LETTERS

Amyloid-like fibrils of ribonuclease A with three-dimensional domain-swapped and native-like structure

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Amyloid or amyloid-like fibrils are elongated, insoluble protein aggregates, formed *in vivo*[1] in association with neurodegenerative diseases or *in vitro*[2] from soluble native proteins, respectively. The underlying structure of the fibrillar or 'cross-β' state has presented long-standing, fundamental puzzles of protein structure. These include whether fibril-forming proteins have two structurally distinct stable states, native and fibrillar, and whether all or only part of the native protein refolds as it converts to the fibrillar state. Here we show that a designed amyloid-like fibril of the well-characterized enzyme RNase A contains native-like molecules capable of enzymatic activity. In addition, these functional molecular units are formed from a core RNase A domain and a swapped complementary domain. These findings are consistent with the zipper-spine model[3] in which a cross-β spine is decorated with three-dimensional domain-swapped functional units, retaining native-like structure.

The discovery that numerous native proteins can be converted to the amyloid-like fibrillar state[4] seems to challenge a central pillar of protein science. This is Anfinsen's 'thermodynamic hypothesis'[5], which states that protein sequence determines a unique structure of lowest free energy. Proteins in the fibrillar, amyloid-like state have common properties[6]: these include an elongated, fibrillar morphology; a 'cross-β' diffraction pattern, indicative of β-strands perpendicular to the fibril axis and β-sheets parallel to the axis; and binding of flat dyes, giving common tinctural properties. From this it has been inferred[2] that "amyloid is a generic structural form of proteins", stabilized by peptide backbone bonding. Is this proposed generic amyloid, in which backbone bonding dominates a second type of structure, in conflict with the thermodynamic hypothesis in which side-chain interactions determine a single stable state for each protein?

The fibrillar nature of the amyloid state has complicated structural studies. Cryoelectron microscopy has been able to resolve helical protofilaments forming the fibril[7,8], and in one case it has been suggested that native domains must unfold in the amyloid form[7]. In some studies (for example, ref. 9), domains seem to decorate the surface of the spine. A currently popular molecular model for the amyloid-like protofibrils depicts part or all of the native protein as refolding into a parallel, left-handed β-helix[10–13]. Another type of model invokes three-dimensional (3D) domain swapping to account for the protein self-specificity of amyloid interactions[14,15] and cohesion. For cystatin amyloid fibrils, domain swapping has been supported by biochemical experiments[16–19]. In short, despite progress, there is as yet little consensus on major questions of amyloid structure, including whether native-like domains are retained or are refolded in the fibrils.

Here we address this question with RNase A, the same protein whose spontaneous refolding in solution led to the original thermodynamic hypothesis[20]. The RNase A molecule has three properties that make it convenient for answering this question. First, it is tightly cross-linked by four disulphide bonds, severely restricting conformational change (Fig. 1a). Second, RNase A forms two types of 3D

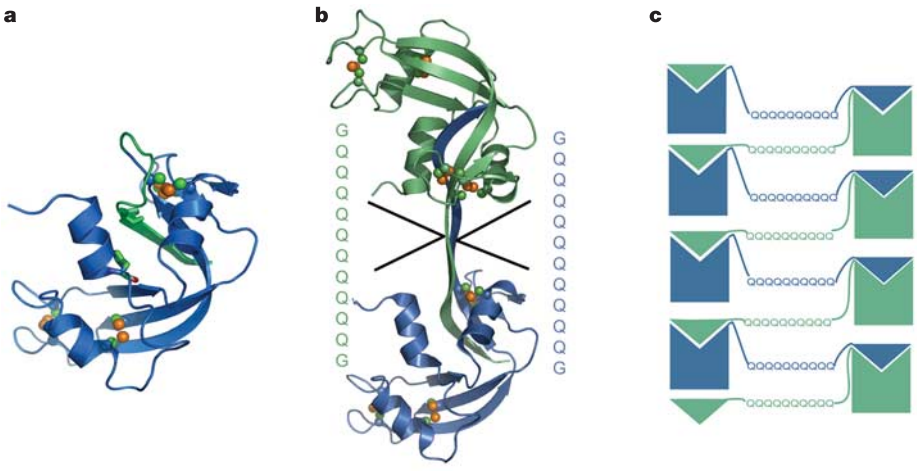

Figure 1 | RNase A monomer and C-terminal domain-swapped dimer and the 3D domain-swapped zipper-spine model. a, The RNase A monomer is stabilized by four disulphide bonds Cys 26–Cys 84, Cys 40–Cys 95, Cys 58–Cys 110 and Cys 65–Cys 72, hindering conformational changes. His 12 in the core of the protein and His 119 on the β-strand that is swapped (shown by sticks) are active-site residues that we mutate to test for activity by complementation. **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 -$GQ_{10}G$-. **c**, Diagram of amyloid-like fibril formation in RNase A with Q_{10} expansion, leading to a runaway domain swap. The Q_{10}-H12A mutants are shown in blue and the Q_{10}-H119A mutants in green. Domain swapping between two mutants complements active sites.

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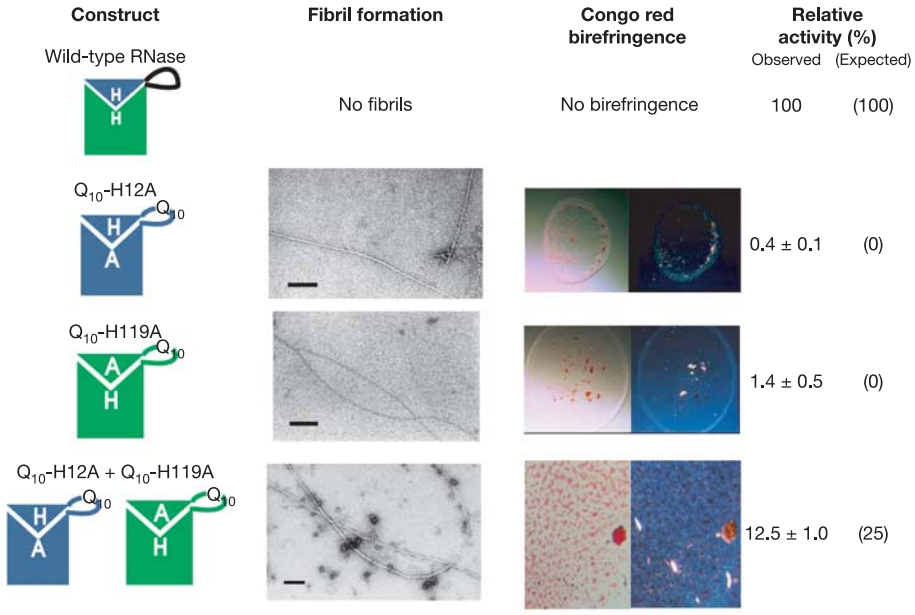

Figure 2 | Properties of the RNase A amyloid-like fibrils. The hinge-loop region of wild-type RNase A that connects the C-terminal β-strand (triangle in the diagrams in column 1) to the protein core is expanded with the -GQ_{10}G- motif to generate amyloid-forming RNase A mutants. Two inactive RNase A mutants are formed by replacing His 12 or His 119 with Ala. Wild-type RNase A does not form fibrils and has a fully functional active site (row 1, columns 2 and 4). The Q_{10}-H12A, Q_{10}-H119A and Q_{10}-H12A + Q_{10}-H119A constructs all form amyloid-like fibrils (column 2) and bind Congo red with the characteristic apple-green birefringence (column 3). The Q_{10}-H12A + Q_{10}-H119A fibrils (row 4, column 4) have significantly higher activity than fibrils of Q_{10}-H12A (row 2, column 4) and Q_{10}-H119A (row 3, column 4) alone. This is a result of complementation of active sites by domain swapping. The expected activity of each of the constructs is given in parentheses. Scale bar (column 2), 200 nm.

domain swaps when freeze-dried from 40% acetic acid[3,21,22]. In one of these (Fig. 1b) it exchanges its carboxy-terminal β-strand with that of an identical molecule. From the structure of the C-terminal swapped RNase A dimer, it has been speculated[3] that if the hinge loop (residues 112–115) connecting the core domain (residues 1–111) with the swapped domain (residues 116–124) were expanded by insertion of an amyloidogenic segment, then a domain-swapped amyloid-like fibril might form, as depicted schematically in Fig. 1c. Third, one of the two catalytic His residues of RNase A (at position 12) is on the core domain of the model (Fig. 1c), whereas the other (at position 119) is on the swapped domain. This segregation of the catalytic His residues on different domains offers the possibility of forming an active, complementary dimer from two inactive RNase A monomers, as explained below.

Before the determination of the structure of RNase A, it was found[21] that when inactive RNase A, alkylated at His 12, was freeze-dried with inactive RNase A, alkylated at His 119, the resulting oligomers had restored activity. The conclusion was that "the

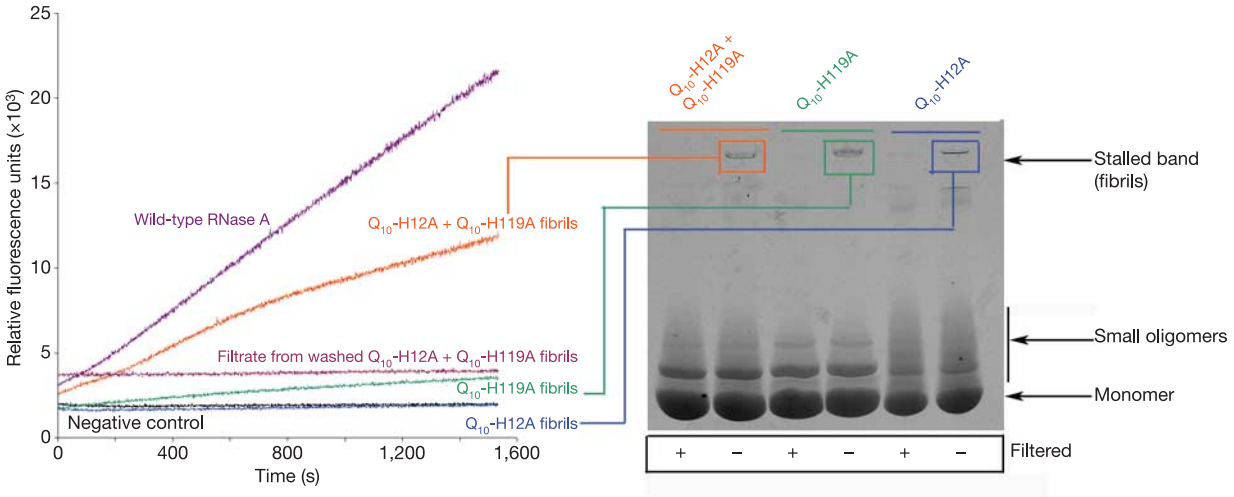

Figure 3 | Fluorescence-based activity assay to examine whether the RNase A amyloid-like fibrils are domain-swapped. The amyloid-like fibrils are separated from smaller oligomers by subjecting the samples to electrophoresis on a non-denaturing gel (right). The fibril-containing stalled bands (confirmed by in-gel Congo red assays) are assayed for activity in solution by a fluorescence assay (left). The slope of each of the traces corresponds to the amount of product formed per unit time. Thus the slope per mg of the enzyme corresponds to the specific activity of the enzyme. The Q_{10}-H12A (blue) and Q_{10}-H119A fibrils (green) have slopes comparable to the negative control (black) and thus a specific activity comparable to background. The Q_{10}-H12A + Q_{10}-H119A sample (orange) has a much steeper slope and hence a significantly higher specific activity (Fig. 2, column 4) than background.

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histidine residues at positions 119 and 12 both form part of the active site of the enzyme", and foreshadowed the discovery of 3D domain swapping by 30 years. Here we use a variation of this experiment, with either His 12 or His 119 replaced by Ala in hinge-loop-expanded RNase molecules, to demonstrate native-like domains and domain swapping in amyloid-like RNase A fibrils.

We converted RNase A to the fibrillar form by expanding the hinge loop after Gly 112 with an insert of ten glutamine residues flanked by a glycine residue on each side ($GQ_{10}G$). When freeze-dried from 50% acetic acid, the constructs with the Q_{10} expansions form fibrils (Fig. 2, column 2) that bind the dye Congo red and show 'apple green' birefringence characteristic of amyloid (Fig. 2, column 3). One of these amyloid-like fibrils (Q_{10}-H119A; Fig. 2, row 3) was subjected to X-ray fibre diffraction and showed the characteristic strong reflection at 4.8 Å and the diffuse reflection at 11 Å resolution (Supplementary Fig. S1). These observations indicate that the RNase A molecules with Q_{10} expansions in the C-terminal hinge loop form amyloid-like fibrils on freeze-drying. RNase A with a GQ_7G or a GNNQQNY (an amyloidogenic motif from the yeast Sup35 prion) expansion also forms amyloid-like fibrils. In contrast, wild-type RNase A did not form fibrils in more than a dozen experiments, as judged by both electron microscopy and silver-stained non-denaturing gels (Supplementary Fig. S2 and Supplementary Table 1); nor did a Gly_9 expansion of RNase A, having nine glycine residues after Gly 112, form fibrils.

The crystal structure at 1.8 Å resolution of the Q_{10}-H119A RNase A before freeze-drying reveals an RNase A molecule virtually identical to the wild type, except that the hinge loop, including the Q_{10} expansion, is disordered and invisible in the electron density (data not shown). This shows that the Q_{10} expansion does not alter the basic features of monomeric RNase A, as expected from the four disulphide bridges that stabilize the monomer.

What happens to the structure of Q_{10}-expanded RNase A on freeze-drying, as fibrils form? One possibility is that the Q_{10} segments stack together to form a β-sheet, with the RNase A molecules otherwise unaltered. A second possibility is envisaged in the zipper-spine model (Fig. 1c)[3]: the C-terminal β-strand of each RNase A monomer breaks its non-covalent bonds with the core domain, exposing the Q_{10} expansion more fully, and the C-terminal β-strand of another molecule swaps in to take its place, forming a domain-swapped functional unit of RNase A. The Q_{10} expansion loops can then stack into a zipper spine[3,23], with the core domains and C-terminal β-strands complementing each other at the periphery of the spine.

We distinguished between these two possibilities by testing the activities of fibrils formed from inactive Q_{10}-H12A RNase A and Q_{10}-H119A RNase A, alone or mixed. The fibrils formed from each of the inactive RNase A molecules are themselves inactive (Fig. 2, rows 2 and 3). However, when these two inactive RNase A constructs are mixed and then freeze-dried, the resulting fibrils now show significant RNase enzymatic activity (Fig. 2, bottom row). This activity must be the result of complementation between RNase A molecules, composite active sites having been formed by domain swapping between the H12A and H119A mutants (as shown schematically in Fig. 1c). That is, the RNase A amyloid-like fibrils are apparently made up of 3D domain-swapped RNase A molecules.

There are two conceivable alternative explanations for the activity attributed to the amyloid-like fibrils, both of which we have ruled out. One alternative is that domain-swapped complementary small oligomers cling to the fibrils in a non-specific manner, resulting in apparently active fibrils. Alternatively, there might be large, non-fibrillar, domain-swapped, active aggregates in the stalled band (Fig. 3, right panel). That is, the stalled band might contain two species: inactive amyloid-like fibrils and active domain-swapped aggregates. From a series of control experiments (see Supplementary Information) we conclude that that activity of the mixed fibrils (Fig. 2, bottom row, and Fig. 3, orange trace) comes from complemented active sites in the fibrils and not from smaller clinging oligomers or larger domain-swapped non-fibrillar aggregates. This means that the RNase A amyloid-like fibrils contain native-like, domain-swapped functional units.

We propose a model (Fig. 4) for the RNase A amyloid-like fibril, based on our biochemical data, suggesting 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 10-glutamine expansion from the hinge-loop segment of RNase A. The β-strands are stacked 4.88 Å apart along the fibril axis (Fig. 4c), as in the crystal structure of the cross-β spine[23]. A 7° twist is introduced between successive Q_{10} segments of the spine to account for the twisted appearance of the fibrils (Fig. 2). Each Q_{10} expansion forms hydrogen bonds to identical segments both above and below within the sheet but not between the sheets. Instead, the Q_{10} side chains from one sheet interdigitate with those from the other sheet, forming what we term a steric zipper[23] (Fig. 4b). The 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.

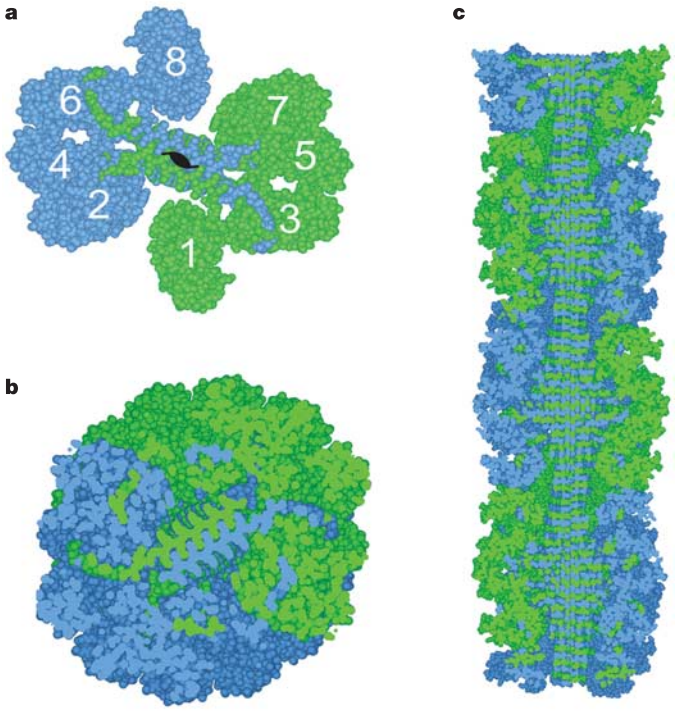

Figure 4 | Domain-swapped zipper-spine model for the RNase A protofibril. **a**, The model is a 'runaway' domain swap between the RNase A monomers, with swaps occurring within one half protofibril but not between half protofibrils. Monomers 1–4 compose half the protofibrillar unit and are coloured as in Fig. 1c to emphasize domain swapping. The C-terminal β-strand of monomer 1 swaps into monomer 2, monomer 2 swaps into monomer 3, and monomer 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 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; the currently available data do not favour one of these models over the other. **b**, The protofibril cross-section reveals the steric zipper, the interdigitation of Gln side chains in the spine of the fibril, modelled on the structure of GNNQQNY[23]. **c**, A cut-away view perpendicular to the fibril axis reveals the stacking of hydrogen-bonded Q_{10} β-strands (4.88 Å apart) in the spine. The spine is largely shielded from solvent by the tight packing of globular domains around the periphery. The fibril model ranges from 100 to 140 Å in diameter, which agrees with the diameter of the fibrils obtained from electron microscopy images (Fig. 2, column 2).

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The model of RNase A amyloid of Fig. 4 is compatible with the thermodynamic hypothesis[5], provided that we take a wider than usual view of what is meant by "the three-dimensional structure of a native protein in its physiological milieu"[5]. In domain-swapped structures, such as the RNase A dimer of Fig. 1b or the model of Fig. 4, RNase A molecules differ considerably in conformation from the monomer (compare the chains in Fig. 1a and Fig. 1b). This is so because the conformations of the hinge loops differ, leading to greatly differing relationships of the core and swapped domains in the two structures. But if we focus on the 'functional unit'[24] consisting of the pair of complementary domains from two RNase A molecules, the structure of the functional unit of the dimer is essentially the same as the structure of the monomer. In fact, the functional unit supports enzymatic activity, taken as the hallmark of the native state in the original experiments[20]. Thus, the functional unit of the domain-swapped molecule can be considered 'native-like' if not actually 'native'. In other words, to the extent that the monomeric and domain-swapped functional units have the same structure, this common structure is determined by the sequence, and the hypothesis holds. We note that others have given evidence that cystatins[16–19] and β_2-microglobulin[14] are domain-swapped, so that the amyloid-like fibrils of these proteins might also be compatible with the thermodynamic hypothesis. Still another amyloid-like fibril, formed from human lysozyme[25], was found to lack enzymatic activity, but the lack of activity would not rule out domain swapping if the hinge loop, which is changed in conformation on swapping, includes one or more active site residues.

Thus, we have designed an amyloid-like fibril of the stable enzyme RNase A with a Q_{10} expansion between its core domain and its C-terminal β-strand. The fibril has RNase enzymatic activity, showing that it contains native-like domains. By mixing two inactive mutant forms of Q_{10}-RNase A we find that activity is restored, showing that domains are swapped in the fibril. 3D domain swapping can account for the protein self-specificity observed in amyloid fibrils and for at least part of the cohesion of the fibril, and part of the kinetic barrier between monomer and fibril[26]. A speculative domain-swapped helical model is proposed for the RNase A fibril, which is compatible with Anfinsen's thermodynamic hypothesis[5] that protein sequence determines the most stable structure. For the RNase A amyloid-like fibril there is no need to invoke a distinctly different, generic structural form of protein, other than for a small segment that forms the β-sheet spine.

METHODS

Strain construction. A wild-type RNase A clone from R. T. Raines was subcloned into the *Sal*I–*Nco*I sites of the pET-32b vector system. The H12A and H119A single mutants were generated by site-directed mutagenesis. The $GQ_{10}G$, GQ_7G and G_9 sequences were inserted into the hinge-loop region (after Gly 112) of the mutant RNase A by two-step insertion polymerase chain reaction. The GNNQQNY expansion was formed by inserting NQQNYGG after Asn 113 (GN corresponds to residues 112 and 113 of the wild-type protein and NQQNYGG corresponds to the inserted sequence). See Supplementary Information for details of expression and purification.

Fibril formation. The purified mutants (pH 7.0) were incubated in 50% acetic acid at concentrations from 0.15 to 1.5 mM, followed by freezing the samples in dry ice and freeze-drying overnight. The freeze-dried samples were resuspended in 30–40 μl of water and stored on the benchtop.

Activity assays. Fibril bands that stalled in the native stacking gel (see the text) were excised and soaked overnight in water at pH 7.0. These solutions were assayed in duplicate for RNase A activity with the fluorescence kit from Ambion. The negative control was the substrate in the assay buffer. The positive control was wild-type RNase A. See Supplementary Information for details.

Model building. The model-building procedures are described in Supplementary Information.

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1. Koo, E. H., Lansbury, P. T. Jr & Kelly, J. W. Amyloid diseases: abnormal protein aggregation in neurodegeneration. *Proc. Natl Acad. Sci. USA* **96,** 9989–9990 (1999).
2. Dobson, C. M. Protein misfolding, evolution and disease. *Trends Biochem. Sci.* **24,** 329–332 (1999).
3. Liu, Y., Gotte, G., Libonati, M. & Eisenberg, D. A domain-swapped RNase A dimer with implications for amyloid formation. *Nature Struct. Biol.* **8,** 211–214 (2001).
4. Sunde, M. *et al.* Common core structure of amyloid fibrils by synchrotron X-ray diffraction. *J. Mol. Biol.* **273,** 729–739 (1997).
5. Anfinsen, C. B. Principles that govern the folding of protein chains. *Science* **181,** 223–230 (1973).
6. Cohen, A. A. & Calkins, E. Electron microscopic observations on a fibrous component in amyloid of diverse origins. *Nature* **183,** 1202–1203 (1959).
7. Jimenez, J. L. *et al.* Cryo-electron microscopy structure of an SH3 amyloid fibril and model of the molecular packing. *EMBO J.* **18,** 815–821 (1999).
8. Jimenez, J. L. *et al.* The protofilament structure of insulin amyloid fibrils. *Proc. Natl Acad. Sci. USA* **99,** 9196–9201 (2002).
9. Kishimoto, A. *et al.* β-Helix is a likely core structure of yeast prion Sup amyloid fibrils. *Biochem. Biophys. Res. Commun.* **315,** 739–745 (2004).
10. Govaerts, C., Wille, H., Prusiner, S. B. & Cohen, F. E. Evidence for assembly of prions with left-handed β-helices into trimers. *Proc. Natl Acad. Sci. USA* **101,** 8342–8347 (2004).
11. Perutz, M. F., Finch, J. T., Berriman, J. & Lesk, A. Amyloid fibrils are water-filled nanotubes. *Proc. Natl Acad. Sci. USA* **99,** 5591–5595 (2002).
12. Pickersgill, R. W. A primordial structure underlying amyloid. *Structure* **11,** 137–138 (2003).
13. Wetzel, R. Ideas of order for amyloid fibril structure. *Structure* **10,** 1031–1036 (2002).
14. Eakin, C. M., Attenello, F. J., Morgan, C. J. & Miranker, A. D. Oligomeric assembly of native-like precursors precedes amyloid formation by β-2 microglobulin. *Biochemistry* **43,** 7808–7815 (2004).
15. Schlunegger, M. P., Bennett, M. J. & Eisenberg, D. Oligomer formation by 3D domain swapping: a model for protein assembly and misassembly. *Adv. Protein Chem.* **50,** 61–122 (1997).
16. Janowski, R. *et al.* Human cystatin C, an amyloidogenic protein, dimerizes through three-dimensional domain swapping. *Nature Struct. Biol.* **8,** 316–320 (2001).
17. Nilsson, M. *et al.* Prevention of domain swapping inhibits dimerization and amyloid fibril formation of cystatin C: use of engineered disulfide bridges, antibodies, and carboxymethylpapain to stabilize the monomeric form of cystatin C. *J. Biol. Chem.* **279,** 24236–24245 (2004).
18. Sanders, A. *et al.* Cystatin forms a tetramer through structural rearrangement of domain-swapped dimers prior to amyloidogenesis. *J. Mol. Biol.* **336,** 165–178 (2004).
19. Staniforth, R. A. *et al.* Three-dimensional domain swapping in the folded and molten-globule states of cystatins, an amyloid-forming structural superfamily. *EMBO J.* **20,** 4774–4781 (2001).
20. Sela, M., White, F. H. & Anfinsen, C. B. Reductive cleavage of disulfide bridges in ribonuclease. *Science* **125,** 691–692 (1957).
21. Crestfield, A. M., Stein, W. H. & Moore, S. Properties and conformation of the histidine residues at the active site of ribonuclease. *J. Biol. Chem.* **228,** 2421–2428 (1963).
22. Gotte, G., Bertoldi, M. & Libonati, M. Structural versatility of bovine ribonuclease A. Distinct conformers of trimeric and tetrameric aggregates of the enzyme. *Eur. J. Biochem.* **265,** 680–687 (1999).
23. Nelson, R. *et al.* Structure of the amyloid spine. *Nature* **435,** 773–777 (2005).
24. Liu, Y. & Eisenberg, D. 3D domain swapping: as domains continue to swap. *Protein Sci.* **11,** 1285–1299 (2002).
25. Booth, D. R. *et al.* Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. *Nature* **385,** 787–793 (1997).
26. Bennett, M. J., Schlunegger, M. P. & Eisenberg, D. 3D domain swapping: a mechanism for oligomer assembly. *Protein Sci.* **4,** 2455–2468 (1995).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The structure of Q_{10}-H119A RNase A has been deposited in the Protein Data Bank with accession code 2APQ. The model of Fig. 4 has been deposited in the Protein Data Bank with accession code 2APU. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to D.E. (david@mbi.ucla.edu).

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