Ribonuclease A suggests how proteins self-chaperone against amyloid fiber formation

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Received 30 August 2011; Revised 17 October 2011; Accepted 17 October 2011

DOI: 10.1002/pro.754

Published online 16 November 2011 proteinscience.org

Abstract: Genomic analyses have identified segments with high fiber-forming propensity in many proteins not known to form amyloid. Proteins are often protected from entering the amyloid state by molecular chaperones that permit them to fold in isolation from identical molecules; but, how do proteins self-chaperone their folding in the absence of chaperones? Here, we explore this question with the stable protein ribonuclease A (RNase A). We previously identified fiber-forming segments of amyloid-related proteins and demonstrated that insertion of these segments into the C-terminal hinge loop of nonfiber-forming RNase A can convert RNase A into the amyloid state through three-dimensional domain-swapping, where the inserted fiber-forming segments interact to create a steric zipper spine. In this study, we convert RNase A into amyloid-like fibers by increasing the loop length and hence conformational freedom of an endogenous fiber-forming segment, SSTSAASS, in the N-terminal hinge loop. This is accomplished by sandwiching SSTSAASS between inserted Gly residues. With these inserts, SSTSAASS is now able to form the steric zipper spine, allowing RNase A to form amyloid-like fibers. We show that these fibers contain RNase A molecules retaining their enzymatic activity and therefore native-like structure. Thus, RNase A appears to prevent fiber formation by limiting the conformational freedom of this fiber-forming segment from entering a steric zipper. Our observations suggest that proteins have evolved to self-chaperone by using similar protective mechanisms.

Keywords: RNase A; amyloid structure; domain-swapping; self-chaperone; cross- β diffraction

Introduction

Amyloid fibers are filamentous aggregates that are associated with neurodegenerative diseases,¹ denatured globular proteins,^{2–4} bacterial inclusion bodies,⁵ and normal cellular functions.^{5–7} Amyloids arise from self-aggregating proteins that contain fiber-forming segments within the native sequence.^{8–10} A survey of genomes shows

Abbreviations: CD, circular dichroism; eSSTSAA, endogenous SSTSAA segment; eSSTSAASS, endogenous SSTSAASS segment; eSYSTMS, endogenous SYSTMS segment; iG6, inserted 6-Gly linker; iSSTSAA, inserted SSTSAA segment; iSYSTMS, inserted SYSTMS segment; MALDI-TOF, matrix assisted laser desorption/ionization–time-of-flight mass spectrometry; RNase A, pancreatic bovine ribonuclease A; sKSRREYGG, substitute KSRREYGG segment; ATP, adenosine triphosphate; Ni-NTA, nickel nitroloacetic acid; ORF, open reading frame; CNS, Crystallography and NMR System.

Additional Supporting Information may be found in the online version of this article.

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Grant sponsor: DOE-BER grant number: DE-FC03-02ER63421; NIH National Institute for Aging grant number: 1R01 AG029430; NSF grant number: MCB-0445429.

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most proteins contain at least one such segment.¹⁰ The well-characterized enzyme bovine pancreatic ribonuclease A (RNase A) contains several of these fiber-forming segments.¹⁰ Many proteins can be induced to enter the amyloid state,³ but RNase A has not been found in amyloid form. RNase A and many other proteins fold *in vitro* to enter their native structures without entering the amyloid state. Here, we investigate the mechanism by which proteins, like RNase A, self-chaperone their folding, avoiding fiber formation, even though they contain fiber-forming segments.

Fiber-forming segments are building blocks of a steric zipper that form the spines of amyloid fibers. A steric zipper is formed from two identical β -sheets whose side chains form noncovalent interactions with each other across a dry interface.⁸ The β -strands within each sheet are held together by backbone hydrogen bonds. As more segments associate with these β -sheets, a steric zipper spine grows, leading to an increase in fiber length along the spine.

Some amyloids have been found to domain-swap, such as prion,¹¹ cystatin,¹² and β -2-microglobulin.¹³ Domain-swapping occurs when molecules form homodimers and higher-order oligomers by exchanging protein domains (Fig. 1). A small swap domain is linked to the core domain of a protein by a flexible hinge loop. When two molecules form open conformations by noncovalent dissociation of their respective swap domains, they both exchange swap domains, and new swap domain-core domain interactions are formed. Such a swap is designated as closed-ended as all functional units are reconstituted in the swap. Open-ended domain swapping can also occur and lead to the formation of higher-order oligomers. Unsatisfied swap domain-core domain interactions drive the recruitment of more molecules by open-ended oligomers, leading to fiber formation.^{14,15}

The steric zipper spine and domain swapping mechanisms have been combined to explain the formation of designed RNase A fibers—a steric zipper is formed by a modified hinge loop that is subsequently immobilized in the spine and domain swapping interactions hold together monomers within a fiber¹⁵ [Fig. 1(d)].

Native RNase A is a stable and dynamic enzyme that is cross-linked by four disulfide bonds.^{16–21} RNase A does not form fibers despite repeated denaturation and renaturation in myriad conditions.^{15,22–24} This is intriguing because RNase A contains segments in its primary sequence that can form fibers when each segment is in isolation from the rest of the RNase A molecule.^{10,25} RNase A can be cleaved by subtilisin to produce S-peptide (the first 20 residues) and S-protein.^{26–28} We have found that S-peptide alone, however, is capable of forming fibers (data not shown).

RNase A forms domain-swapped oligomers by lyophilization in 50% acetic acid.²⁹ The three-dimen-

sional (3D) structures of RNase A dimers show that domain-swapping occurs when either an N-terminal α -helix (residues 1–15) or a C-terminal β -strand (residues 116–124) is exchanged between the two monomers (Protein Data Bank [PDB] IDs: 1F0V and 1JSO).^{30,31} Intermolecular noncovalent bonds that form between the swap domain and the core domain hold the dimers together and the native fold of RNase A is maintained [Fig. 1(c,g)]. These swap domain-core domain interactions are identical to those in monomeric RNase A. Trimers can also form by different combinations of N-terminal α -helix and C-terminal β -strand swapping.³² Both domain-swapped dimers and trimers of RNase A retain catalytic activity.³³ Two histidine residues, His12 and His119, in the catalytic site are situated in separate domains. His12 lies in the N-terminal swap domain, while His119 lies in the C-terminal swap domain [Fig. 1(a)]. These His residues and a third catalytic residue, Lys41, maintain close proximity to each other in domain-swapped RNase A molecules (Fig. 1). Thus, catalytic activity is maintained in RNase A oligomers by virtue of domain-swapping.

Previously, we found that insertion of short, fiber-forming segments of amyloid disease-related proteins into the C-terminal hinge loop of RNase A converts RNase A into the amyloid state [Fig. 1(d,e)].^{15,34} An atomic model was built to show that a fiber can accommodate domain-swapped RNase A around a steric zipper spine.¹⁵ These designed RNase A fibers contain functional RNase A molecules in their native structure as shown by maintenance of ribonucleolytic activity.

The goal of this work is to explore why fiber-forming segments of the RNase A sequence, previously found to form fibers when isolated from the rest of the enzyme,¹⁰ fail to convert RNase A to a fiber form. We find that conformational flexibility is essential for these segments to form steric zippers to drive the enzyme into the amyloid state.

Results

Duplication of endogenous fiber-forming segments and insertion of them in the C-terminal hinge loop causes RNase A to form fibers

RNase A enters the fiber state when fiber-forming segments are inserted in the C-terminal hinge loop.^{15,34} Native RNase A does not form amyloid-like fibers, even when denatured, despite six fiber-forming segments in its sequence.¹⁰ To test for fiber formation, two such segments,¹⁵ SSTSAA²⁰ and ⁷⁵SYSTMS⁸⁰, were duplicated and inserted between Gly112 and Asn113 in the flexible C-terminal hinge loop (residues 112–115). These segments form fibers on their own, confirming prediction of fiber formation by the Rosetta–Profile method.¹⁰ The segments

Figure 1. Domain-swapping is a possible mechanism for fiber formation. When concentrated in mild acid, native RNase A forms domain-swapped dimers by exchanging a domain, called the swapped domain, with an identical molecule. The N-terminal domain (residues 1–15) and hinge loop (residues 16–22) are colored green; the C-terminal domain (residues 116–125) and hinge loop (residues 112–115) are orange; the core domain is blue. A closed monomer [shown in (a)], can form a C-terminally domain-swapped dimer [shown in (c)], by first breaking the noncovalent interactions of its C-terminal domain, forming an open monomer (b).³⁰ The C-terminal domain is connected to the rest of the RNase A molecule by a hinge loop. Intermolecular noncovalent bonds between the swapped domain and the core domain hold the dimer together, forming two functional units, both with active sites. Insertions of fiber-forming segments in this hinge loop may facilitate intermolecular steric zipper interactions that lead to fibers by the formation of runaway domain-swapped oligomers (d,e). This model of fiber formation was proposed for an RNase A variant with a 10-Gln insertion.¹⁵ Similarly, RNase A may form domain-swapped dimers and runaway domain-swapped oligomers by swapping its N-terminal domain instead, as shown in (f–i). In this work, we made two 6-Gly insertions in the N-terminal hinge loop of RNase A, shown in (f). Lengthening of this hinge loop allows RNase A to form amyloid-like fibers *via* a runaway domain-swapped mechanism (h,i). Residues sandwiched by the Gly insertions form a steric zipper in these RNase A fibers.

were inserted in the hinge loops of two RNase A constructs. To permit protein expression in *Escherichia coli*, the inserts were placed in inactive mutants, RNase A H119A and H12A (Fig. 2). The C-terminal hinge loop allows native RNase A to form domain-swapped oligomers.³¹ Previously, expansion of this hinge loop by insertion of a 10-Gln amyloid forming segment facilitated the formation of domain-swapped amyloid-like fibers [Fig. 1(d,e)].¹⁵ In this work, SSTSAA (Rosetta energy score of -22.5) and SYSTMS (Rosetta energy score of -24.7) were each inserted in the hinge loop, expanding it by six residues [Fig. 1(g)]. As anticipated, these C-terminal-inserted SSTSAA (^CiSSTSAA) H119A and C-terminal-inserted SYSTMS (^CiSYSTMS) H12A constructs form fibers [Fig. 2(b) and Materials and Methods section]. ¹⁵SSTSAASS²² was also inserted in the hinge loop and this expanded construct forms fibers as well [Fig. 2(b)].

Insertion of nonfiber-forming segments in the C-terminal hinge loop does not cause RNase A to form fibers. For example, insertion of KSRREY, a segment from an ATP-dependent RNA helicase with a Rosetta energy score -14.7 kcal/mol, and hence predicted not to form steric zippers, did not cause RNase A to form fibers even after 3 months [^CiKSRREY H119A construct in Fig. 2(b)]. Insertion of the expanded KSRREYGG segment also did not cause RNase A to form fibrils [^CiKSRREYGG H119A construct in Fig. 2(b)]. In addition, a 9-Gly insertion in this hinge loop was previously found not to form fibers after 3 months as well.¹⁵ As a precaution, all fiber-forming samples were checked for the presence of S-peptide because S-peptide is capable of forming fibers. All samples examined for fiber formation in this work were analyzed by matrix assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF) for the presence of this proteolysis product. No S-peptide was detected in any fiber-forming sample (not shown), confirming that all fibers observed were formed by full-length RNase A variants.

An endogenous fiber-forming segment causes RNase A to form fibers when made more flexible by inserted Gly linkers

We tested the extent of additional flexibility required for fiber formation, with Gly inserts around endogenous segments in the N-terminal hinge loop. Pairs of 1–6 Gly residues were inserted between Asp13 and Ser14, and Ser22 and Ser23, to confer extra flexibility to the endogenous ¹⁵SSTSAASS²² (denoted eSSTSAASS) segment [Figs. 1(f) and 2(a)]. Two Gly residues inserted to sandwich eSSTSAASS [^NiG2-eSSTSAASS-iG2 H119A construct in Fig. 2(b)] were sufficient for fiber formation but one Gly on each side of eSSTSAASS was not. Insertion of 3–6 Gly residues that sandwich eSSTSAASS also caused RNase A to form fibers. Fibers of ^NiG6-eSSTSAASS-

Figure 2.

Figure 3. RNase A amyloid-like fibers have cross- β diffraction pattern. RNase A fibers resemble amyloids by displaying cross- β diffraction pattern with reflections at 4.8 Å and 8–11 Å. RNase A constructs are represented with bar diagrams and colored as in Figure 2. A cross- β diffraction pattern was observed for C iSSTSA H119A fibers (a) and N iG2-eSSTSAASS-iG2 H119A fibers (b). 4.8 Å is the distance between strands within a β -sheet while 8–11 Å is the distance between two β -sheets that come together to form a fiber spine.^{35,36} These distances correspond to those observed in the 3D structure of the SSTSA peptide steric zipper.²⁵ N iG6-eSSTSAASS-iG6 H119A fibers had reflections at 4.8 Å and 9 Å (c) representing distances within the β -rich structure of fibers.

iG6 H119A are shown in Figure 2(b). When eSSTSAASS was replaced by nonfiber forming KSRREYGG, the new N iG6-sKSRREYGG-iG6 H119A construct did not form fibers for 3 months [Fig. 2(b)]. Similarly, two Gly residues inserted to sandwich the endogenous 15 SSTSA²⁰ (eSSTSA) subsegment [N iG2-eSSTSA-iG2 H119A construct in Fig. 2(b)] caused fiber formation; no fibers were observed for 3 months when KSRREY substituted SSTSA between the two Gly insertions [N iG2-sKSRREY-iG2 H119A construct in Fig. 2(b)]. With the increased flexibility provided by the insertion of two Gly on both of its sides, eSSTSAASS is able to drive fiber formation.

Previously, we observed that fibers with different amyloid-related inserts vary in persistence

length, width, and tendency to tangle or align.³⁴ In this work, inserted Gly residues flanking eSSTSA and eSSTSAASS do not appear to grossly influence fiber morphology. The fibers appear long, with an average diameter of 110 Å, and do not particularly tangle or bundle.

RNase A fibers bind Congo red and display birefringence characteristic of amyloid

The RNase A fibers described above bind Congo red and display varying intensities of yellow to green birefringence when observed between cross polarizers [Fig. 2(b)]. Because amyloid fibers display this birefringent property upon Congo red staining,^{35,36} our observations indicate that these RNase A fibers have defined

Figure 2. RNase A constructs form amyloid-like fibers when its endogenous fiber-forming segments are placed in flexible loops. (a) The 3D structure of RNase A is colored for various segments of interest. The N-terminal domain (residues 1–15) is green while the C-terminal domain (residues 112–115) and hinge loop (residues 116–125) are orange. The endogenous fiber-forming segments 15 SSTSA²⁰ (eSSTSA) and 75 SYSTMS⁸⁰ (eSYSTMS) are magenta and red, respectively. A star marks the site of insertion in the C-terminal hinge loop between G112 and N113. Arrows point to Gly insertions surrounding eSSTSA and eSSTSAASS. (b) RNase A constructs are represented with bar diagrams in the left column. Segments are colored as in (a). All constructs are in an inactive H12A or H119A background. The second column shows electron micrographs with 200 nm scale bars. The third and fourth columns show Congo red binding by corresponding RNase A constructs when observed in bright field or between cross polarizers. Scale bars for Congo red images are 100 μ m. eSSTSA and eSYSTMS are two endogenous fiber-forming segments.²⁵ RNase A forms fibers when SSTSA, SSTSAASS, or SYSTMS is duplicated by insertion in the C-terminal hinge loop. The inserted SSTSA, SSTSAASS, and SYSTMS RNase variants are C iSSTSA H119A, C iSSTSAASS H119A, and C iSYSTMS H119A. When nonfiber-forming segments, KSRREY and KSRREYGG, were each inserted in the C-terminal hinge loop, the engineered C iKSRREY H119A and C iKSRREYGG H119A variants did not form fibers even after 3 months and 4 weeks, respectively. RNase A also forms fibers when eSSTSAASS is sandwiched by 2–6 Gly. Only results for two-Gly and six-Gly on each end of eSSTSAASS (N iG2-eSSTSAASS-iG2 H119A and N iG6-eSSTSAASS-iG6 H119A) are shown. RNase A did not form fibers for 3 months when SSTSAASS was substituted with KSRREYGG. Native RNase A (not shown) and the H12A (not shown) and H119A inactive variants do not form fibers. All constructs that form fibers bind Congo red and display varying degrees of yellow and green birefringence when observed between cross polarizers. Arrowheads point to fibers and areas of dye binding and corresponding birefringence.

molecular structure that resemble amyloids. Birefringence is not observed in the H119A, ^CiKSRREY H119A, ^CiKSRREYGG H119A, and ^NiG6-sKSRR EYGG-iG6 H119a constructs that do not form fibers.

RNase A fibers display the cross- β diffraction pattern with steric zipper dimensions

RNase A fibers (^CiSSTSA H119A and ^NiG2-eSSTSAASS-iG2 H119A) display the cross- β diffraction pattern typical of amyloid with reflections at 4.8 Å and 8–11 Å [Fig. 3(a,b)]. For comparison, the 3D structure of the SSTSA segment, which we determined previously (PDB ID: 2ONW), shows that this peptide forms two β -sheets that mate across a steric zipper interface.²⁵ Reflections at 4.8 Å in the fiber diffraction image correspond to the spacing between β -strands within a sheet in the atomic structure.^{37,38} Fiber diffraction reflections at 8–11 Å correspond to the spacing between two β -sheets across a steric zipper interface.^{37,38} Fibers of ^NiG6-eSSTSAASS-iG6 H119A also display the amyloid signature with 4.8 Å and 9 Å distances as observed in corresponding reflections in x-ray diffraction experiments [Fig. 3(c)].

RNase A fibers are composed of N-terminal domain-swapped molecules that retain native functional units

RNase A maintains enzymatic activity after the ^NiG6-eSSTSAASS-iG6 constructs are converted into fiber form. His12, Lys41, and His119 are residues in RNase A required for ribonucleolytic activity.^{28,39–41} Both H119A and H12A mutants of the iG6-eSSTSAASS-iG6 construct are inactive. Oligomers and fibers that may result from the mixing and lyophilization of inactive H119A and H12A mutants have restored ribonucleolytic activity due to reconstituted active sites by domain-swapping.^{28,29,33} When equimolar amounts of both inactive mutants are perfectly mixed together, only one of four possible active sites [marked with a purple star in Fig. 4(b)] can have the native combination of catalytic residues for activity. Resulting fibers of mixed ^NiG6-eSSTSAASS-iG6 were separated from monomers and small oligomers by native gel electrophoresis [Fig. 4(c)]. A dot blot of purified fibers probed with anti-RNase A polyclonal antibodies confirms that the fibers observed by electron microscopy consist of RNase A molecules. Purified fibers were assayed for specific activity. Mixed ^NiG6-eSSTSAASS-iG6 fibers [purple trace in Fig. 4(a)] show a specific activity of $13 \pm 0.12\%$, while the H119A [blue trace in Fig. 4(a)] and H12A [green trace in Fig. 4(a)] inactive mutants show specific activities of $5.9 \pm 2.3\%$ and $2.7 \pm 0.14\%$, respectively. RNase A fibers contains monomers that domain-swap *via* their N-terminal domains. The fibers would be inactive in the absence of domain swapping.

Appropriate negative controls were performed to confirm that the observed enzymatic activity was not an artifact of extraneous nucleases [Fig. 4(a)]. To confirm that the activity attributed to the mixed fibers was not due to active oligomers that may nonspecifically associate with the fibers, an active mix of prefiber H119A and H12A ^NiG6-eSSTSAASS-iG6 oligomers was added to inactive ^NiG6-eSSTSAASS-iG6 H12A fibers and subjected to native gel electrophoresis. Fibers purified from the stacking gel remained inactive (Supporting Information Fig. 2). Fibers extracted from the native gels are free of nonspecifically-associated monomers and small oligomers.

Discussion

The segment SSTSA is sufficient to drive RNase A into fibers when properly placed

From earlier studies, we know that the insertion of certain segments of six or more residues are sufficient to force RNase A into fiber form, when that segment has high propensity for fiber formation and is positioned within the C-terminal hinge loop.^{15,34} Here, we find that RNase A forms fibers when the SSTSA segment of RNase A is duplicated and inserted in the C-terminal hinge loop (^CiSSTSA H119A in Fig. 2). This suggests that the expanded ^CiSSTSA segment forms a steric zipper, leading to an amyloid-like fiber. Similarly, in the N-terminal hinge loop, the endogenous SSTSAASS (^NeSSTSAASS) segment likely enters a steric zipper when it gains greater conformational flexibility than available in its native state. Flexibility is gained when at least two Gly residues were inserted on each end of the segment to lengthen the N-terminal hinge loop (Figs. 1 and 2). We previously found that multiple Gly residues do not form steric zippers.¹⁵ Therefore, only the residues of ^NeSSTSAASS contribute to fiber formation in the inserted-Gly constructs.

Gly insertions that sandwich eSSTSAASS cause structural changes in RNase A that lead to fiber formation. The difference in midpoints of guanidine denaturation, C_M , of monomeric native RNase A and of monomeric ^NiG2-eSSTSAASS-iG2 H119A is 0.5M (Supporting Information Fig. 1). This may be attributed to weakened hydrogen bonding interactions involving the N-terminal hinge loop (between D14 and T17 as well as A19 and H48) presumed to be incumbent upon the loop's expansion. RNase A is destabilized when Gly residues are inserted to sandwich eSSTSAASS, disrupting the closed interface between the monomer and its swap domain and allowing it to access conformational states that lead to fiber form.

Complete unfolding of a protein is not required for amyloid formation

Amyloid formation does not preclude a protein's ability to retain its native structure. RNase A is

Figure 4. Constructs containing six-Gly inserts form amyloid-like fibers, which are domain-swapped via the N-terminal domain and display enzymatic activity. H12, K41, and H119 are residues in RNase A required for ribonucleolytic activity. The H119A and H12A mutants of the N_i G6-eSSTSAASS-iG6 construct are inactive. When equimolar amounts of both inactive mutants are mixed together, oligomers and fibers may form via domain-swapping with reconstitution of active sites. Only one of four possible active sites [marked with a purple star in (b)] will have the right combination of catalytic residues for activity. Resulting fibers of mixed N_i G6-eSSTSAASS-iG6 were separated from monomers and small oligomers by native gel electrophoresis [labeled “+” in (c)]. Fibers that stalled in the stacking gel were extracted and blotted on a PVDF membrane and probed with anti-RNase A polyclonal antibodies (c). These purified fibers were assayed for ribonucleolytic activity with a fluorescence-based activity assay for 60 min. Results for the first 35 min of the assay are shown in (a)—the slope of each trace represents the amount of cleaved product over time with respect to the amount of enzyme present. Specific activities were calculated with values from 10 to 30 min. Mixed N_i G6-eSSTSAASS-iG6 fibers (purple trace) showed significantly higher specific activity than either of the inactive fibers alone. Mixed N_i G6-eSSTSAASS-iG6 fibers showed specific activity of $13 \pm 0.12\%$ while the H119A (green trace) and H12A (blue trace) inactive mutants each showed specific activities of $5.9 \pm 2.3\%$ and $2.7 \pm 0.14\%$, respectively [shown in panel (b)]. Water (red trace) and reaction buffer (black trace) also were assayed and had no activity from extraneous ribonucleases. All samples were assayed in triplicate. Specific activity for all samples were compared with native RNase A (yellow trace). Error bars represent standard error of measurements.

catalytically active and natively folded within fibers (Fig. 4) in addition to participating in amyloid structure (Fig. 2). A domain-swapped steric zipper spine model was previously proposed for RNase A with a 10-Gln insertion in the C-terminal hinge loop [Fig. 1(d) shows a 2D depiction].¹⁵ C-terminal domain-swapping across the fiber spine can occur because the expanded hinge loop-spine is able to

accommodate globular RNase A functional units tethered to the spine without steric hindrance. In this work, we show that the N_i G6-eSSTSAASS-iG6 fibers have ribonucleolytic activity because of N-terminal domain-swapping (Fig. 5). Domain-swapping interactions alone are insufficient for amyloid fiber formation, which is why native RNase A is incapable of forming fibers.

Figure 5. A zipper spine model of N-terminally domain-swapped NiG6 -eSSTSAASS-iG6 RNase A fibers. The β -strands of the central spine contain the sequence SSTSAASS, with coordinates adapted from the crystal structure of SSTSAA. There are two central sheets composed of the SSTSAAS sequence and functional units surrounding. Each sheet is composed of parallel strands; the two sheets are antiparallel to each other. Panel (a) shows the eight RNase A molecules that comprise the asymmetric unit of the fiber. The N-terminal swap domain is green, the core domain is blue, and the C-terminal swap domain is orange. Colors match the scheme in Figure 1. Panel (b) shows the same view of the asymmetric unit—RNase A molecules from one sheet are colored in two shades of blue; molecules from the other sheet are colored two shades of orange. Four of the functional units are intersheet swaps (comprised of orange and blue molecules), four are intrasheet swaps (comprised of dark and light orange molecules or dark and light blue molecules). In panel (c), a segment containing 56 RNase A molecules is shown with a vertically oriented fiber axis. RNase A molecules are colored as in panel (b) to show domain-swapping. Half of the fiber is cut away, revealing lengthwise-cross section. The zipper spine that is partly concealed by globular RNase A domains surrounding it are evident in this view. The model contains closed dimer swaps between molecules at extreme ends of the cross section in panel (b). Runaway domain-swaps occur among the RNase A monomers running the length of the fiber for the molecules packing closest to the two faces of the spine sheets.

We propose a runaway N-terminal domain-swapped fiber model for NiG6 -eSSTSAASS-iG6 RNase A [Figs. 1(h) and 5]. In this model, the eSSTSAASS segments adopt a steric zipper spine by stacking 4.8 Å apart in parallel, in-register sheets, and two sheets mate together face-to-face, in a central, self-complementary pair, as observed in the crystal structure of the six-residue segment, SSTSAA (PDB ID: 2ONW) [Fig. 5(a)]. There are two RNase A molecules per 4.8 Å step because a single RNase molecule occupies a 4.8-Å step in each of the two sheets in the steric zipper. N-terminal, domain-swapping connects functional units on opposite ends of the sheets evident in the two colors of each functional unit represented in [Fig. 5(b)].

Although eSSTSAASS segments participate in amyloid structure, they do not perturb the native fold of RNase A. This is facilitated by the six-Gly insertions on each side of eSSTSAASS. In fact, we found the NiG2 -eSSTSAASS-iG2 fibers [Fig. 2(b)] had insignificant enzymatic activity (Supporting Information Fig. 3). The two-Gly insertions do not make the segment long enough to maintain globular domains by domain-swapping. This result supports our model of an in-register fiber spine with homogenous composition, consisting two RNase A molecules per 4.8 Å step. Our observation supports the conclusion of others that a protein is not required to completely denature to enter the amyloid state.^{42–46}

RNase A prevents fiber-forming segments from entering steric zippers by limiting segment length, consequently restricting conformational freedom

Apparently, RNase A has evolved to prevent its fiber-forming segments from entering steric zippers by restricting the flexibility of those segments. Genome-wide analyses of *E. coli*, *Saccharomyces cerevisiae*, and *Homo sapiens* and analysis of a nonredundant set of PDB structures (based on 50% sequence identity) predicted fiber-forming segments in almost all proteins.¹⁰ Although 14–15% of ORFs were predicted to have fiber-forming segments, most proteins are not known to form amyloid fibers in physiological conditions.¹⁰ It was found that 95% of the fiber-forming segments in proteins deposited in the PDB are buried, which reveals a major mechanism for protection against fiber formation [10]. However, for segments that are solvent-exposed, proteins must somehow protect their fiber-forming segments from entering steric zippers.

In the case of RNase A, eSSTSAA does not drive native RNase A into amyloid-like fibers even though it lies in the most flexible region of the protein.^{47–49} RNase A's first 20 amino acids includes eSSTSAA and is predicted to participate in the final folding steps of RNase A.⁵⁰ The sidechains of eSSTSAA are solvent-exposed and do not participate in noncovalent interactions in RNase A structure.^{19,51} eSSTSAA is exposed until RNase A is completely folded, yet RNase A does not form fibers in denaturation and refolding studies.^{15,22–24,34} The native sequence of RNase A prevents eSSTSAA from forming steric zippers by keeping the N-terminal hinge loop short. Although native RNase A can form small oligomers *via* N-terminal domain-swapping, fibers have never been observed. Steric zippers are absent in native intermolecular domain-swapping interactions.^{30,32} eSSTSAA segments in domain-swapped oligomers cannot interact with each other because the native N-terminal hinge loop is too short. Insertion of 4 Gly in the N-terminal hinge loop caused RNase A to form fibers [Fig. 2(b)]. Six Gly are predicted by the Rosetta-Profile method to be unfavorable in steric zipper formation (not shown). eSSTSAA gains extra flexibility because inserted Gly residues allow it to sample new conformations that were previously unavailable in the native fold, including the steric zipper. One general mechanism for protection against fiber formation may be to limit flexibility of fiber-forming segments such that identical segments from two different monomers do not have the opportunity to interact. Proteins may have evolved to limit segment lengths, striking a balance between optimal protein folding for function and protection against fiber formation.

Materials and Methods

Design of constructs

All RNase A constructs are in the pET-32b vector. The ^CiSSTSAA H119A, ^CiSSTSAA H12A, ^CSSTSAASS

H119A, and ^CKSRREYGG H119A constructs were obtained by QuikChange site-directed mutagenesis (Stratagene). SSTSAA, SYSTMS, SSTSAASS, and KSRREYGG were each inserted between G112 and N113 in the RNase A C-terminal hinge loop. The ^NiG6-sKSRREYSS-iG6 H119A construct was also obtained by QuikChange. All other constructs were produced by GenScript Corporation. Native bovine pancreatic RNase A was obtained from Sigma.

Protein expression and purification

All RNase A constructs except for ^CiKSRREYGG H119A were expressed and purified as described elsewhere.³¹ Briefly, BL21-CodonPlus(DE3)-RIPL, an *E. coli* strain with extra copies of argU, ileU, proL and leuW tRNA genes (Agilent Technologies), and Terrific Broth were used for overnight protein expression. Cells were harvested and lysed by sonication. Proteins were purified from supernatant by Ni-NTA affinity with increasing imidazole gradient. His-tags were cleaved by enterokinase (Invitrogen) digestion and removed by Ni-NTA affinity.

For ^CiKSRREYGG H119A, cells were harvested and lysed with an EmulsiFlex (Avestin) and pellets were resuspended in 6M guanidinium hydrochloride buffer. The protein was purified in 8M urea buffer by Ni-NTA affinity and refolded by step-wise dialysis with decreasing urea concentration. This protocol was used previously to refold RNase A constructs with 10-Gln or ABeta 1-42 inserts. Refolded RNase A variants were determined to have the same fold as native RNase A by x-ray crystallography¹⁵ and circular dichroism (CD; not shown).

Fiber formation

Purified RNase A proteins were prepared for fiber formation as previously described in Sambashivan *et al.*¹⁵ Samples were concentrated to 7–20 mg/mL, as determined by absorbance at 280 nm with dilutions in 6M Gdn-HCl and 100 mM Tris pH 8. Samples were incubated in 50% (v/v) acetic acid, lyophilized, and resuspended in water to a final apparent concentration of 35 mg/mL. Samples were stored at room temperature and analyzed overtime by electron microscopy for fiber formation. Samples were also analyzed by MALDI-TOF (Micromass-Waters) for proteolysis.

Electron microscopy

Protein samples were pelleted by centrifugation and rinsed with several rounds of water. Resuspended pellets were applied to hydrophilic 400-mesh, carbon-coated formvar films, mounted on copper grids (Ted Pella) and stained with 1–2.5% uranyl acetate. Samples were examined with a Hitachi H-7000 transmission electron microscope (TEM) at 75 keV or a Phillips CM120 TEM at 120 keV.

Congo red assay

Congo red assay was carried out as described in Teng and Eisenberg.³⁴ RNase A protein aggregates were pelleted by centrifugation at 14,000g and rinsed with water. Each pellet was resuspended in 100 μ L of 0.1 mg/mL Congo red, 150 mM NaCl, 10 mM Tris pH 8, and incubated for 30 min at room temperature. Samples were pelleted again by centrifugation and rinsed with water to remove excess Congo red. Samples were dried on silanized cover slips and examined with an optical microscope in bright field and between cross polarizers.

Fiber preparation for x-ray diffraction

RNase A fibers were prepared as described in Ivanova *et al.*⁵² with modifications. Fibers were harvested by centrifugation at 14,000g and rinsed with water. Each rinsed pellet of fibers was then resuspended in 10 μ L of water. Each sample was then placed between the fire-polished ends of two silanized glass capillaries and allowed to dry in a closed petri dish at room temperature. Diffraction images were recorded at APS beamline 24ID-C equipped with ADSC Quantum 315 CCD detector and on a Rigaku FR-E x-ray generator equipped with R-Axis HTC imaging plate detector.

CD measurements

CD spectra of RNase A variants were measured in 100 mM NaCl, 10 mM Tris pH 8.0, and increasing concentrations of Gdn-HCl pH 8.0, containing 125 μ M RNase A. A cuvette of 1 cm path length was used and measurements were recorded at 222 nm and 25°C in a Jasco CD spectrophotometer with a Peltier temperature control attachment. Each melting curve was normalized to the maximum and minimum points with this equation: Relative ellipticity = $1 + (y - y_{\max})/(y_{\max} - y_{\min})$.

Activity assay

Activity of RNase A fibers was assayed as described in Sambashivan *et al.*¹⁵ All surfaces and utensils were cleaned with RNaseZap (Applied Biosystems) and RNase-free water. RNase A fibers that stall in the native stacking gel were harvested by excision of the stacking gel and subsequent soaking of the gel in RNase-free water. Protein concentrations were measured with the Micro BCA Protein Assay Kit (Thermo Scientific). Enzymatic activities were assayed with RNaseAlert Lab Test Kit (Applied Biosystems). Measurements were recorded with the SpectraMax M5 Microplate Reader (Molecular Devices).

3D domain-swapped zipper spine

The model takes its spine from the crystal structure of SSTSA (residues 15–20 of bovine pancreatic

RNase A; PDB ID 2ONW).²⁵ That is, the spine contains two β -sheets composed of parallel, in-register SSTSA β -strands running perpendicular to the fiber axis (Fig. 5). The two sheets are related by a twofold screw axis that is coaxial with the fiber and positioned midway between the sheets. The spine corresponds closely to the crystal structure of SSTSA. The globular domains of the RNase A molecules originated from PDB entry 2E3W.⁵³ Molecules were manually oriented and positioned around the spine to: (1) avoid steric overlap with other molecules in the fiber; (2) maintain proximity to the spine allowed by the six-Gly inserts that connect the spine to the globular domain; and (3) allow domain-swapping between RNase A molecules. Eight molecules compose the asymmetric unit of the fiber. The symmetry operation used to generate the fiber from the asymmetric unit was a -20° rotation and an 18.8Å translation, which corresponds to a 5° rotation for every 4.7Å step along the fiber axis. The rotation was introduced to simulate the gentle twist frequently observed in other amyloid fibers by electron microscopy. Inspection of clashes among fiber-symmetry related molecules was facilitated by the program O.⁵⁴ It continuously updates orientations and positions of symmetry related molecules, while probing prospective orientations and positions of the asymmetric unit. In some of the molecules, two to three additional residues of the globular domain were perturbed from their native fold to connect the globular fold with the Gly inserts. These perturbations did not disrupt the three native disulfide bonds or the active site geometry. Domain-swapping takes place between molecules within the same sheet and between sheets. In the domains closest to the spine's faces, the swapping is "runaway" along the length of the fiber. In the domains closest to the spine's termini, the swapping is closed. This crude model was then energy minimized using the program CNS⁵⁴ with van der Waals, electrostatic, and hydrogen bonding terms.⁵⁵

Conclusions

RNase A is converted to amyloid-like fibers when an endogenous fiber-forming segment in the N-terminal hinge loop is given extra flexibility by virtue of Gly residue insertions that sandwich the segment. A combination of x-ray diffraction and biochemical experiments demonstrate that the RNase A fibers contain a steric zipper spine, as predicted for this segment, and also contain domain-swapped RNase A molecules which retain native-like structure. Our findings show that participation in amyloid structure does not prevent a protein from being natively folded; only a small protein segment is necessary for formation of the steric zipper spine of the fiber.

A segment that has high propensity for amyloid formation is necessary for forming a fiber, but is

insufficient on its own. In the earlier work, we found that RNase A contains several segments that form amyloid-like fibers when in isolation from the rest of the protein.¹⁰ These segments do not drive native RNase A to form fibers. Here, we find that if one of these segments is given additional conformational freedom by sandwiching it between pairs of Gly residues, that this slightly expanded RNase A molecule does enter the amyloid state. Such a segment must have sufficient flexibility to conform to the tight packing constraints of a steric zipper structure, in which identical segments from neighboring molecules stack 4.8 Å apart in hydrogen-bonded sheets and the sheets mate together in self-complementary pairs. This suggests that proteins have evolved, not to rid themselves of all segments that have sequences capable of forming steric zipper spines of fibers, but rather to protect those segments by either burying them or giving them insufficient flexibility to form fibers.

Acknowledgments

The authors thank Stuart A. Sievers and Yanshun Liu for discussion and also Magdalena Ivanova, Daniel Anderson, Neil P. King, Martin Phillips, and Mark Arbing for experimental advice.

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