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Article

Amyloid fibrillation rate differs greatly among single-disulfide variants of hen lysozyme

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Four molecular species of hen lysozyme single-disulfide variants, each retaining one of the four native disulfide bonds (Cys6-Cys127, Cys30-Cys115, Cys64-Cys80, or Cys76-Cys94) spontaneously form fibrils that are morphologically similar to the amyloid protofibril of hen lysozyme all-disulfide-deficient variant. The fibrillation rate of the single-disulfide variants, however, differs among each other by as much as three orders of magnitude. Rapid-fibrillating species are those which have previously been shown to have submolecular structures comparatively stable and localized. The slow-fibrillating species retains the Cys6-Cys127 disulfide bond, indicating that the N- and C-terminal regions should be sterically separated from each other for efficient fibrillation to occur. The proximity of N- and C-terminal regions imposed by the Cys6-Cys127 disulfide bond was also shown to result in a fibril of a slightly decreased diameter.

Keywords: Hen Lysozyme; Disulfide bond; Amyloid fibril; Unfolded state

Introduction

Amyloid fibrillation is generally believed to take place via partially unfolded conformer of protein [1]. A comparative survey of amyloidogenic conformation of globular proteins and that of natively unfolded proteins has shown that it is relatively unfolded one and shares structural properties with a pre-molten globule state [2]. In order to clarify the property of the amyloidogenic conformation, it is helpful to study the fibrillation of a set of protein variants with various extent of intramolecular structures existing at different peptide regions [3].

Formation of disulfide bond is generally coupled with that of protein structure [4]. A series of disulfide variants of hen lysozyme have been constructed and their intramolecular structures were characterized [5-8]. Among these, the 0SS variant, which lacks all the four disulfide bonds and is nearly unfolded under native solution conditions, has been shown to spontaneously form amyloid fibrils [9].

Here, four molecular species of single-disulfide variants, 1SS, each having one of the four native disulfide bonds in hen lysozyme, Cys6-Cys127, Cys30-Cys115, Cys64-Cys80, and Cys76-Cys94 (Fig. 1), were employed to find how individual native disulfide bonds and accompanying submolecular structures affect the fibrillation process. All the four 1SS variants are relatively unfolded, belonging to the pre-molten globule state [2], and have only unstable submolecular structures of differing size and stability [6]. The results show highly differential fibrillation kinetics, acceleration or deceleration altogether by three orders of magnitude, among the four 1SS variants, and a slight change in the fibril

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The abbreviations used are: 0SS, hen lysozyme variant which lacks all the four disulfide bonds; 1SS (6-127) ((30-115), (64-80), (76-94)), hen lysozyme single-disulfide variant which retains the disulfide bond Cys6-Cys127 (Cys30-Cys115, Cys64-Cys80, Cys76-Cys94, respectively); β_2m, β2-microglobulin

structure of the slow-fibrillating 1SS variant.

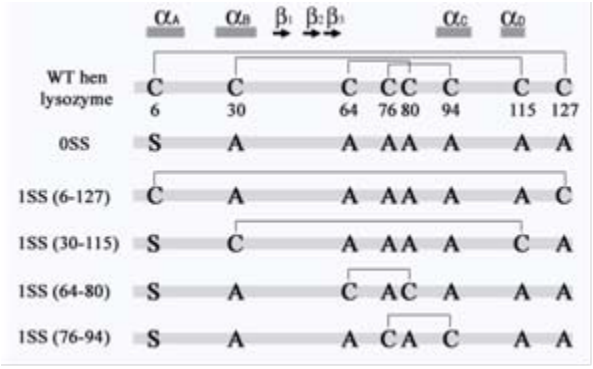

Fig. 1. Disulfide bridging patterns in the four 1SS variants. C, A, and S denote cysteinyl, alanyl, and seryl residues, respectively. Thin lines indicate disulfide bridging between cysteinyl residues. WT hen lysozyme and 0SS are also shown for reference. The Cys6-Cys127 bond connects extreme N- and C-terminal regions; Cys30-Cys115 resides in the α-domain in the native structure of WT lysozyme and connects α-helices B (α_B) and D (α_D); Cys64-Cys80 resides in the β-domain that contains β_1, β_2, and β_3 strands; Cys76-Cys94 is located at the interface between the α- and β-domains.

Materials and Methods

Hen lysozyme 1SS variants were produced in *E. coli* and chromatographically purified as described previously [6,10]. For fibril formation, freeze-dried 0SS powder was firstly dissolved in pure water. Then, the solution was mixed rapidly with buffer and salt solutions to a desired final composition to start the fibrillation reaction. Atomic force microscopy (AFM) observations were carried out on an SPI-3800 scanning probe microscope (Seiko Instruments Inc.) under a cyclic contact mode as described [11]. High-performance size exclusion chromatography (SEC) was carried out on a TSK GSW column (7.5 mm i.d. × 7.5 cm, Tosoh; a nominal Mr exclusion limit of 1×10^5) with an elution solution of 20 mM sodium acetate, pH 4.0, 10 mM sodium sulfate used at a flow rate of 1.0 ml/min. Far-UV CD spectra were taken on a J-820 spectropolarimeter (Japan Specroscopic) with a cell of 0.1-mm optical path length temperature-controlled at 25 °C as described [12]. Small-angle X-ray Scattering (SAXS) measurements of the fibril solutions were carried out at RIKEN structural biology beamline I (BL45XU) at SPring-8 in Harima, Japan, with X-ray beam of 0.9-Å wavelength and a camera length of 2245 mm and a typical exposure time of 1 sec with a temperature-controlled (25 °C) cell of 50 μL capacity [13].

Results

Single-disulfide variants form amyloid-like fibrils morphologically similar to the 0SS fibril

The all-disulfide-deficient variant of hen lysozyme, 0SS, spontaneously forms amyloid fibrils under mildly acidic conditions [9]. The 0SS fibril is about 2 nm in height when observed under atomic force microscopy, and has a nodular and winding morphology typical of amyloid protofibrils [11]. Here, we found that four molecular species of hen lysozyme 1SS variants also formed fibrils spontaneously (Fig. 2). Morphologies of the 1SS fibrils as observed under AFM were similar to each other as well as to that of 0SS fibril, and their height was about 2 nm in common. In close inspection, however, minor differences existed such as frequent occurrence of ring forms in 1SS (30-115) and short assemblies in 1SS (64-80).

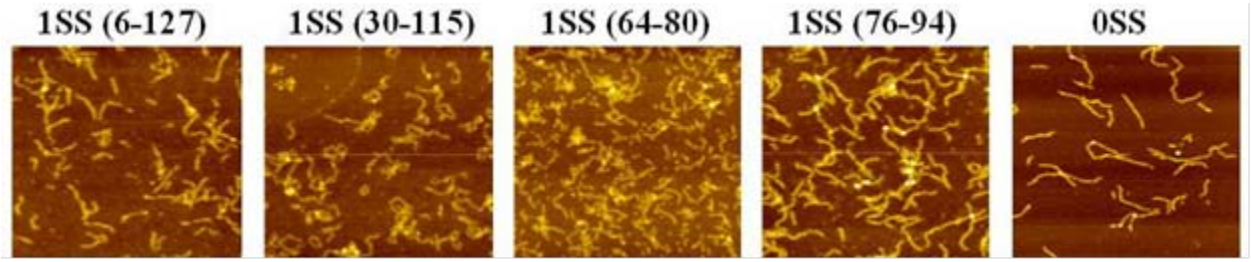

Fig. 2. AFM images of the fibrils of the 1SS-variants and 0SS. Fibrils were formed by incubating 5 mg ml^{-1} each variant protein in 20 mM sodium acetate, pH 4.0, 50 mM NaCl at 25 °C for four days (1SS (6-127)) or one day (1SS (30-115), 1SS (64-80), 1SS (76-94), and 0SS). All images are 1 μm × 1 μm in an observation window size.

Fibrillation kinetics varies greatly among the four 1SS variants

The fibrillation kinetics of each 1SS variant was monitored with high-performance SEC (Fig. 3).

The 1SS variants, initially existed in their monomer states and eluted at the total bed volume of the SEC column, spontaneously self-associated into polymer forms, which eluted early around the void volume of the column, well-separated from the monomer peak. The progress of the association was quite different among the 1SS variants. It took tens of hours in one variant, while minutes in some others (Fig. 4). The apparent association rate constant (Table 1) differed widely by more than three orders of magnitude among the four 1SS variants: High in 1SS (30-115) and 1SS (64-80), intermediate in 1SS (76-94), which was similar to 0SS control (apparent association rate constant of 1.3 hr^{-1}), and very low in 1SS (6-127).

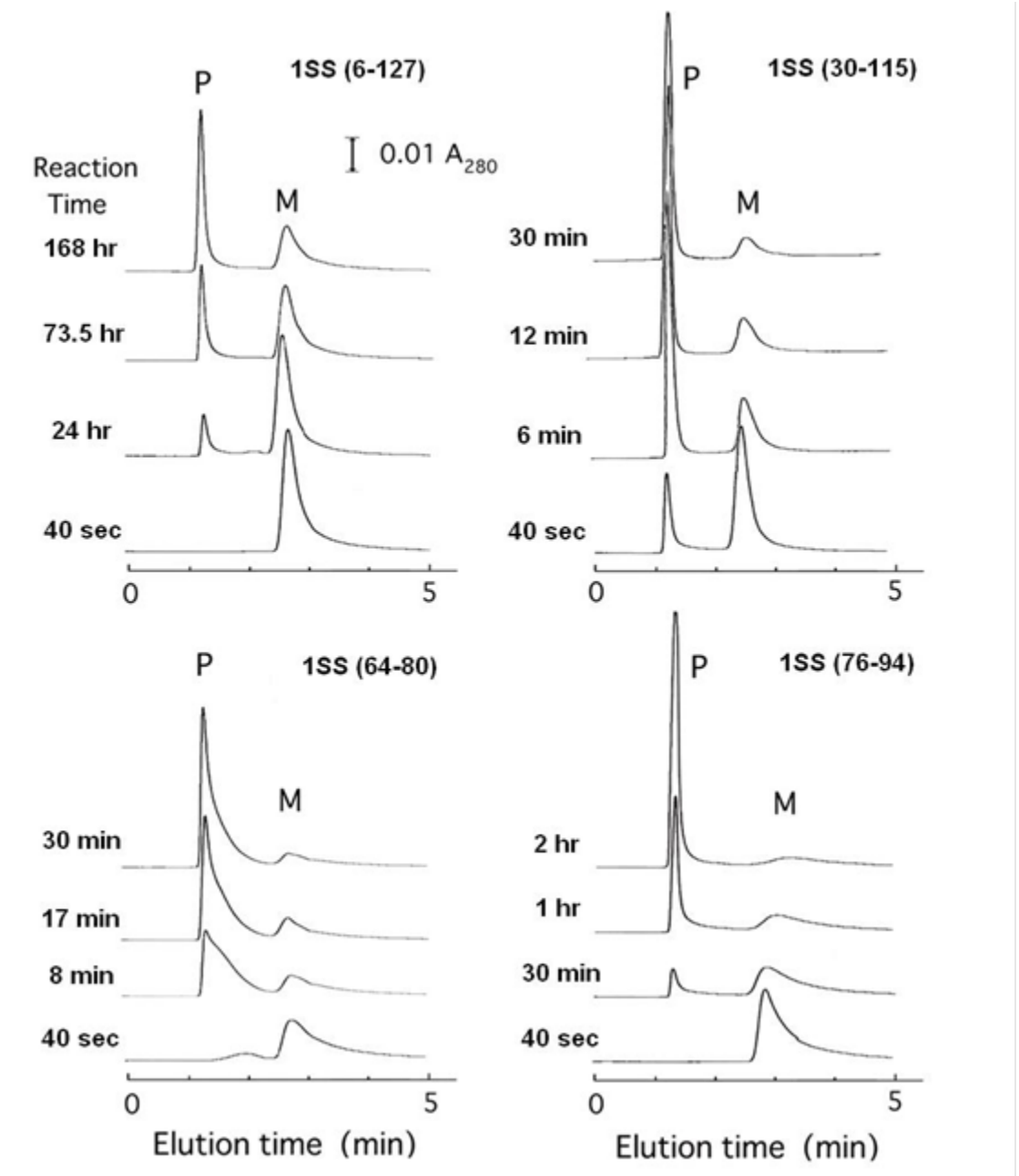

Fig. 3. Changes in SEC elution profiles of the 1SS variants with the progress of fibrillation. Each 1SS solution, 4 mg ml^{-1} in 20 mM sodium acetate, pH 4.0, 50 mM NaCl, was incubated at 25 °C. At each incubation period labeled to each profile aliquot of the solution containing ten µg protein was subjected to SEC. P and M denote polymer and monomer peaks, respectively.

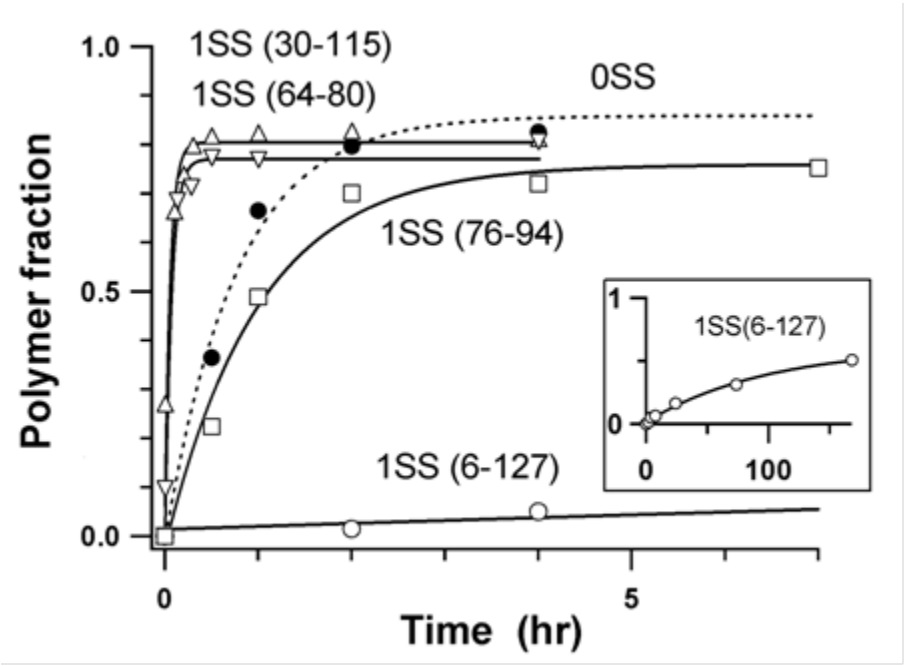

Fig. 4. Time course of the increase in polymer fraction in each 1SS fibrillation reaction. The fractional amount of polymer and that of monomer were quantified from the area of the elution peaks shown in Fig. 3: 1SS (6-127) (circle), 1SS (30-115) (up triangle), 1SS (64-80) (down triangle), 1SS (76-94) (square). As a control, those for 0SS (filled circle) were included. Apparent association rate constant was obtained with a regression of the time course to a single-exponential function of time (full lines for 1SS variants, and a dotted line for 0SS). Data points at up to 168 hrs (inset) were included in the regression for 1SS (6-127).

Table 1. Fibrillation properties and thermodynamic parameters of the 1SS variants.

Variant	Apparent association rate constant (hr^{-1})	Fibril diameter[a] (nm)	Intrinsic stability of submolecular structure[b] (kJ mol^{-1})
1SS (6-127)	0.010	6.9 ± 0.2	−17.9
1SS (30-115)	17	8.2 ± 0.2	−16.6
1SS (64-80)	14	7.8 ± 0.1	−15.2
1SS (76-94)	1.0	8.0 ± 0.1	−18.7

[a] Diameter = $2 \times \sqrt{2} \times$ (Cross-sectional radius of gyration at infinite dilution).
Mean and SD for three fibril samples are shown.
[b] Taken from ref [6].

The association of each of the 1SS variants was accompanied by β-structure formation: The mean residue ellipticity at 217 nm deepened from −7,000 ~ −8,000 deg cm^2 $dmol^{-1}$ in the monomeric state to −12,000 ~ −15,000 deg cm^2 $dmol^{-1}$ in the polymeric state (not shown). The diversity in the kinetic rate of β-structure formation among the four 1SS variants closely paralleled with that in the rate of the increase in the polymer fraction as observed in SEC: rapid in 1SS (30-115) and 1SS (64-80), and very slow in 1SS (6-127).

Slow-fibrillating 1SS variant has a slightly decreased fibril diameter

It is frequently noted that the width of fibrils as seen under AFM does not necessarily represent the actual diameter of the fibril in solution because of the mechanism of scanning-probing itself. Instead of the width, the height under AFM is usually taken to represent the diameter of the fibril. All the four 1SS variants and 0SS employed here showed a fibril height of about 2 nm in common under AFM. However, the highly differential fibrillation rate observed among the 1SS variants prompted us to investigate possible differences in fibril morphology in high resolution with SAXS measurements (Fig. 5).

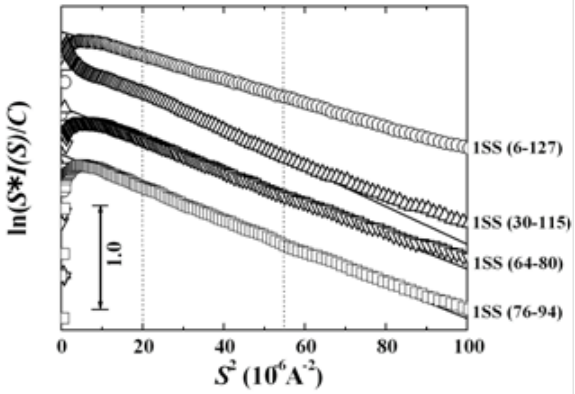

Fig. 5. Cross-sectional Guinier plots of SAXS profile of the 1SS variant fibrils. Cross-sectional radius of gyration of fibril at infinite dilution is obtained from the slope of the cross-sectional Guinier plots, $\ln(S \times I(S)/C)$ vs S^2, by using data points in the region, 20×10^{-6} A^2 < S^2 < 55×10^{-6} A^2 , shown in dotted lines: *S*, momentum transfer (=2(sin(θ))/λ, where 2θ is a scattering angle and λ the wavelength); *I(S)*, scattering intensity; *C*, protein weight concentration. The plots were displaced along the ordinate for clarity. Plot symbols are the same as in Fig. 4. There was no clear protein concentration dependency in *I(S)/C*. Fibrils were formed by incubating 5 mg ml^{-1} protein in 20 mM sodium acetate, pH 4.0, 50 mM sodium chloride at 25°C for 75 days.

Information about fibril radii can be obtained from cross-sectional Guinier plots of SAXS intensity [14]. A slightly shallow slope of the plot for 1SS (6-127) fibril as compared to those of the other three 1SS variants indicates that the fibril diameter is decreased specifically for this variant. This is not due to the comparatively high amount of unassociated monomers of 1SS (6-127) coexisting with its fibrils (Fig. 3), since 1SS (6-127) fibril solutions of different incubation periods (3 and 75 days), and therefore of different amounts of coexisting monomers, gave the decreased value of the cross-sectional radii that are closely similar to each other, which is rationalized from much higher contribution to scattering of high-molecular weight fibrils compared to monomers. Quantitatively (Table 1), while the other three 1SS fibrils has a diameter of about 8 nm in common, 1SS (6-127) has a slightly decreased diameter of about 7 nm. We note that both of these values are quite larger than the value, 2 nm, observed as a height of fibrils under AFM. The fibrils may be so flexible as to be flattened under AFM.

Discussion

Intricate effects of single disulfide bond incorporation on the fibrillation of lysozyme

The present study showed highly differential effect of the incorporation of different single disulfide bonds on the formation of protofibrils of the intrinsically unfolded hen lysozyme all-disulfide-deficient variant. Although the effect was large on the apparent association rate, all the four lysozyme 1SS variants fibrillate spontaneously to fibrils of nearly the same morphology in low resolution. In this respect, the effects were not so profound compared with those reported for β2-microglobulin (β_2m) [15,16], which contains a single disulfide bond in its native form. The fibril of β_2m single-disulfide form is thick, tight and straight, quite different from the thin, curled filaments of the disulfide-absent (reduced) protein, although the morphology changes with pH and salt-concentration as well [17-19]. The less profound effect observed here for lysozyme is probably due to the fact that all the four lysozyme 1SS variants only contain unstable submolecular structures [6], close in structural level to intrinsically unfolded 0SS and distant from the fully structured 4SS native form. Interestingly, WT lysozyme is known to form thick and straight fibrils under harsh solution conditions that lead partial proteolysis or under the aid of seeding [20,21], similar to β_2m WT 1SS form. Thus, the present 0SS to 1SS variant system of lysozyme, and possibly 2SS and 3SS variant systems to be undertaken in future, are suited to investigate intricate mechanistic details of the effect

of the introduction of disulfide bonds on fibrillation at various levels of protein structures.

Relationship between the fibrillation efficiency and the stability of submolecular structures

The present study has shown that 1SS (30-115) and 1SS (64-80) fibrillates rapidly while 1SS (6-127) very slowly, compared with 0SS fibrillation. Simple interpretation of the kinetic result is that in the transition state between monomeric and fibrillar states of 0SS, residue 30 is sterically near to residue 115, residue 64 is also near to residue 80, but residue 6 is at a distance from residue 127. In energetic terms, however, the present finding that two of the four 1SS variants fibrillate faster than 0SS is surprising since introduction of native disulfide bonds is thought to stabilize native structures and therefore disfavor amyloid fibrillation, which is generally thought to involve misfolded structures.

We have previously analyzed the denaturant-concentration dependence of an effective concentration of protein cysteinyl thiol groups that participate in each native disulfide bond, and shown that only local and unstable submolecular structures exist in all of the four lysozyme 1SS variants [6]. When the thermodynamic parameter values obtained previously for each 1SS variant are compared with the rate of amyloid fibrillation obtained here, we clearly see that the rapid-fibrillating species have comparatively high intrinsic stability of submolecular structures: 1SS (30-115) and 1SS (64-80) rank second and first, respectively, in the stability. (Note that the stability of the submolecular structures in Table 1 is shown with the unfolding free energy change: less negative values indicate higher stability) On the other hand, the moderate- and slow-fibrillating species, 1SS (76-94) and 1SS (6-127), rank fourth and third, respectively, in the intrinsic stability. Thus, relatively stable protein submolecular structures which are formed coupled with native disulfide-bond formation are able to enhance the fibrillation. It is interesting that the two disulfide bonds, Cys30-Cys115 and Cys64-Cys80, that enhance fibrillation, have also been shown to be important for the formation of native secondary structures spatially close to them [8]. Thus, it appears that starting from a completely unfolded state, submolecular structure formation proceeds to a certain structural level in which both monomolecular folding and intermolecular fibrillation are favored.

Separation between N- and C-terminal regions is necessary for efficient fibrillation

The fibrillation is decelerated in 1SS (6-127) by two orders of magnitude, while it is accelerated in 1SS (30-115) by one order of magnitude. Considering the previous results that the 2SS variant containing both of these two disulfide bonds establishes the native tertiary structure similar to that of the α-domain of WT lysozyme [7,22], the mutually opposing effect observed here on fibrillation of these two disulfide bonds is remarkable, and emphasizes the differential mechanism between the native folding and amyloid fibrillation of lysozyme. The extremely slow fibrillation of 1SS (6-127) cannot be explained with its relatively low intrinsic stability alone. It is most probably due to the fact that it has the largest size of substructure among the 1SS variants [6]. Such a large structure should be on the pathway to lead global native folding and therefore depresses fibrillation. The inhibitory role of Cys6-Cys127 disulfide bond, which connects N- and C-terminal regions of lysozyme, is reminiscent of the role of N- and C-terminal β-strands of β_2m that protect native folding against fibrillation [23]. The depressed fibrillation and the slight but significant decrease in the fibril diameter specifically observed in 1SS (6-127) indicate that N- and C-terminal regions are sterically separated from each other in a natural form 0SS fibrils. The imposed proximity of N- and C-terminal regions in 1SS (6-127) on the one hand strongly decelerates fibrillation, and on the other hand forces protein peptide segments to change their steric arrangement in fibrils.

It may be argued that the extremely slow fibrillation of 1SS (6-127) is related with a possibly long nucleation period involving a nearly first-order reaction of conformational (or covalent-bond) change of protein as a rate-determining step. In such a case, the length of a lag period and the overall association rate will not strongly depend on the total protein concentration, as actually observed for the fibrillation of WT lysozyme [24]. We repeated the same sort of fibrillation reaction as shown in Fig. 3 at various 1SS variant concentrations. The

association rate did strongly increase in powers of the total protein concentration with an exponent of two to three (not shown). The linear dependence of logarithm of the association rate on that of total protein concentration observed for all the four 1SS variants and 0SS conforms to an existing theory of "nucleation-elongation" type polymerization reaction [25]. Thus, there does not exist serious difference in the spontaneous nucleation mechanism among the 1SS variants. The high variation in the apparent association rate among them derives from the difference in the intrinsic rate for the elementary step of binding of monomers to polymer ends and development of intermolecular β-structure.

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