallography or NMR has been difficult. A notable property common to amyloid protein deposits is that fibrils of different origins show similar biophysical and ultrastructural characteristics. In all cases, amyloid fibrils are highly

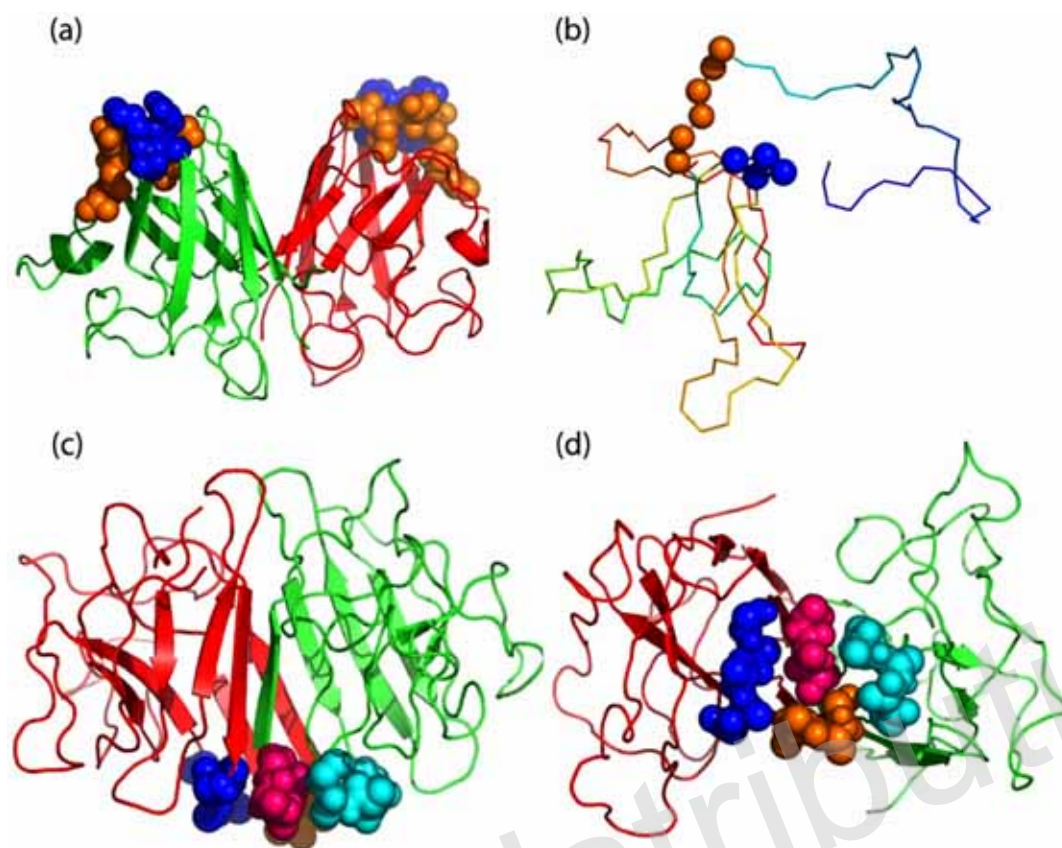


Fig. (2). Putative mechanism of Cu/Zn superoxide dismutase (SOD) aggregation involving both self-association of amyloidogenic fragments and domain-swapping-like mechanisms. (a) Native SOD dimer, contacts between key residues are highlighted in orange and blue. (b) A destabilized intermediate misfolded state of SOD identified by our studies in which key interactions are disrupted, (c) and (d) two views of an alternative SOD dimer formed by the self-association of two destabilized SOD monomers. Residues highlighted in orange interact with cyan residues in the other monomer (domain swapping-like) instead of forming intra-monomer contacts with the corresponding residues show in blue. Also, residues highlighted in orange interact with the same region from the other monomer highlighted in pink (self-association of amyloidogenic fragments). Figure adapted from Ref. [109].

ordered molecular assemblies with a diameter of 7–10 nm, as reflected by a typical X-ray fiber diffraction pattern of 4.6–4.8 Å on the meridian [87]. Additionally, various spectroscopic methods have shown that all fibrillar amyloid assemblies are predominantly in β -sheet conformation [88].

The fact that small peptides, as short as four residues in length, can form fibrils has been used to obtain structural information about amyloid-like topologies [89]. It was found by Eisenberg and co-workers that a seven-residue sequence from the yeast prion protein Sup35 (GNNQQNY) was able to form micro-crystals [90]. An atomic structure of this micro-crystalline material shows a double β -sheet, with each sheet formed from parallel segments stacked in register. Side chains protruding from the two sheets form a dry, tightly self-complementing steric zipper, bonding the sheets [90]. Within each sheet, every segment is bound to its two neighbouring segments through stacks of both backbone and side-chain hydrogen bonds. Although it is unclear if the topology found in micro-crystals is the same as that in the insoluble fibrils, the structure provides insight into possible seeding and propagation mechanisms for self-assembly and growth. Other topologies based on solid-state NMR, molecular dynamics and electron microscopy have been proposed and

include β -helices, β -zippers, β -hairpins [91]. However, current studies on insoluble fibrils have not been able to definitively identify any one of these β -topologies as the building block of the insoluble aggregate.

Structures of soluble oligomers formed on- or off-pathway to fibril formation have been studied using atomic force microscopy, small angle neutron scattering and electron microscopy, and a diversity of topologies have been identified [92–94]. These topologies include protofibrillar intermediates, including spherical protofibrils which anneal to form chains and pore-like annular species (amyloid pores) [95–98]. In vitro, all of the protofibrillar species seem to be consumed by fibril formation at the end of the aggregation reaction. An antibody raised against the spherical oligomers of one peptide, the A β -peptide, cross-reacts with oligomers of several other proteins, suggesting that the oligomers, like the amyloid fibrils, may also share a common structural epitope [99]. These studies suggest that the topologies adopted in the soluble oligomers and fibrils are different from each other, and different techniques measure the topology of different species on the reaction pathway.

The differences between the topology of the oligomers and insoluble aggregates are not well-understood at high

structural resolution. Based on H/D exchange, Wetzel and co-workers suggested that the β -sheet elements comprising the amyloid fibril are already present in protofibrils, but that they are expanded (into adjacent residues) upon the formation of mature amyloid – thereby providing additional stabilization [100]. It is also likely that soluble oligomers, although structurally similar to mature fibrils on average, are much more conformationally flexible and heterogeneous.

OLIGOMERS AND CYTOTOXICITY

The existence of a causative link between protein aggregation and cytotoxicity in neurodegeneration remains controversial. While aggregation is usually co-incident with cell death, it is not clear if aggregation is the cause of cytotoxicity or an effect, i.e., it may represent a protective response of the cell which depletes the pool of the cytotoxic soluble protein. The literature on toxicity and its relationship to aggregation is vast, and an exhaustive treatment of this literature outside the scope of the current review, but we will mention some proposals for toxicity. Evidence has accumulated that soluble oligomeric species, rather than the insoluble fibrils, may cause cytotoxicity. Toxicity may also arise by aberrant interactions with and/or sequestration of key cellular components, such as transcription factors and the proteosomal machinery [101]. Another mechanism of oligomer-induced cytotoxicity is the permeabilization of membranes [102], which disrupts ion-homeostasis and results in cell death [103,104]. While membrane permeabilization is known to occur by the spontaneous formation of oligomeric ion-channels in mixtures of disease-associated peptides and artificially reconstituted membrane bilayers in vitro [102,105], no evidence for ion-channels has been found in in vivo studies. It has been suggested that oligomers may form pores in membranes which are similar to those formed by bacterial toxins [106,107]. Thus, several scenarios linking aggregation and cytotoxicity have been proposed, and cytotoxicity may well be multi-factorial involving simultaneously more than one mechanism of cell death. It is clear, however, that aggregation is an important event during neurodegeneration.

CONCLUSIONS

The underlying mechanism of polypeptide aggregation is an intense area of investigation, with possible therapeutic applications in human diseases. We review the observations of several investigations and propose that there exist three mutually overlapping scenarios for the mechanism of protein aggregation: edge-strand mediated assembly, domain swapping and self-association of amyloidogenic fragments. We propose that a combination of these driving forces causes the assembly of misfolded/unfolded proteins into soluble oligomers. Some studies suggest that these soluble oligomers are the toxic species in a set of neurodegenerative diseases. These soluble oligomers then further self-associate into large, insoluble fibrils in a nucleation-elongation mechanism. Understanding the molecular mechanism of amyloid formation will be crucial in designing rational structure-based therapies for human diseases involving protein aggregation.

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