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Conformational Change and Assembly through Edge β Strands in Transthyretin and Other Amyloid Proteins

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ABSTRACT
Numerous diseases are characterized by the formation of insoluble, amyloid protein fibrils. Intensive investigations are beginning to unravel the detailed molecular and structural principles that underlie the spontaneous formation of these fibrils. The amyloid protein transthyretin serves as an excellent system for dissecting the conformational changes and ensuing subunit−subunit associations that lead to amyloid. One working model for tranthyretin amyloid involves the exposure of an “unprotected” edge β strand, followed by symmetric assembly of subunits to give head-to-head and tail-to-tail protofibrils. The models and principles emerging from studies on transthyretin lead to connections to other amyloid systems.

Introduction

Protein misfolding and aggregation have been implicated in a growing number of human diseases. These include Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, prion diseases, and numerous others, with each pathology being related to the aggregation and deposition of a particular protein in various tissues and organs.[1,2] Despite the possibility of common underlying molecular mechanisms, the proteins involved are diverse and exhibit a range of structural properties and aggregation behaviors. Among this group of aggregating proteins, the term amyloid is assigned to those that assemble into essentially linear extracellular filaments and that satisfy other criteria such as the ability to bind and shift the spectral properties of certain hydrophobic dyes and a strong scattering of X-rays at an angle corresponding to a 4.7 Å molecular spacing.[3]

One theme that is shared across diverse amyloid systems is the presence of a conformational change in the native protein, leading to new non-native subunit−subunit associations and revealing non-native epitopes in the misfolded form of the protein.[4,5] In vitro studies of amyloid formation are generally conducted under varied destabilizing conditions, such as low pH, increased temperature, lyophilization, and mild to moderate chemical denaturation. Such conditions promote the conformational changes that lead to aggregation. The conformational changes are generally understood to involve an increase in β-sheet structure, which drives the association of subunits to form a fibril composed of β strands running perpendicular to the long fibril axis and connected by hydrogen bonding along the direction of the fibril axis. The importance of β-sheet structure is supported by multiple lines of reasoning. Foremost, amyloid fibrils share certain diagnostic features in their X-ray-scattering patterns, such as the 4.7 Å axial reflection noted above, which is diagnostic for ordered β-sheet structure[3,6] (Figure 1). Circular dichroism studies also demonstrate an increase in β-sheet conformation.[7,8] In addition, computational studies have suggested that proteins may be prone to amyloid formation if they contain regions that appear at the amino acid sequence level to favor β-sheet formation but which exist in the native protein structure in a non-β configuration.[9] Various studies have therefore made it clear that amyloid fibrils arise from new β-sheet interactions between subunits, but the details of the conformation changes and the new interactions that result are not understood in detail.

Because the formation of fibrils is a common feature of amyloid diseases, the structural basis for assembly is of significant interest. In several amyloid systems, the formation of mature, well-ordered fibrils appears to occur by a multistep process involving individual protofibrils or even smaller oligomeric assembly intermediates. In some cases, there is evidence that toxicity may be more closely linked to assembly intermediates than to the mature fibrils.[10,11] Nonetheless, assembly appears to be a key element, and therefore, whether toxicity arises at the stage of the oligomer or the mature fibril, a structural view of the molecular interactions between subunits will be essential in understanding these systems.

Structural studies on amyloid span a wide range of length scales, and at the electron microscopy (EM) scale, such studies have revealed amyloid fibrils having diverse supramolecular architectures. Fibrils formed from different amyloid proteins have grossly similar morphologies, with diameters typically in the 70−130 Å range, but there are significant variations. Mature fibrils often appear to be composed of thinner protofibrils in lateral association, but the protofibrils differ in number and arrangement

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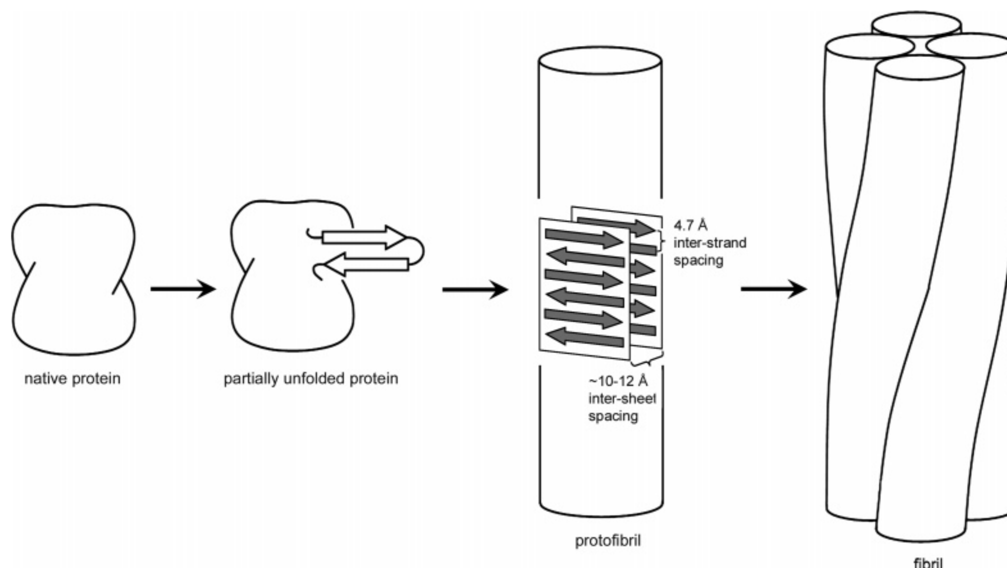

FIGURE 1. Rough model for amyloid formation. Some of the details are either unknown or vary between different amyloid proteins. The exact nature of the conformational changes that take place in the native proteins is unknown. Whether individual proteins contribute part or all of their structures to the β sheet of the protofibril is unknown. X-ray-scattering studies support the basic cross-β organization of β strands and sheets within protfibrils, but whether the strands run parallel or antiparallel is unknown or variable. In cases where protofibrils aggregate further, the number of protofibrils per fibril varies.

between different amyloid proteins. The number of protofibrils appears to vary from two to six, and some protofibrils twist around each other to form rounded fibrils,[12] while others associate into flat, ribbon-like fibrils.[13,14] The wide variation between distinct amyloid fibrils seen at the EM scale suggests that, if there are unifying molecular mechanisms for amyloid formation, they prevail over a shorter length scale and will require investigations at that level.

At the finest length scale, peptide studies are beginning to reveal critical information. Nelson et al.[15] recently reported the crystal structure of a peptide fragment, GNNQQNY, from the yeast prion protein Sup35. The arrangement of peptide molecules in the crystal leads to important implications for the molecular interactions in amyloid fibrils. Hydrogen bonding between peptide molecules, each in an extended β-strand conformation, produces an indefinitely long β spine, reminiscent of models for amyloid protofibrils. In addition to the expected backbone interactions, very tight amino acid side-chain packing is seen between peptides, and two parallel sheets are so tightly packed that the space between them is nearly anhydrous. Numerous other peptide-based studies have contributed to an understanding of the structural and sequence basis for amyloid formation.[16−19]

Peptide studies provide critical clues about how certain residue types might interact at an amyloid subunit−subunit interface, but numerous questions arise subsequently about how full-length amyloid proteins assemble into amyloid. Which parts of these full-length amyloid proteins are involved in the kinds of interactions implicated by peptide studies? What kinds of conformational changes are required for larger proteins to interact in the ways implied by peptide studies? Are massive conformational rearrangements of native proteins required, or might more subtle changes be sufficient? To achieve the next step in understanding, the atomic rearrangements and interactions between full-length amyloid proteins must be dissected.

Transthyretin (TTR)

The most common inherited amyloid disease in humans is caused by point mutations in the plasma protein TTR.[1] TTR is a homotetrameric plasma protein consisting of 127 residues and measuring 55 kDa in its tetrameric form. The biological function of TTR is to carry thyroxine (T4) hormone, mainly in cerebrospinal fluid and also to bind retinol-binding protein in the blood, thereby transporting vitamin A (retinol). TTR is found in a fibril form in several disease states, including familial amyloid polyneuropathy (FAP), familial amyloid cardiopathy (FAC), central nervous system selective amyloidosis (CNSA), and senile systemic amyloidosis (SSA). Fibrils in the genetic diseases FAP, FAC, and CNSA are composed of full-length TTR, with more than 80 known point mutations that enhance amyloidogenicity.[20,21] In SSA, which affects over 25% of the population over 80 years of age, fibrils are composed of a mixture of full-length TTR wild-type and C-terminal fragments formed mainly by trypsin cleavage after residue K49 prior to fibril formation.[22]

A number of features have made TTR an attractive system for amyloid studies. The crystal structure of TTR is known at atomic resolution.[23] This has provided a valuable framework for understanding the conformational changes and interactions that lead to amyloid. Methods for forming TTR fibrils in vitro have been developed,[24] making it possible to study the thermodynamics and kinetics of TTR amyloid formation for native, mutant, and engineered TTR constructs, as well as complexes of TTR

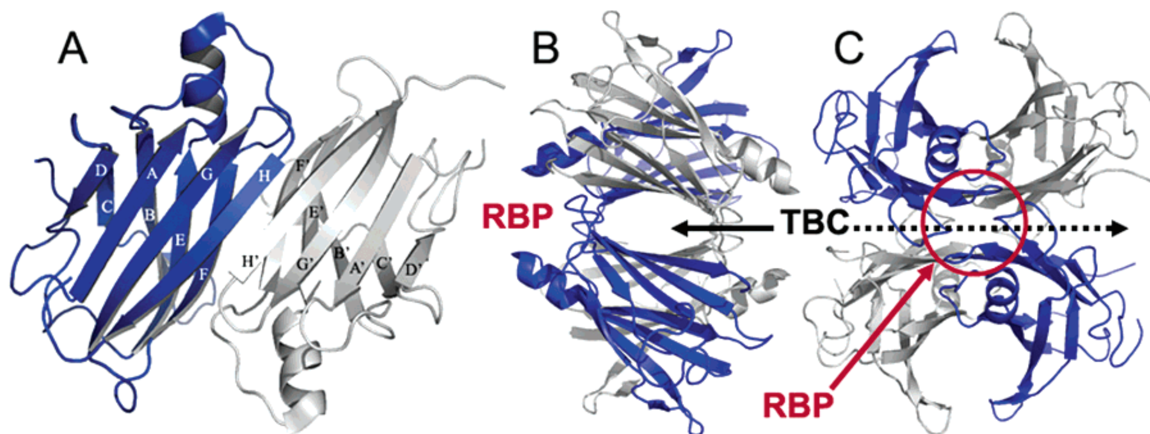

FIGURE 2. Three-dimensional structure of the amyloid protein, TTR. (A) Tightly held TTR dimer is stabilized by the formation of continuous β sheets between strands labeled F and F′ and H and H′ from distinct monomers. (B) Native TTR tetramer is shown looking through the thyroxine-binding channel (TBC). DAGH sheets of both dimers line the channel. The retinol-binding pocket (RBP) is noted in red on the sides. (C) Same tetramer is shown rotated 90° about the vertical axis. The RBP is now in front (circled in red), and the TBC runs horizontally through the center (dashed line).

with drugs designed to prevent destabilization and subsequent amyloid formation (refs 25 and 26 and references therein). Finally, a compelling feature of TTR is its preponderance of β-sheet structure in its native form. The arrangement of β sheets in TTR, including sheets that span two monomers interacting across a dimer interface, suggests that this protein might be able to assemble into amyloid fibrils without having to undergo massive rearrangements. This could make structural analysis of the fibril state more tractable and could also address key questions regarding the magnitude of conformational changes that occur during amyloid formation.

TTR is a tetramer of the dihedral type, meaning it is composed of a pair of dimers. An extensive interface between two monomers is created by the F and H β strands of one subunit forming continuous β sheets with like strands from the other subunit of the dimer, with strands interacting in antiparallel fashion as they pass by the axis of 2-fold molecular symmetry (Figure 2). Each TTR monomer resembles a sandwich of two four-stranded β sheets. Two monomers come together to produce a dimer consisting of two eight-stranded intermolecular β sheets, which can be labeled as DAGHH′G′A′D′ for one sheet and CBEFF′E′B′C′ for the other. The full TTR tetramer is created by weaker interactions between the two dimers,[27] with contacts occurring through back-to-back hydrophobic interactions of the AB and GH loops. A central thyroxine-binding cavity is formed in the space between the DAGH sheets of each of the four subunits in the tetramer. The hydrophobic pocket formed at the tetrameric contacts is the site of the interaction with retinol-binding protein.

Because wild-type TTR is soluble in the native state, aggregation must be triggered by a series of events that initiate destabilization of the soluble form. The stabilization and destabilization of the TTR tetramer has been studied deeply by Kelly et al.[27−30] Stabilization of the tetrameric form strongly inhibits amyloid formation. A known trans-suppressor mutation in TTR (T119M), which rescues the amyloidogenic mutation V30M, is located in the region of the protein that forms the tetramer contacts and prevents dissociation of the tetramer.[28] Dissociation of the tetramer appears to be the rate-limiting step in fibril formation, followed by conformational changes within the subunits, leading to downhill polymerization.[29,31] The data indicate that tetramer disassembly must precede fibril formation. However, the nature of the conformational changes that follow tetramer disassembly and the subsequent subunit interactions that lead to polymerization are yet to be determined. These questions are the subject of much study.

Conformational Changes in TTR

Although the crystal structures of several amyloidogenic mutants of TTR (in their soluble tetrameric forms) generally reveal only minor structural differences compared to the wild type,[32] many studies show that significant conformational changes occur in TTR prior to protofibril formation. H/D exchange[33] and spin-labeling[34] experiments show that internal β strands remain protected from the solvent in fibrils, while the CD region (the region encompassing strands C and D at the edge of the native β sheet) dislocates to expose strands B and A, respectively. Antibody studies show that epitopes present in TTR fibrils are not accessible in native TTR, and conversely, epitopes present in the native TTR are not accessible in TTR fibrils.[35,36] Structural and biophysical studies confirm the mobility and conformational variability of the CD region.[37−39]

The outer edge β strands C and D of TTR in the monomeric β sandwich have different structural and functional roles than the interior strands. The inner β strands contact their neighboring strands through hydrophobic side-chain interactions that allow the strands to stick together within the sheet architecture. Edge strands protect these hydrophobic surfaces and prevent aggregation while exposing a hydrophilic surface to the solvent. Strategies to deal with the problem of sticky edge strands in natural β sheets include short outer strands, loops covering the end of the sheet, inward pointing side chains

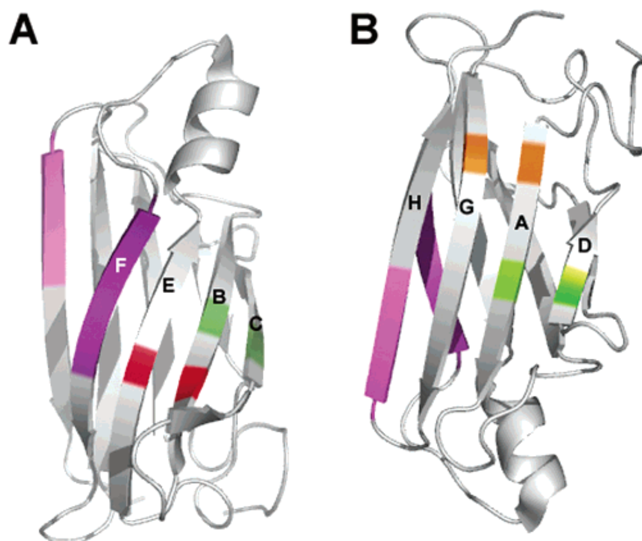

FIGURE 3. Location of engineered disulfide pairs in the TTR monomer, which were constructed to probe the requirement for movement in amyloid formation. (A) The CBEF sheet of the monomer is shown with paired cysteine mutations indicated in red (strand B/E, 33:70) and green (strand C/B, 46:31) (B) The DAGH sheet of the monomer is shown with paired cysteine mutations indicated in orange (strand A/G, 12:105) and lime (strand A/D, 15:54). The F and H strands involved in the native dimeric interface are shown in purple.

Table 1. Effects of *β*-Strand Cross-Linking on TTR Amyloid Formation

β strands	residues	effect
E tacked to B	33:70	same as the wild type
A tacked to G	12:105	same as the wild type
C tacked to B	46:31	delayed fiber formation
D tacked to A	15:54	delayed fiber formation

in the center of the outer strand, and proline bulges to prevent unwanted intermolecular interaction.[40] There are two sets of edge strands in a TTR monomer *β* sandwich. One set (strands F and H) forms the native dimeric interface through association with the same strands from a neighboring monomer, creating continuous *β* sheets. The other set (C and D strands) are short edge strands flanked by covering loops. Mutations in the short edge strands and loops tend to facilitate TTR amyloid formation.[41−43] The most severely amyloidogenic mutations, E54 and L55[43,44] on the D strand, were observed in different conformations in two different crystal forms,[39] further emphasizing the structural variability of this region. Along with those that destabilize the tetramer, mutations affecting the CD region constitute an important class of amyloidogenic mutations.

Recently, we have investigated the dependence of the fibril formation rate on the ability of edge strands C and D to move away from their native positions. Preliminary data suggest that tacking the mobile C or D edge strands to their adjacent neighbor strands (B or A) via disulfide bonds results in delayed formation of protofibrils, whereas tacking the non-edge strands (B or A) to their more interior neighboring strands (E or G) has no effect on the rate of fibril formation (Figure 3 and Table 1; J. Laidman, unpublished data). The results provide further evidence that (1) the mobility of the C and D edge strands is essential to protofibril assembly and (2) core strands may remain associated during assembly of the protofibril.

The earlier observation that deleting the D strand[45] results in an amyloidogenic protein raises the possibility that the entire CD region might be dispensable for fibril formation. We therefore sought to assess the effects of removing the CD region from TTR to see if a truncated form of the protein might assemble into amyloid-like fibrils with similar morphology to the wild-type protein. To address these questions, a TTR construct was created by deleting the CD region, residues R34−V65, and replacing it with the short flexible linker GGGSGGG. According to the design, the *β* strands B and A, formerly protected by strands C and D, would become exposed as edge strands. The protein aggregated in inclusion bodies when expressed recombinantly in *Escherichia coli* but could be purified in its unfolded form. Although not unexpected, the insolubility of the truncated TTR makes it difficult to assess the degree to which the protein retains the remainder of its native structure and, therefore, the degree to which its behavior can be related to the behavior of wild-type TTR. Nonetheless, the behavior of the truncated form is notable. Although the protein becomes insoluble upon removal of the denaturant, at pH 7, it does not form amyloid fibrils (G. J. Forse, unpublished data). As is the case for native TTR, acidic conditions are required for amyloid formation. The truncated protein was able to form fibrils within a week at pH 4.75, and its amyloidogenicity was enhanced by the presence of up to 1 M urea. The fibrils bound thioflavin T and showed *β*-sheet character by circular dichroism. Their morphology under EM was that of irregular linear aggregates in a matted arrangement as is sometimes seen in wild-type TTR amyloid. The observation that fibrils can be formed by a variant of TTR entirely lacking the edge *β* strands further argues that conformational change or complete disordering of that region is likely a key event preceding the formation of new subunit−subunit associations.

The need for caution should be recognized in interpreting experiments, including those from our group, involving labeled or cross-linked proteins in their aggregated or fibril states. Chemical and genetic perturbations could lead to aggregated structures that differ from those formed by wild-type proteins. In addition, spectroscopic studies of intermolecular distances in the amyloid state are complicated by the difficulty of obtaining structurally homogeneous fibril preparations. Our experiments on amyloid fibrils of various TTR constructs have been conducted on samples that appear by EM to be similar to the wild type but which are decidedly heterogeneous in structure.

Possible Models for TTR Subunit Interactions

Several models have been proposed for how edge *β*-strand alterations in TTR might lead to assembly and fibril formation. These models include the *β*-slip mechanism, domain swapping, and assembly through newly exposed

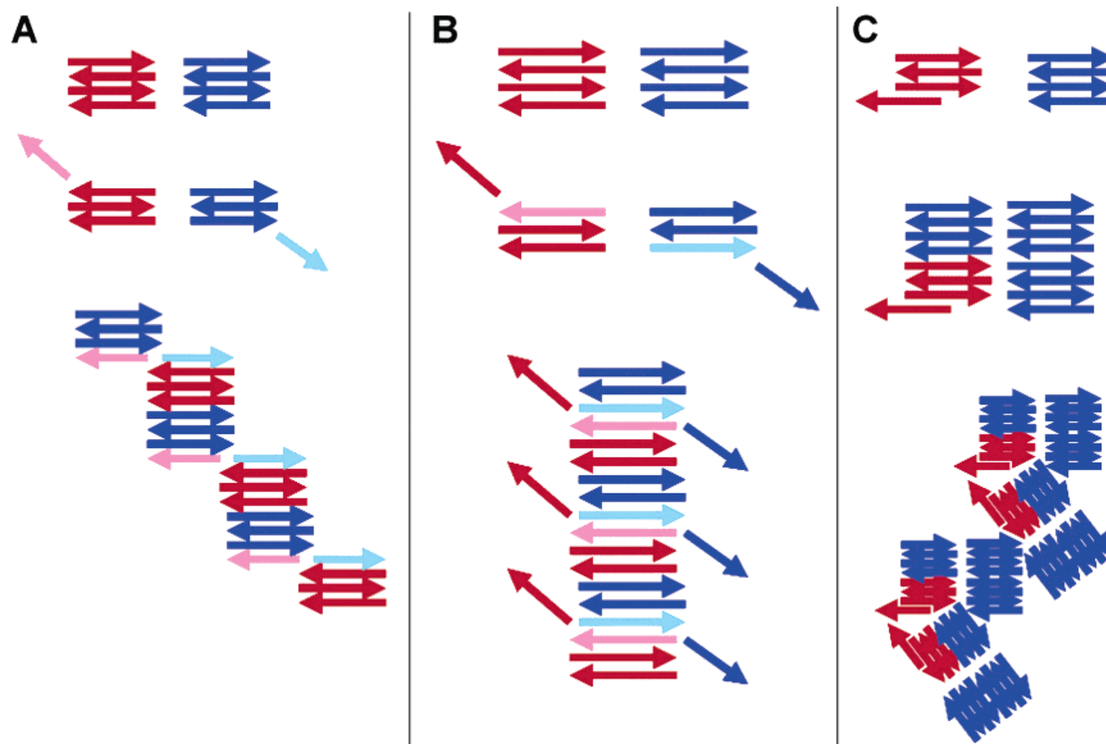

FIGURE 4. Schematic representation of different TTR fibril formation models. Groups of four β strands are intended to represent a native β sheet in the TTR monomer. (A) A domain-swapping model is illustrated in which a domain from one monomer (pink and light blue arrows) unfolds and then reforms native-like interactions with the corresponding region of the next monomer. No specific data are available at the present time to argue for domain swapping in TTR. (B) A model is shown in which a gain of interaction between two molecules is made possible by the displacement of an edge β strand (red and blue) to expose new sheet edges (pink and light blue).[34,49] (C) A "β-slip" model is shown in which a β strand misaligns in the tetramer (shifted red arrow), revealing a new region that can interact with a neighboring tetramer, leading to filament growth.[42] In the three models presented, assembly of an extended fibril involves native-like interactions that are preserved between two subunits of a dimer (red and blue).

β-sheet edge strands (Figure 4). In 2000, Eneqvist et al.[42] introduced the β-slip model based on revelations from the crystal structure of an engineered triple mutant of TTR (G53S/E54D/L55S) with mutations located in the D β strand. A dramatic shift in the positioning of the D strand was observed in the crystal structure, with a shift of residues 58–60 to the position normally occupied by residues 53–55. The location of the C strand remained the same, although the CD loop became longer and the DE loop became shorter. This slipped conformation of the D strand allowed new crystal contact interactions including hydrogen bonding of the BC loop, D strand, and DE loop with the retinol-binding sites of neighboring molecules to occur.[42] The β-slip model proposes that amyloidogenic mutants of TTR can undergo a β-strand register shift similar to the one observed in the engineered triple mutant. It is proposed that subunits could assemble by repeating the event in which an unstable slipped edge locks in place as an adjacent tetramer contacts the slipped tetramer at its retinol-binding site (Figure 4C). The authors note that two triplets of tetrameric building blocks fit together well and can form the nucleus of a helical protofibril. This model implies building a protofibril from tetrameric building blocks.

Another model for amyloid fibril formation is domain swapping, in which a domain or part of one protein dislodges from its monomeric subunit and then reforms equivalent interactions with another subunit by displacing its corresponding domain or part (Figure 4A). This mechanism could propagate to form filaments. Domain-swapping mechanisms of this type have been proposed in prion fibrils[46,47] and in some amyloid-like fibrils.[48] Although no direct evidence exists that TTR participates in domain swapping, the dislocated edge strands could in principle interact with another similarly destabilized subunit.

Finally, models based on edge exposure[40] fit well with much of the data on TTR. In a model of this type put forward by our group,[34,49] displacement of an edge strand (e.g., strand C) causes an inner strand (e.g., strand B) to become exposed, after which the newly exposed strand interacts with another similarly exposed inner strand from another molecule to form a continuous β-sheet structure (Figure 4B). Such a model is supported by electron paramagnetic resonance (EPR) studies by Serag et al.,[34,49] wherein cysteine mutagenesis and site-specific spin labeling were used to analyze the proximity of certain residues in the fibril state of TTR. Experiments on a series of cysteine mutants showed that, upon fibril formation, residues in the inner β strand B come into close proximity to equivalent B-strand residues in another molecule. The data are consistent with the formation of an antiparallel β interaction between the two B strands, with residue 31 being closest to the new symmetry axis introduced by the interaction (Figure 5).

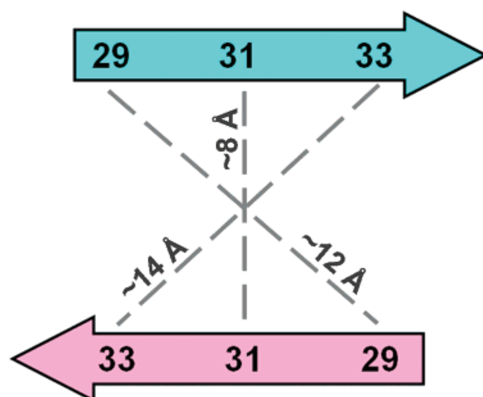

FIGURE 5. Spatial proximity between the B *β* strands of two TTR subunits in the amyloid state, as observed in EPR experiments by Serag et al.[34] The data support the formation of a new head-to-head interaction between subunits, with the axis of symmetry running near residue 31. As discussed in the text, this model requires displacement of the native edge strand C to expose strand B. The coloring corresponds to Figure 4B. This figure was adapted from Serag et al.[34]

One notable element of the edge exposure model proposed for TTR is the formation of a new symmetric dimer interaction between molecules. While this kind of interaction provides for a new connection between molecules, it does not by itself lead to indefinite fibril assembly. Because a symmetric dimer interaction does not propagate itself, additional interactions between molecules are required to form an extended filament. A key feature of TTR is that the native oligomeric structure suggests such additional interactions. If the native dimeric interface in TTR (i.e., involving strands F and H) is retained at least to some extent in the fibril state, then its combination with the new nonnative interface (i.e., involving strands B) would produce a linear arrangement of indefinite length.[49] Thiol-specific cross-linking and EPR data support the idea that a native *β*-sheet interface between F strands may be at least partially retained in the TTR fibril. A double cysteine mutant (89C/96C) in which the F strands were doubly cross-linked across the native dimer interface was competent to form fibrils. It was possible to show using EPR data that residues in strand F (e.g., residues 89 and 94), which are at the dimer interface in the soluble form of TTR, show similar spin−spin interactions in the fibril form[34,49] (Figure 5). The model that results implies the construction of TTR fibrils from dimeric species resembling the native dimer in certain respects. Whether a dimeric intermediate could be involved in TTR formation remains a point of discussion,[50−54] but the possibility is supported by evidence for disassembly of native TTR through a dimeric intermediate[49,55,56]

The combination of two symmetric interactions (i.e., head-to-head and tail-to-tail) as a potential mechanism for amyloid formation was first discussed by Serag et al.[34,49] It has since been invoked in other amyloid systems (see below). In addition, synthetic protein fibrils have been made from designed proteins following this principle of combined dimeric interfaces.[57]

Similar Principles in Other Amyloid Proteins

Some of the features present in models for TTR fibril formation are also seen in studies on other amyloid proteins. Connections can be drawn between TTR and other systems in which considerable native structure appears to be retained, edge *β* strands are involved in new interactions, and symmetric (i.e., head-to-head or tail-to-tail) interfaces are formed. Recent structural studies on *β* 2 microglubulin (B2M) provide a compelling example.[58] The crystal structure of a B2M mutant reveals a conformational rearrangement in which a short edge *β* strand flips over to undergo a one-residue register shift. The rearranged segment becomes a longer *β* strand, which pairs with another copy of itself in another monomer to create a new symmetric, dimeric interaction between subunits. Additional interactions at the other end of the B2M molecule that would be required to form a filament have not been characterized yet.

Head-to-head and tail-to-tail interactions leading to filaments have been demonstrated in at least two other systems. Human superoxide dismutase (SOD) is a *β*-sheet-rich protein that forms a dimer in its native form. Numerous mutations in SOD have been linked to ALS (Lou Gehrig's disease), and protein aggregation has been explored as an underlying mechanism.[59] A crystal structure of a SOD mutant has suggested that rearrangements at the edge of the molecule can lead to a new dimeric interaction between molecules. Two such dimeric interactions generate a protofibril following ideas described above for TTR.[34,49] The yeast prion protein Sup35 has also been shown to form fibrils using symmetric subunit interactions. Individual cysteine residues were labeled with pyrene maleimide, and symmetric interactions were observed by excimer formation by proximal pyrene molecules.[60] Those experiments parallel the use of EPR with site-directed spin labeling in earlier experiments on TTR.[49]

The amyloid protein cystatin C, which is a monomer in its native form, has also been shown to form dimers.[61] In this system, dimers are formed by a domain-swapping mechanism. Although the dimeric form is a symmetric structure created by the exchange of equivalent segments between two monomers, a model for cystatin amyloid has been proposed in which the domain swapping leads not to dimers but, instead, to a propagating swapping mechanism.[62] This type of runaway domain swapping has been demonstrated by Sambashivan et al. in fibrils formed by a variant of RNaseA, which was engineered to create an amyloid-like *β* spine upon domain swapping.[48]

Concluding Remarks

Successful strategies for inhibiting amyloid formation are likely to involve either stabilizing the native conformation of amyloid proteins or destabilizing their aggregated states. For TTR, Kelly et al. have demonstrated success with the first strategy.[25,28] The likelihood of success in applications of the second strategy will be increased by an understanding of the structural basis for amyloid formation.

Several issues point to the importance of understanding amyloid assembly at the level of detailed atomic interactions. The variation in organization of different amyloid proteins at the scale of the mature multiprotofibril fibril suggests that the unifying principles of amyloid formation may lie principally at the level of subunit interactions between individual protein molecules. Furthermore, increasing evidence supports the biological relevance of oligomeric assembly intermediates.[10,11] The difficulty of achieving atomic-resolution detail from EM and fiber diffraction highlights the role to be played by various methods able to provide information at the near-atomic level. X-ray crystallography studies on short amyloidogenic peptides are revealing important clues about how particular amino acid types might interact in amyloid fibrils,[15] although generalizing the results of peptide studies to full-length amyloid proteins is a challenging problem. In addition to various spectroscopic approaches,[18,63] methods aimed at mapping amino acid side-chain proximities in the amyloid state are providing information about subunit associations in larger proteins.[33,34,60,64]

Chemical cross-linking, EPR spin labeling, and excimer labeling have proven fruitful in examining subunit interfaces in fibrils of TTR[34,49] and Sup35.[60] In these two systems as well as others, analyses of non-native subunit−subunit interfaces have revealed symmetric (e.g., head-to-head) molecular arrangements, which in combination could lead to extended filaments. Among the various pitfalls of such studies is the likely presence of myriad atomic interactions in the aggregated state, including interactions within and between protofibrils[55] and even between larger fibrils. A more definitive view of subunit interactions could come from crystallographic studies on defined oligomeric assembly intermediates, if molecular species consisting of several subunits could be isolated. This strategy has been applied with some preliminary success to the structure determination of filamentous F-actin,[65] suggesting that efforts along these lines might be fruitful in studies of amyloid as well.

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