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The Structural Biology of Protein Aggregation Diseases: Fundamental Questions and Some Answers

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ABSTRACT

Amyloid fibrils are found in association with at least two dozen fatal diseases. The tendency of numerous proteins to convert into amyloid-like fibrils poses fundamental questions for structural biology and for protein science in general. Among these are the following: What is the structure of the cross-β spine, common to amyloid-like fibrils? Is there a sequence signature for proteins that form amyloid-like fibrils? What is the nature of the structural conversion from native to amyloid states, and do fibril-forming proteins have two distinct stable states, the native state and the amyloid state? What is the basis of protein complementarity, in which a protein chain can bind to itself? We offer tentative answers here, based on our own recent structural studies.

Amyloid and Amyloid-Like Fibrils

This Account focuses on fundamental questions of protein science that are posed by amyloid fibrils and offers some tentative answers based on our recent research. At the same time, we recognize that there is much complementary research in the field, some of which is summarized in other papers in this issue, as well as elsewhere.[1–11]

Protein aggregation diseases are pathologies accompanied by the deposition of aggregated proteins. The most prevalent aggregation diseases are the amyloid diseases,[12] associated with elongated, unbranched protein fibrils. To be defined by pathologists as an amyloid disease, the fibrils must be deposited extracellularly and must bind the dye Congo Red, giving an "apple-green" birefringence.[12] As of 2005, Alzheimer's disease and some 24 others have been found to satisfy this stringent definition.[12]

Biochemists and biophysicists consider a wider range of protein fibrils to be amyloid-like. The biophysical study of amyloid fibrils has revealed that they display common properties, in addition to their morphology and tendency to bind Congo Red. These other properties include the so-called cross-β X-ray diffraction pattern. This pattern consists of an X-ray reflection at $\sim$4.8 Å resolution along the fibril direction and another X-ray reflection at $\sim$10–12 Å resolution perpendicular to the fibril direction.[13–15]

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This pattern reveals that the fibrils contain β-sheets parallel to the fibril axis with their extended protein strands perpendicular to the axis. Another property of amyloid fibrils is that they form from their constituent protein molecules with cooperative, nucleation-dependent kinetics.[16] Because the fibrils associated with other diseases, such as Parkinson's and Huntington's (intracellular fibrils), display many of the same physical characteristics as the fibrils of the officially designated amyloid diseases,[17] biophysical chemists often refer to these fibrils also as amyloid-like fibrils.

It has been known for decades that normal globular proteins can be transformed into amyloid-like fibrils. These are fibrils that resemble those found in amyloid diseases but are not themselves associated with pathologies. In 1935, the pioneering biophysicist William Astbury stretched poached egg white and found that it exhibited the cross-β diffraction pattern.[13] More recently, numerous globular proteins have been converted to amyloid-like fibrils by removing them from their native temperatures and pH values.[18-21]

Fundamental Questions

The finding that numerous proteins can convert from their native structures to amyloid-like fibrils having common properties raises several fundamental questions for structural biology and for protein science in general:

(1) What is the structure of the cross-β spine, the molelcular feature that gives rise to the cross-β diffraction pattern, which is common to all amyloid and amyloid-like fibrils?

(2) Do amyloid-forming proteins have two distinctly different stable structures, their native state and the amyloid state? Can the amyloid state retain aspects of the native structure?

(3) What is the nature of the conversion of a protein from its native structure to the amyloid state?

(4) Is there a sequence signal for the formation of amyloid-like fibrils, or is the structure a generic backbone structure, where side chains do not contribute to the specificity of the structure?[22]

(5) What is the origin of protein self-complementarity, in which a protein binds strongly to itself, as in amyloid-like fibrils?

We provide tentative answers to these fundamental questions below, based on recent structural studies.

Microcrystal Structure of the Cross-β Spine of Amyloid-Like Fibrils

We chose the yeast prion Sup35p for X-ray diffraction studies of the cross-β spine because past work had shown that its fibril formation is the basis of protein-based inheritance and prion-like infectivity.[23-27] Based on earlier genetic studies that localized the amyloid-forming ability of Sup35p to the N-terminal segment,[28,29] we were able to identify a seven-residue peptide of sequence GN-NQQNY that forms amyloid-like fibrils with all of the common properties.[30] These properties include an elon-

gated, unbranched shape, the binding of the dye Congo Red with birefringence of a green-yellow hue, exhibition of the cross-β diffraction pattern, and lag-dependent aggregation of monomers into fibrils. Later we found that fibrils are formed by its subfragments NNQQNY and NNQQ.

All three of these short peptides formed microcrystals as well as fibrils, enabling the determination of crystal structures. The dimensions of these microcrystals never exceeded $\sim$50 μm $\times$ 4 μm $\times$ 4 μm, despite much effort to enlarge them. Fortunately, advances in microcrystallography[31] now make it possible to collect diffraction data from sturdy crystals of this size, and we were able to determine structures for GNNQQNY and Zn-NNQQNY to 1.8 and 1.3 Å resolution, respectively.[32] These high-resolution structures offered, for the first time, objective, refined atomic models for the cross-β spine of amyloid fibrils. While here we emphasize the results from this study, important contributions to understanding amyloid structure have come from other methods. These include solid-state NMR studies of various fibrils by the groups of R. G. Griffin,[33] D. G. Lynn,[34] R. Tycko,[35,36] and B. Meier;[9,37] EPR studies of fibrils by the groups of W. Hubbell, T. O. Yeates,[38] and R. Langen;[39] hydrogen/deuterium exchange studies by of the groups of Y. Goto[40] and R. Riek;[9,10] mutagenesis studies by the group of R. Wetzel;[11,41] electron microscopy studies by the groups of H. R. Saibil[42,43] and U. Aebi;[44] spectroscopic studies by the groups of S. Radford[45] and S. Lindquist;[5] X-ray fiber diffraction studies by the groups of L. Serpell,[2] E. Atkins,[1] and K. Namba;[4] and peptide design work by the group of L. Serrano.[46] Elsewhere we have reviewed the various models for amyloid that have come from this body of work by many investigators.[47]

The structure for the cross-β spine formed from GN-NQQNY is shown in Figure 1. The structure is essentially the same as that of Zn-NNQQNY (not shown here) in which the Zn ion occupies the position of the N-terminal glycyl residue in the longer peptide. Each GNNQQNY peptide is extended and forms one strand of a parallel, in-register β-sheet, which extends upward and downward for the entire length of the elongated crystal. Each β-sheet is paired with a second sheet around a completely dry interface that we call the steric zipper of the cross β-spine. These two sheets are related by the 2_1 axis shown in the figure: that is, one sheet can be superimposed on the other by a rotation about the axis by 180° and translation along the axis of one-half the interstrand spacing of 4.8 Å. The 2_1 symmetry relationship brings identical faces together so that the glutamine (Q) and asparagine (N) side chains protruding from the two sheets are tightly intermeshed, forming the steric zipper, as shown in Figure 1d.

This tight, dry interface between the two sheets of a pair-of-sheets motif is different in character from the crystal contacts between one of these pair-of-sheet motifs and its surrounding pairs (Figure 1c). The latter are wet interfaces, resembling the intermolecular contacts in protein crystals: they contain water molecules, and there are few contacts made between protein atoms in different
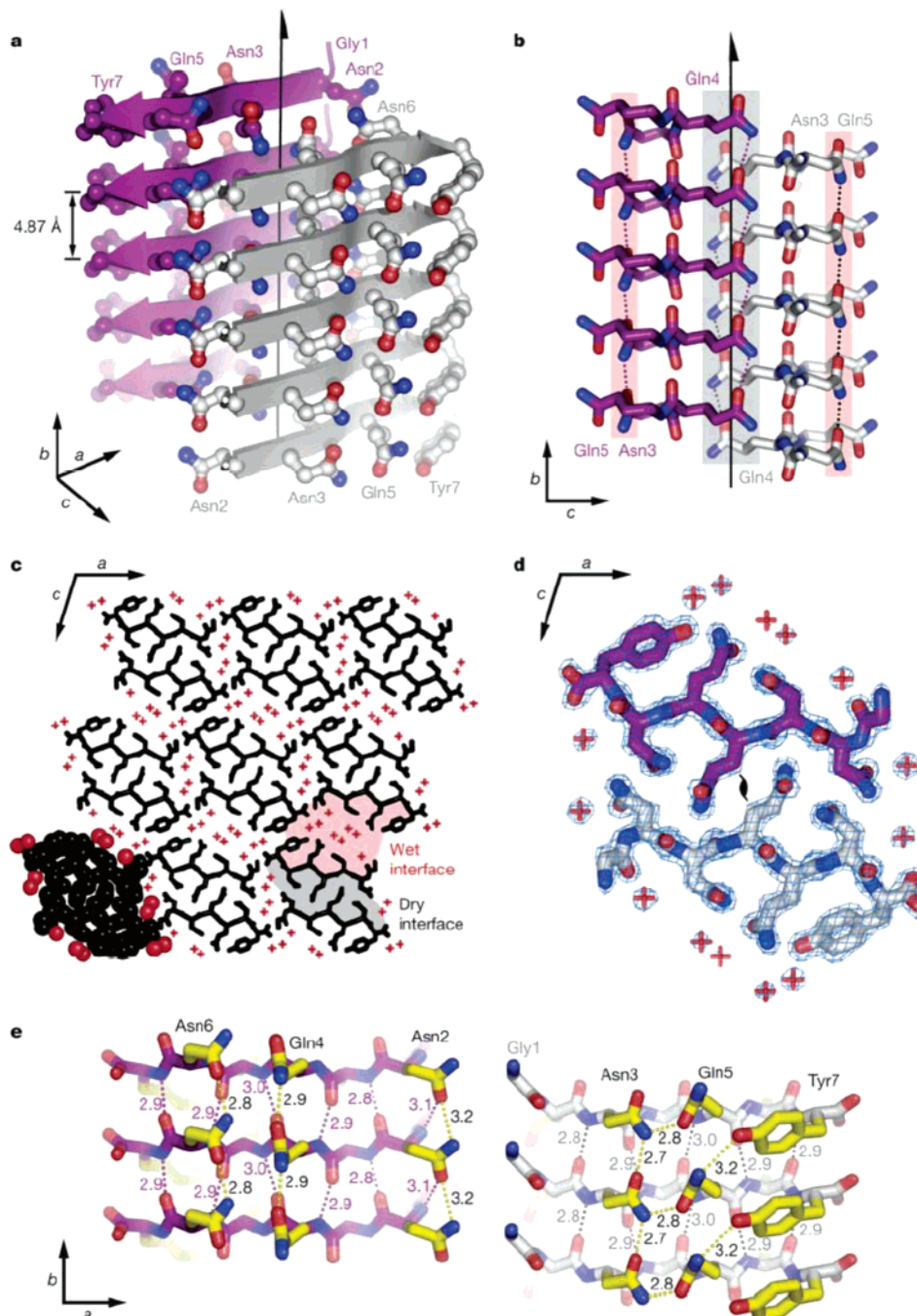
FIGURE 1. Structure of GNNQQNY. Panel a depicts the pair-of-sheets structure, showing the backbone of each β-strand as an arrow with side chains protruding. The dry interface is between the two sheets, and the wet interfaces are on the outside surfaces. Side chains Asn2, Gln4, and Asn6 point inward, forming the dry interface. The 2_1 screw axis of the crystal is shown as the vertical line. It rotates one of the strands of the near sheet 180° about the axis and moves it up $^1/_2 \times 4.87$ Å so that it is superimposed on one of the strands of the far sheet. Panel b shows the steric zipper viewed edge on (down the a-axis). Note the vertical shift of one sheet relative to the other, allowing interdigitation of the side chains emanating from each sheet. The amide stacks of the dry interface are shaded in gray at the center, and those of the wet interface are shaded in pale red on either side. Panel c shows the GNNQQNY crystal viewed down the sheets (from the top of panel a, along the b-axis). Six rows of β-sheets run horizontally. Peptide molecules are shown in black, and water molecules are red plus signs. The atoms in the lower left unit cell are shown as spheres representing van der Waals radii. Panel d shows the steric zipper. This is a close-up view of a pair of GNNQQNY molecules from the same view as panel c, showing the shape complementarity of the asparagine and glutamine side chains protruding into the dry interface. $2F_o - F_c$ electron density is shown, and the position of the central screw axis is indicated. Panel e provides views of the β-sheets from the side (down the c-axis), showing three β-strands with the interstrand hydrogen bonds. Side-chain carbon atoms are yellow. Backbone hydrogen bonds are shown by purple or gray dots and side-chain hydrogen bonds by yellow dots. Hydrogen bond lengths are noted in Å. The views of the interfaces are close to the views of panel a. The left-hand set is viewed from the center of the dry interface; the right-hand set is viewed from the wet interface. Note the amide stacks in both interfaces. Carbon atoms are purple or gray, oxygen is red, and nitrogen is blue, unless noted otherwise. Reprinted with permission from *Nature* (http://www.nature.com), ref 32. Copyright 2005 Nature Publishing Group.

pairs. Thus we regard a pair of β-sheets mating in a dry steric zipper as the fundamental structural motif of the cross-β spine.

The view of the pair-of-sheets unit shown in Figure 1d is down the sheets. Notice the tight interdigitation of the glutamine and asparagine side chains from positions 2, 4, and 6 of each strand with the same side chains from the mating sheet. A measure of the structural complementation of one protein surface with another is given by the S_C parameter of Lawrence and Colman,[48] a quantity that can vary from 0 up to 1 for perfect complementation of two surfaces. The two surfaces of the mating sheets in GNNQQNY have a value of 0.86, considerably higher than the tight surfaces between proteases and their protein inhibitors, which receive an average value of S_C of 0.73 ± 0.03 (ref 48) or of the average value of S_C of 0.66 ± 0.02 for the binding surfaces of antibodies to their antigens. In short, the surfaces of the two mating sheets are unusually tight-fitting as shown by the black van der Waals representation in the lower left unit cell of Figure 1c.

The GNNQQNY peptides in one sheet form no hydrogen bonds to the peptides in the mating sheet, but each forms 11 hydrogen bonds to the identical molecules above and below it in the same sheet. Five of these are backbone N−H⋯O hydrogen bonds, and five are hydrogen bonds between side chains. Notice that the side chain amide groups of each molecule are oriented in the same direction as those above and below, creating columns of hydrogen bonds running up and down the sheets. These are reminiscent of the "polar zipper" hydrogen bonds proposed for polyglutamine fibrils by Perutz et al.[49,50] and Sikorski and Atkins.[1]

A view of the pair of sheets in the direction down the strands is given in Figure 1b. This shows the interdigitation of the side chains of the glutamine residues, meshing much like the teeth of the zipper. Because of their tightly complementary fit, we term this the steric zipper. Notice that the steric zipper is completely dry. The water molecules in the crystal are found on the outer surface of the pair-of-sheets motif.

Energetics of the Cross-β Spine

Knowing the structure of the cross-β spine allows us to understand some of the energetics and kinetics of formation. The structure suggests three levels of organization within the fibrils. The first level is the alignment of individual GNNQQNY molecules to form a β-sheet. The second level is the mating of two sheets, forming the pair-of-sheets structure with its dry interface. The third level winds the pair-of-sheets structures around each other to form a fibril. The noncovalent forces involved in this third level are probably weaker than those driving the formation of the first two levels.

β-sheets form rapidly[51,52] and reversibly, so we assume that the individual sheets form more rapidly than the pairing of sheets. The pairing is likely to be slower because the amide side chains must acquire their proper rotamers to allow interdigitation with the opposite sheet. We believe the entropy reduction accompanying this step creates the barrier to fibril formation, which is seen in the lag-dependent cooperative formation. Once a nucleus of the cross-β spine has formed, other molecules can be added more rapidly, provided the concentration of monomers is high enough. We have argued from the structure that the nucleus for the pair-of-sheets structure is about four molecules, so that the transition-state complex on the path to the nucleus is approximately three molecules.[32] From crude energy calculations,[32] we estimate that the free energy of forming this complex is ∼8 kcal mol⁻¹ of monomer. Thus if there are three molecules in the transition-state complex, the barrier is ∼24 kcal mol⁻¹. A transition-state barrier on the order of 24 kcal mol⁻¹ is substantial enough to make nucleation a rare event and hence to give an appreciable lag time before fibers start to grow.

Our crude analysis of the standard free energy change of the order of +8 kcal mol⁻¹ for the addition of a monomer to the growing of the nucleus[32] suggests that fibrils are not "intrinsically stable" in the sense of having a sizable negative free energy change for addition of monomers to the fibril. According to this analysis, the fibrillar state becomes the stable state only at high monomer concentration. If this is so, fibril formation from monomers is different from the formation of an oligomeric enzyme from its monomers: in the latter case, there is a more negative free energy change, so that oligomerization from monomers takes place at lower monomer concentrations. There is, however, a kinetic barrier to cross either in the formation or the dissolution of cross-β spine. This means that once formed, amyloid structure could take a long time to dissolve, even when the monomer concentration is reduced, because the molecules would be kinetically trapped in the fibril state.

Designed Amyloid-Like Fibrils of Ribonuclease A Have a Three-Dimensional, Domain-Swapped, Native-Like Structure

To gain insight into the structural changes that take place when entire proteins enter amyloid-like fibrils, we designed an amyloid-like fibril based on ribonuclease A (RNase A, shown in Figure 2a). We selected RNase A because it is well characterized, it is tightly cross-linked by four disulfide bonds, and it forms a domain-swapped dimer when concentrated in acetic acid.[53−55] Domain swapping is a mechanism for forming oligomers by the exchange of protein domains. The swapped domain is in some cases a helix or β-strand and in other cases an entire tertiary domain and is linked to the rest of the protein by a segment of chain called the hinge loop. From the structure of the RNase A dimer, shown in Figure 2b, we reasoned[53] that expanding the hinge loop connecting the core domain with the swapped domain by inserting an amyloidogenic segment might permit the formation of a domain-swapped amyloid-like fibril. A schematic diagram of such a structure is shown in Figure 2c. It contains a cross-β spine in the center, in this case formed from an
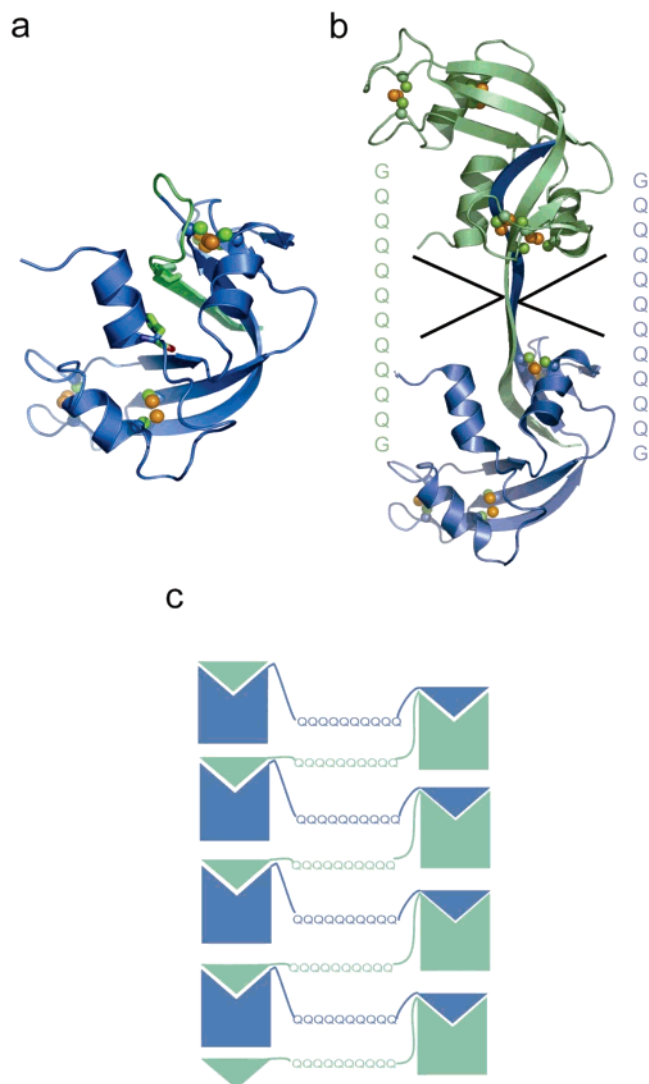
a

b

c

G
Q
Q
Q
Q
Q
Q
Q
Q
Q
Q
G

G
Q
Q
Q
Q
Q
Q
Q
Q
Q
Q
G

FIGURE 2. Ribonuclease A monomer and domain-swapped C-terminal dimer and the 3D domain-swapped zipper-spine model. Shown in panel a, the ribonuclease A monomer is stabilized by four disulfide bonds between Cys26 and Cys84, Cys40 and Cys95, Cys58 and Cys110, and Cys65 and Cys72, hindering conformational changes. His12 in the core of the protein and His119 on the β-strand that is swapped (shown by sticks) are active site residues mutated to test for activity by complementation. Shown in panel b, the C-terminal domain-swapped dimer is formed by exchanging the C-terminal β-strands between two monomers. The hinge loop (residues 112–115) has been expanded by inserting the sequence $-GQ_{10}G-$. Panel c shows the schematic model for amyloid-like fibril formation in RNase A with Q_{10} expansion, leading to a runaway domain swap. In blue are the Q_{10}-H12A mutants and in green the Q_{10}-H119A mutants. Domain swapping between two mutants complements active sites. Reprinted with permission from *Nature* (http://www.nature.com), ref 56. Copyright 2005 Nature Publishing Group.

expansion of 10 glutamine residues. At the periphery of the spine are RNase A molecules, each formed from a core domain and a complementing C-terminal β-strand from another RNase A molecule. If such a structure forms, we would expect that it would be fibrous and would contain native-like domains capable of enzymatic activity.

These expectations were fulfilled by the construction of such an expanded RNase A molecule.[56] RNase A

molecules into which we inserted a Q_{10} expansion in the C-terminal hinge loop in fact formed amyloid-like fibrils that bind Congo Red and display the cross-β diffraction pattern. RNase A with a GQ_7G expansion or with an expansion of the Sup35p sequence GNNQQNY also forms amyloid-like fibrils. In contrast, wild-type RNase A did not form fibrils, nor did a polyglycine (G_9) expansion. In short, amyloidogenic sequences inserted into the C-terminal hinge loop of RNase A produce amyloid-like fibrils.

Are functional RNase A molecules formed by domain-swapping in these fibrils? To answer this question, we measured the enzymatic activities of mutant RNase A fibrils, both alone and mixed. For more than 40 years it has been known that the active site of RNase A contains two histidyl residues: His12, in the core domain, and His119, in the C-terminal β strand.[54] This strand is swapped into the core of a second molecule in the domain-swapped dimer shown in Figure 2b. We used this old finding to establish that domains are swapped in the fibrils. The mutant RNase A molecules H12A and H119A were separately prepared for RNase with Q_{10} expansion. Both of these molecules form fibrils but lack enzymatic activity, because neither has a complete active site. When the two molecules are mixed and fibrils are formed, about $^1/_8$ of the wild-type activity returns. We presume this activity of the mixed fibril comes from complemented active sites, which have both H12 and H119. If the reconstitution of the active sites were perfect, then we would expect $^1/_4$ of full activity. Presumably there are losses because of imperfect refolding and imperfect stoichiometry of mixing. Nevertheless, the observed activity is enough to conclude that there are active RNase A molecules in the fibrils and that these are active because of domain swapping.

Because enzymatic activity is perhaps the best measure of native structure, we are forced to conclude that the RNase A fibrils contain molecules in their native state and that the fibril contains both a cross-β spine and domain-swapped molecules. Based on this conclusion, we built a hypothetical atomic model for the RNase A amyloid-like fibril shown in Figure 3, which contains domain-swapped functional units. In this model, the spine of the structure is a twisted pair of antiparallel β-sheets. Each β-strand is the Q_{10} insertion in the hinge-loop of RNase A. These polyglutamine β-strands are stacked 4.8 Å apart along the fibril axis (Figure 3c). A slight twist is introduced between successive segments of the spine to be consistent with the measured twist of the fibrils. Each Q_{10} segment forms hydrogen bonds to identical segments above and below within each sheet but not between sheets. The two sheets are held together by a steric zipper of the Q_{10} side chains (Figure 3B). This model depicts the native fold of RNase A as being essentially retained with only a small segment of the protein (the Q_{10} hinge loop) forming the cross β-spine. Evidence of native-like character in the fibril forms of Ure2p[57−59] and β2-microglobulin[60] has been uncovered in other laboratories.
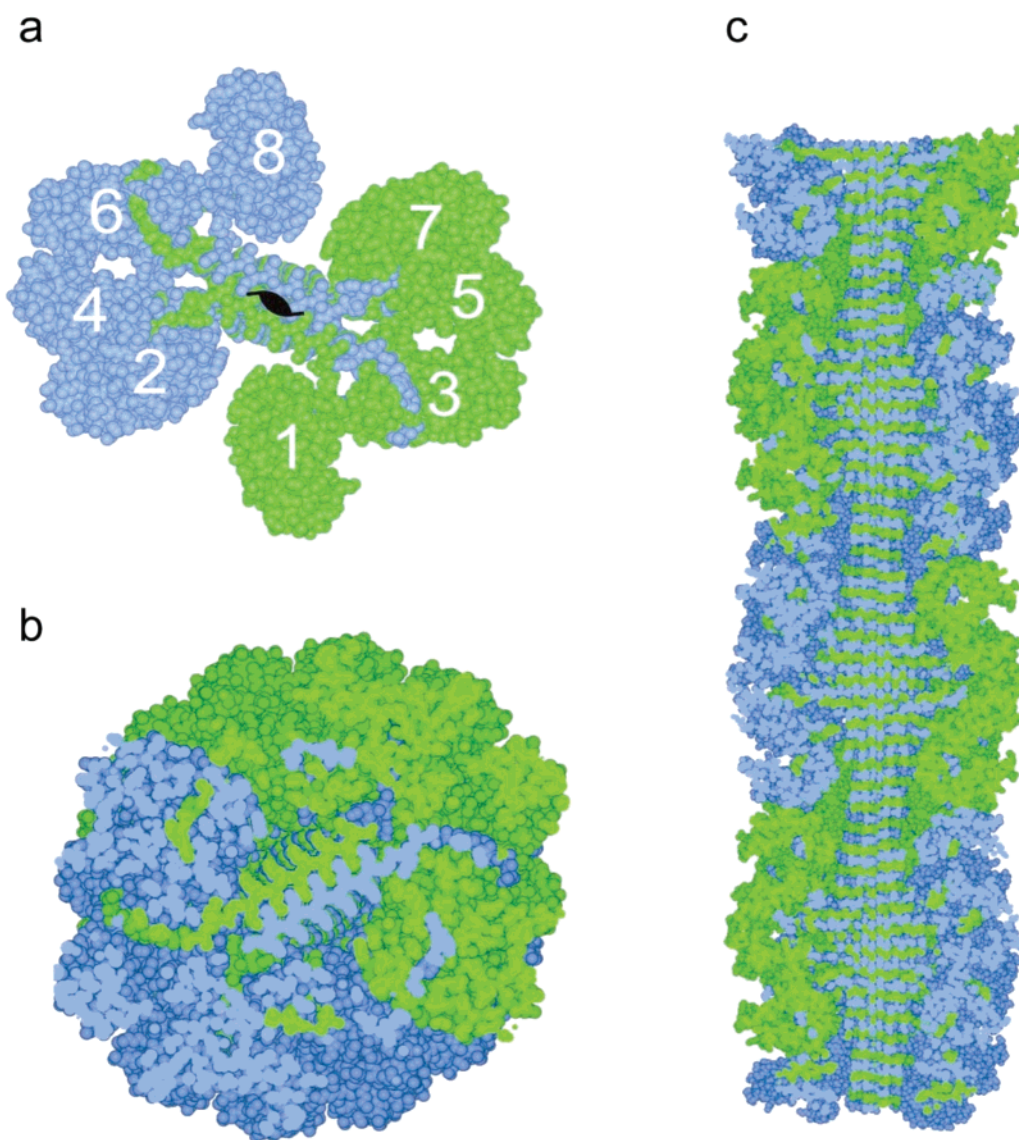
FIGURE 3. Domain-swapped zipper-spine model for the RNase A protofibril. Shown in panel a, the model is a "runaway" domain swap between the RNase A monomers with swaps occurring within one half protofibril but not between half protofibrils. Monomers 1–4 compose half the protofibrillar unit and are colored as in Figure 1c to emphasize domain swapping. The C-terminal β-strand of monomer 1 swaps into 2, 2 swaps into 3, and 3 swaps into 4, rising along the axis of the fibril. Q_{10} segments from these monomers form one antiparallel β-sheet in the spine. Monomers 5–8 form the other β-sheet, related to the monomers 1–4 by a 2_1 axis along the fibril. Eight RNase A monomers comprise the asymmetric unit of the fibril. A similar model can be built from domain-swapped dimers, and currently available data do not favor one of these models over the other. Shown in panel b, the protofibril cross-section reveals the steric zipper, the interdigitation of glutamine side chains in the spine of the fibril, modeled on the structure of GNNQQNY. Panel c shows the protofibril model in longitudinal cross-section. The zipper spine is seen at the center of the protofibril. Reprinted with permission from *Nature* (http://www.nature.com), ref 56. Copyright 2005 Nature Publishing Group.

Summary and Tentative Answers to the Fundamental Questions

Based on the studies described above, we offer tentative answers to the fundamental questions raised at the start of this review:

1. What is the structure of the cross-β spine common to all amyloid and amyloid-like fibrils? Both GNNQQNY and NNQQNY form fibrils and microcrystals, each with their β-strands perpendicular to the long axis. The atomic-level structures of the two microcrystals offer objective pictures of the cross-β spine. In both structures, the spine consists of a pair of β-sheets mated tightly together with their side chains intermeshed in what is termed a steric zipper. Each peptide forms one β-strand of a sheet. The sheets are parallel with strands in register. Also the two sheets are in register. There seems to be no fundamental reason that other amyloid structures need to be built of parallel sheets; they could instead be built from antiparallel sheets. Nor is there any basic reason that the sheets need to be in register with one another; they could slip along the strand directions. The more fundamental feature appears to be the dry steric zipper motif.

2. Do amyloid-forming proteins have two distinctly different stable structures, their native state and the amyloid state? In the case of the designed amyloid-like fibrils of RNase A, the native and amyloid states resemble each other in that both contain native-like functional units. In the amyloid form, the functional units are domain-swapped RNase A units made up of complementary domains from two RNase A molecules. The argument for native-like domains rests on the observation of enzymatic activity in the fibrils. So at least in the example of the RNase A fibrils, there is no fundamental structural change between the native and fibrillar states, except that the hinge loop that links each RNase A core domain to its swapped domain participates in the cross-β spine. Evidence for retention of native-like structure has also been demonstrated by others for fibrils of Ure2p[58,61] and β2-microglobulin.[60] To the extent that native-like structures are found in general as part of amyloid-like fibrils, the changes in structure would be mainly confined to the segments forming the steric zipper.

3. What is the nature of the conversion of a protein from its native structure to the amyloid-like fibrillar state? In the case of the RNase A fibrils, the process of conversion would start with a breaking of noncovalent bonds between the C-terminal β-strand of the enzyme and the core, and then its swapping into the core of a second molecule. This would expose the Q_{10} expansion loops, which would be free to stack into a cross-β spine.

4. Is there a sequence signal for the formation of amyloid-like fibrils, or is the structure a generic backbone structure? The tightly complementary cross-β spine with the intermeshing side chains suggests that spine formation would be sequence-dependent, favoring sequences that can intermesh over those that cannot. Thus low complexity sequences (such as those containing many asparagine and glutamine residues) would be favored. Highly charged sequences for residues in the inward-pointing positions would be disfavored. Additional support for the importance of protein sequence in fibril formation has come from both computational[62] and experimental[46,63,64] studies.

5. What is the origin of protein self-complementarity, in which a protein binds strongly to itself, as in amyloid-like fibrils? Our work to date has revealed three types of self-complementarity in amyloid-like fibrils. The first is the steric zipper of the cross-β spine. It seems likely that this motif will be found in other amyloid-like fibrils. The second is the domain swap. A domain swap is not necessary for the formation of a fibril, but it may be found in some instances.[3,56,60,65] Domain swapping has the feature that it is protein specific. That is, a domain swap can occur only with a molecule having an identical domain. Thus domain swapping can account for the observation that amyloid fibrils tend to be formed from a single protein. If instead amyloid structure were independent of side chains and had a generic backbone structure, there would be no reason that amyloid fibrils could not contain more than a single type of protein. The third type of complementarity we have seen is the stacking of side chains in "polar zippers", such as shown in Figure 1b. Side chain stacking, with hydrogen bonding between side chains in strands on top of one another, can arise only for parallel, in-register β-sheets with side chains capable of stacking, such as asparagine and glutamine. So this type of complementarity is not a necessary feature of amyloid, and we would expect to find it only with sequences containing "stackable" residues, such as asparagine and glutamine.

We expect that new features of amyloid structures will be revealed in future atomic-level structures and that these structures will give us a more detailed and general picture of features involved.

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