Enhancement of Misfolding and Amyloid Fibrillation by Native Disulfide Bonds

Hideki Tachibana[1,2] and Ryohei Kono[3]

Abstract

Native disulfide bonds are generally thought to assist correct folding, and therefore to depress misfolding. Here, the effect of the presence of individual disulfide bonds on the amyloid fibrillation of hen lysozyme was investigated by using a set of disulfide-variant proteins: 0SS, an all-disulfide-deficient variant; 1SS, single-disulfide variants; 2SS, double-disulfide variants; 3SS, triple-disulfide variants; and 4SS (WT). The protein conformation in a monomeric state covered by this set of variants ranges from global unfolding, through various local folding, to a global folding. Under the solution conditions not favoring spontaneous fibrillation, seeded fibrillation reactions were carried out using sonicated WT lysozyme fibril fragments as seeds and the disulfide variants as reactants for fibril-elongation, and monitored with thioflavine T fluorescence, CD spectroscopy, and scanning probe microscopy. Contrary to the general expectation, disulfide bonds C64-C80 and C76-C94 enhanced amyloid-fibrillation indicating that these native disulfide bonds have a positive role in the misfolding into regular intermolecular β-structures and fibrillation. The other native disulfide bonds C6-C127 and C30-C115 depressed the fibrillation reaction.

Key words : amyloid fibrillation, disulfide bond, protein misfolding, lysozyme variant

1. Introduction

Protein self-association into fibrils of μm-length with regular tertiary arrangement of the β-secondary-structured segments and their deposition in tissues or organs are diagnostics of a number of amyloid diseases [(1)]. A prerequisite for amyloid fibrillation is protein misfolding, which is brought about by exposure of peptide segments that have a high propensity for both β-structure and hydrophobic aggregation and absence of appropriately organized intramolecular peptide segments that could be present in the case of native folding and could accept or pair with the exposed β-segment. Such exposure is caused by partial [(2,3)] or full [(4,5)] unfolding of protein, which is realized *in vivo* by cleavage of peptide chains with specific proteinases or interaction with various endogenous substances, and *in vitro* by chemical denaturants or physically denaturing conditions. In simplified systems of amyloid fibrillation such as hen lysozyme, modelled on naturally occurring amyloidogenic variants of human lysozyme [(2)] with reduced thermodynamic stability, proteins with its disulfide bonds cleaved or synthetic peptides that cover highly amyloidogenic regions have been used [(4,6,7)] since their unfolded state can be achieved under native solution conditions, which enabled the use of a wide variety of experimental conditions with additives.

We have produced in *E.coli* a set of disulfide-deficient variants of hen lysozyme in order to dissect an otherwise highly cooperative folding and unfolding reactions: 0SS, an all-disulfide-deficient variant [(8)]; 1SS, single-disulfide variants [(8)]; 2SS, double-disulfide variants [(9)]; 3SS, triple-disulfide variants [(10,11)]; and 4SS, WT or its recombinant protein [(12)]. Starting from the disulfide-deficient unfolded state, protein disulfide bond formation couples with the formation or stabilization of protein structures [(13)]. Each disulfide bond is coupled to varying degrees with the formation of submolecular structures of protein, which can be probed only through specifically designed experimental analyses employing disulfide variant proteins [(8,12-15)]. It is also coupled with indirect stabilization of the folded state through an entropic destabilization of the unfolded state, which is generally predictable from the loop size of the polypeptide chain closed with the disulfide bond [(16,17)]. Although the formation of nonnative disulfide

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[1] Department of Biotechnological Science, Kinki University, Wakayama 649-6493, Japan

[2] High Pressure Protein Research Center, Institute of Advanced Technology, Kinki University, Wakayama 649-6493, Japan

[3] Department of Strategic Surveillance for Functional Food and Comprehensive Traditional Medicine, Wakayama Medical University, Wakayama, 641-0012, Japan

bonds (hereafter we use "native" to indicate the disulfide-bridging between a pair of cysteinyl residues that is identical to the one found in the native protein) is observed in certain proteins in the initial stage of folding and contributes indirectly to the acceleration of overall folding reaction, native folding is usually assisted by the formation of native disulfide bonds [13].

The contribution of disulfide bonds to protein folding described above is largely concerned with the folding in a monomeric protein state. In the case of multimeric protein association, the effect of disulfide bond has barely been paid general attention because almost completely folded proteins usually participate in the association through non-covalent interactions or are connected with intersubunit disulfide bonds which do not appear to be coupled with additional formation of protein secondary or tertiary structure. The formation of amyloid fibrils, by contrast, involves protein unfolding and misfolding into β-structures as described earlier, and is subject in principle to the formation of disulfide bonds. Here, simple expectations will be that the native disulfide bond formation promote native folding as in the case of a monomolecular folding event, and therefore will depress amyloid fibrillation which intrinsically involves misfolding. Contrary to this expectation we have reported that in the case of spontaneous formation of amyloid protofibrils, i.e. without the addition of amyloid seeds, although a native disulfide bond C6-C127 decelerates the fibrillation reaction hundred-fold or so, native disulfide bonds C64-C80 and C30-C115 accelerates it ten-fold or more [18]. In this study, employing sonicated-fragments of WT lysozyme amyloid fibrils and various molecular species of disulfide variants of lysozyme as seeds for fibrillation and reactants for fibril-elongation, respectively, we show that also in the case of a seeded-fibrillation system some native disulfide bonds enhance amyloid-fibrillation reaction significantly.

2. Materials and Methods

2.1 Protein samples

The disulfide-deficient variants of hen lysozyme (Fig. 1) were prepared as described previously [8-12]. Briefly, the variants were produced in *E.coli* as inclusion bodies, solubilized and chromatographically purified under reducing solution conditions, reoxidized (except for 0SS) with glutathione redox system or with air-oxidation at a considerably low protein concentration to avoid protein aggregation and at a selected temperature in the presence of appropriate concentration of glycerol which favors an enhanced population of a native disulfide isomer, concentrated with reversed-phase resins, purified with large-scale reversed-phase chromatography, and freeze-dried. Wild-type hen lysozyme (six times recrystallized) was obtained from Seikagaku Kogyo.

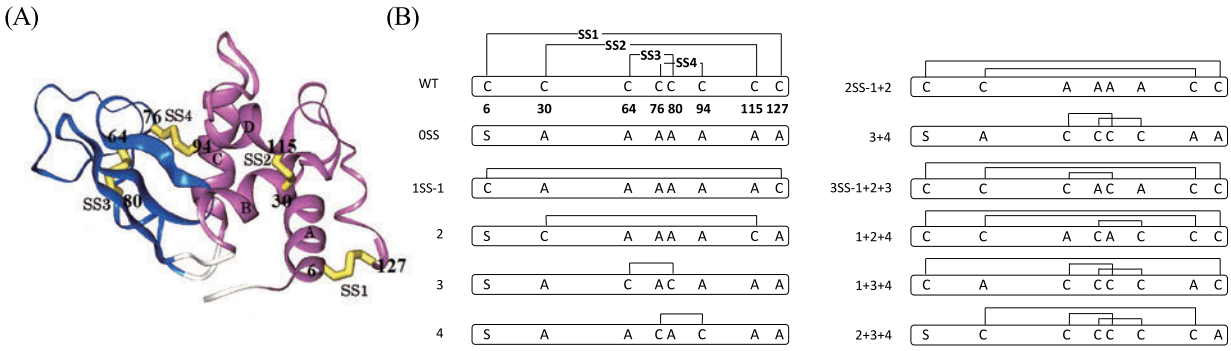

Fig. 1. (A) Natively folded structure of hen lysozyme represented with ribbon-diagram (PDB 6LYZ). The residue numbers for eight cysteines and their bridging to form disulfide bonds (yellow sticks) SS1 (C6-C127), SS2(C30-C115), SS3(C64-C80), and SS4(C76-C94) are shown. Red and blue regions represent α- and β-domains respectively. In the α-domain, four helices (A through D) are indicated. (B) Schematic diagrams showing the native disulfide bridging pattern of WT and a set of disulfide variants of hen lysozyme used in this study. 0SS, 1SS, 2SS, and 3SS means zero-, single-, double-, and triple-disulfide bonds retaining variants, respectively, followed by the identity of the retained disulfide bonds, which had been confirmed with peptide-mapping. There exist four other 2SS-variants, which were not used here. C, S, and A denote Cys, Ser, and Ala residues respectively. Expression was not observed, in our hands, for the genes that contain Ala codon at the sixth amino acid residue. The secondary structures in each variant have been described [19].

2.2 Seeded-fibrillation

The seeds for amyloid fibrillation were prepared by sonicating 4.0 mg mL^{-1} WT lysozyme second-generation amyloid fibrils [20] in 80 mM NaCl, pH 2.2, for multiples of 15-min period at an output of about 140 W on a Branson S-250A sonifier attached with a cuphorn temperature-regulated at 20 °C. Seeded-fibrillation was carried at a total protein concentration of 4.0 or 5.0 mg mL^{-1} in 20 mM GlycineHCl, pH 2.6, 50 mM NaCl, in which the mixing ratio, in weight, of the WT amyloid-fibril seed to lysozyme disulfide-deficient variant monomeric protein was 1:19, and proceeded at indicated temperature for the indicated period of time. Under the solution conditions, spontaneous fibrillation hardly takes place.

2.3 Monitoring and quantification of fibrillation

Atomic force microscopy observation was carried out on a scanning probe microscope SPI-380 (Seiko Instruments Inc.) under a dynamic-force cyclic-contact mode at a frequency 110 – 150 kHz with a cantilever DF20 (SII NanoTechnology). The incubated fibril solution was diluted hundred-fold, ten μL of which was applied onto freshly cleaved mica surface, rinsed with pure water, which was blotted sideways, and left to dry. Far-UV CD spectra of the fibril solution, not diluted, were taken on a J-820 spectropolarimeter (JASCO Co., Tokyo) using a quartz cuvette of 0.1-mm optical path length. A mean residue molecular weight value 110.85 for WT lysozyme was used throughout the variants for the sake of simplicity. The extent of fibrillation was monitored with thioflavine T fluorescence measurements, in which 4 μL of the fibril solution was mixed with 396 μL of 20 μM thioflavine T working solution in 20 mM potassium phosphate, pH 7.4, 100 mM sodium chloride, and fluorescence emission spectra were taken with an excitation wavelength of 450 nm, and the fluorescence intensity maximum which was usually at 482 nm, F_{482}, was plotted below.

3. Results

3.1 Preferential fibrillation of variants containing SS3 (C64-C80) and SS4 (C76-C94)

Our supplementary experiments using thioflavine T fluorescence have clarified pH- and salt concentration-dependence of the seeded-fibrillation of WT lysozyme (not shown): it occurred efficiently at pH 1.5 and 2.2, moderately at pH 3.0, and inefficiently at pH 3.6 and 4.0, in parallel with the decrease in the population of unfolded state with the increase in pH to neutral region; the increase in NaCl concentration from 4 to 80 mM increased the apparent rate of seeded-fibrillation from about 0.01 hr^{-1} to 1.0 hr^{-1} at pH 2.2 and 4.0 mg mL^{-1} total protein concentration. Higher ratios of seed to monomer resulted in proportionally higher apparent rates of fibrillation as theoretically predicted [21]. A too higher ratio, however, is expected to obscure possible difference among the fibril-elongation reactions by individual disulfide variants. All these considerations led to the choice of the reaction conditions as described in subsection 2.2.

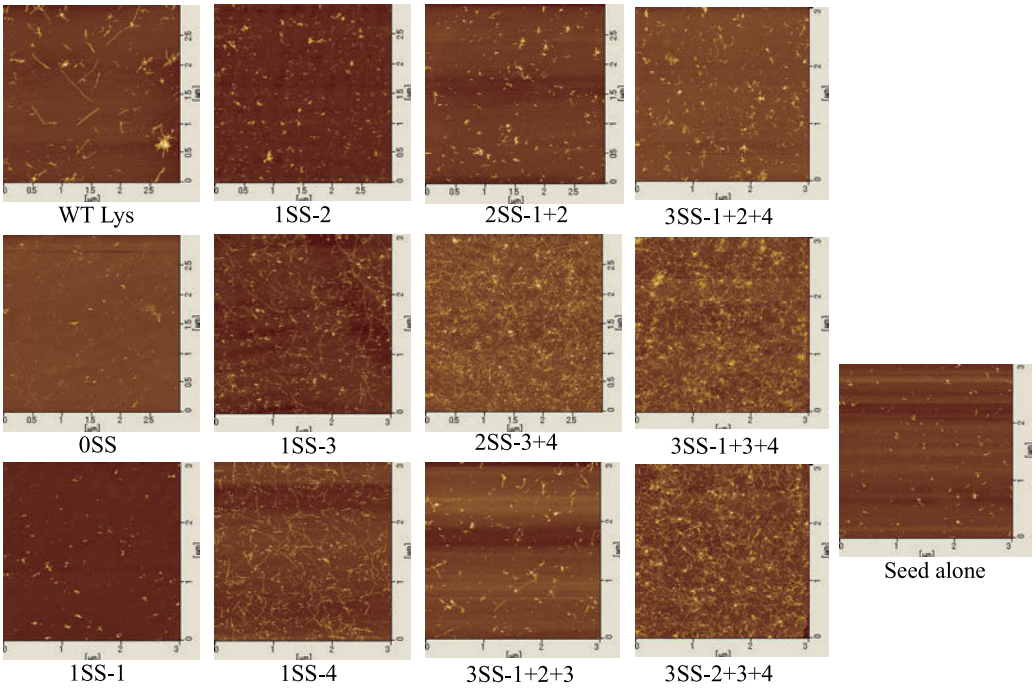

Fig. 2. AFM images of the cross-seeded-fibrillation products. Fibrils were formed at 57 °C for 18 hrs. The observation window size is 3 μm × 3 μm.

Seeded-fibrillation indeed occurred under not exactly homologous pairs of seed and monomer, i.e. WT lysozyme and disulfide variants. A large number of fibrils were formed for the variants that contain SS3(C64-C80) and/or SS4(C76-C94) (Fig. 2). The amount of fibrillation was further increased by the presence of both SS3(C64-C80) and SS4(C76-C94), while it was contrastingly low in 0SS, 1SS-1, 1SS-2, and 2SS-1+2. The inhibitory effect of SS1(C6-C127), or SS2 (C30-C115) appears to be passive in that the presence of either of them did not significantly reduce the amount of fibrils as seen in 3SS-1+3+4 and 3SS-2+3+4. The simultaneous presence of both SS1(C6-C127) and SS2(C30-C115), however, did reduce the fibrillation as seen in 3SS-1+2+3 and 3SS-1+2+4 in comparison with 1SS-3 and 1SS-4, respectively. The thickness of the fibrils, as determined from the height measurement, were 0.5 to 2 nm, and increased to 4 nm with prolonged incubation periods. The fibril morphology was not thin and highly winding, unlike that of 0SS [22] or 1SS species [18].

3.2 Preferential formation of β-structure in the variants containing SS3 (C64-C80) and SS4 (C76-C94)

Formation of β-structure is one of the characteristics of amyloid fibrillation. Appearance of a single negative band centered around 217 nm in far-UV CD spectrum is indicative of β-structure formation, while the existence of two troughs around 208 and 222 nm is of α-structure. As shown in Fig. 3, the products of seeded-fibrillation of WT, 1SS-3, 2SS-3+4, 3SS-1+3+4, and 3SS-2+3+4 developed β-structures, while those of 0SS, 1SS-1, 2SS-1+2, 3SS1+2+4 did not develop β-structures, and those of 1SS-2, 1SS-4, and 3SS-1+2+3 gave CD spectra of in-between characteristics. Among the variants that did not well develop β-structure, 0SS, 1SS-1, 1SS-2 and 2SS-1+2 showed a negative trough around 200 nm, which indicates that a considerable fraction of random coil conformation, originally existed in the starting reactants, ie. monomeric variant proteins, remained unorganized into regular secondary structures. These variants coincide with those that showed poor fibrillation on AFM (Fig. 2). Thus, we conclude that the native disulfide bonds SS3(C64-C80) and SS4(C76-C94), have positive contribution to amyloid fibrillation.

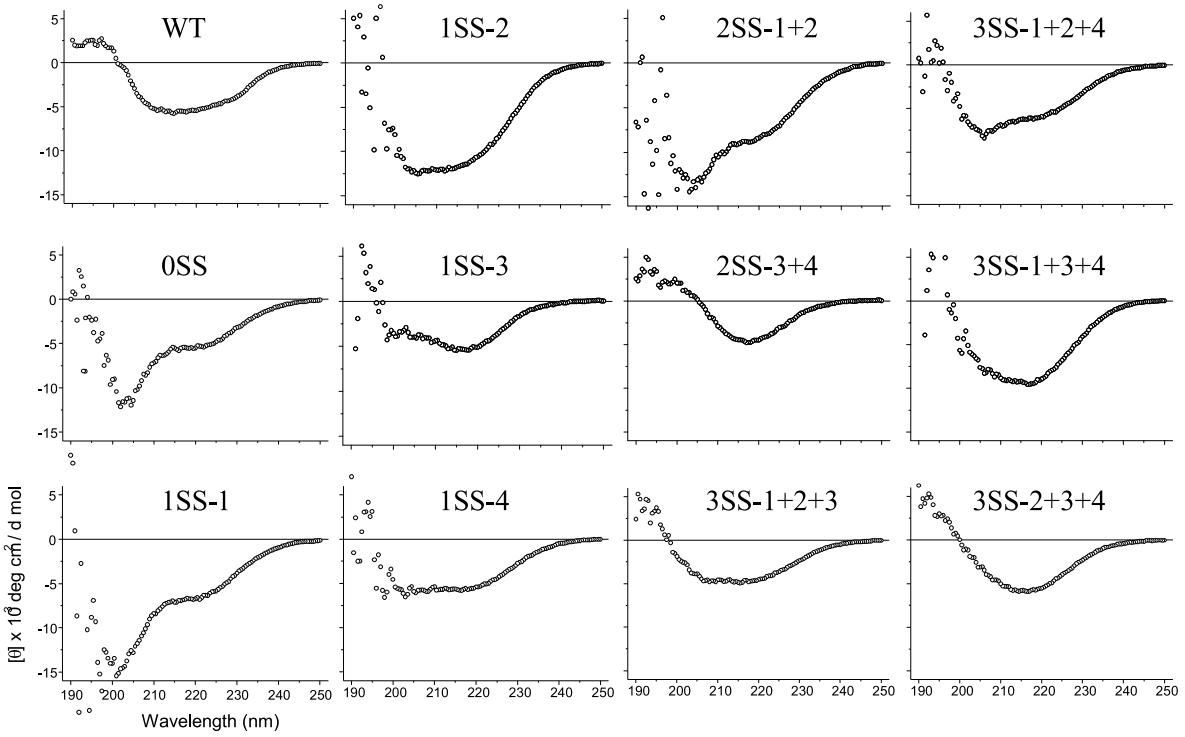

Fig. 3. Far-UV CD spectra of the product of seeded-fibrillation. Fibrils were formed at 57 °C for 24 hrs, and the spectra were taken at RT. Protein concentration was 5.0 mg mL^{-1} throughout.

3.3 Disulfide bond preference and temperature-dependence of fibrillation

In order to quantify the differences in the effect of individual disulfide bonds on amyloid fibrillation and also to see its temperature-dependence we carried out thioflavine T fluorescence measurements on the produced fibril solutions (Fig. 4). The positive contribution of SS3(C64-C80) and SS4(C76-C94) was clearly demonstrated in the results among the four 1SS variants; their synergistic effect is prominently shown in the results of 2SS and 3SS variants. This preference for SS3(C64-C80) and

SS4(C76-C94) is apparent already at moderate temperature such as 25°C (dark green-colored bars in Fig. 4). The seeded-fibrillation of WT lysozyme was enhanced with increasing temperature: this is of course due to its reduced stability at higher temperature. The seeded-fibrillation of all the four 3SS variants was also enhanced with increasing temperature due to the same reason. However, in 2SS variants, too high temperature reduces the fibrillation amount: for instance, there exists a temperature optimum at 41 °C in 2SS-3+4. The situation is same for the temperature-dependence of 1SS variants. 2SS and 1SS variants have reduced stability compared with 3SS variants or WT, and the appearance of temperature optimum suggests that the unfolded state itself does not suffice for amyloid-fibrillation to proceed efficiently.

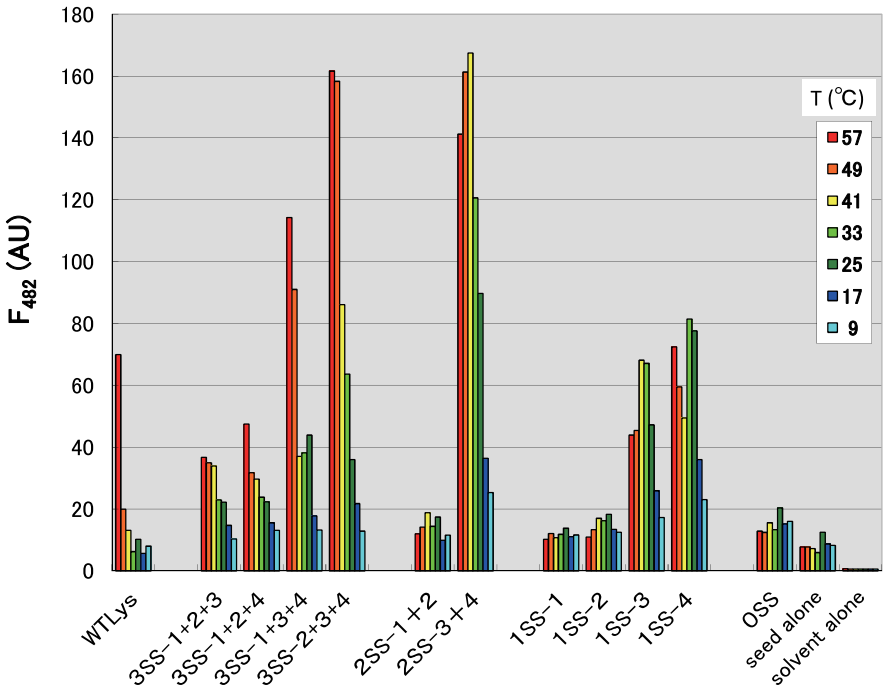

Fig. 4. Thioflavine T fluorescence intensity for the product solution of seeded-fibrillation carried out for 24 hrs at a protein concentration of 4.0 mg mL^{-1} and temperature points indicated with a color code. Negative controls for seed alone and solvent alone were also included. The F_{482} value of negative control for spontaneous fibrillation without seeds (not shown) was 1–3.5 for 1SS and 0SS variants, and 1– 2 for other protein species.

4. Discussion

Clear differences in the effect on amyloid fibrillogenesis between α-domain and β-domain disulfide bonds were demonstrated in this study. Disulfide bonds SS3(C64-80) and SS4(C76-94) enhance amyloid fibril formation while SS1(C6-C127) and SS2(C30-C115) depress it. The positive effect of the native disulfide bonds SS3(C64-80) and SS4(C76-94) means that, although the unfoldedness of protein with the cleavage of all the disulfide bonds is sufficient for amyloid fibrillation of hen lysozyme [4] and moreover the formation of native disulfide bonds is generally thought to positively contribute almost exclusively to the folding into a correct native state, the presence of native disulfide bonds does contribute in certain cases positively to the misfolding and fibrillation. This is somewhat surprising, but understandable when we recall that amyloid fibrillation is not merely a random aggregation of unfolded protein, but is a phenomenon of aggregation into ordered structures.

We can compare the present results on seeded-fibrillation with our previous report [18] on spontaneous protofibril formation. On the one hand, there exists parallelism: SS3(C64-C80) enhances both seeded- and spontaneous fibrillation reactions, and SS1(C6-C127) depresses both reactions. On the other hand, there exist differences: SS4 (C76-94) enhanced seeded-fibrillation, while it neither accelerates nor decelerates spontaneous fibrillation; SS2(C30-C115) depresses seeded-fibrillation, while it accelerates spontaneous fibrillation. The difference in the morphology of produced fibrils between the two reaction schemes, “straight and thick” versus “winding and thin”, indicates the difference in the molecular architecture of the fibrils, detailed future analyses of which will clarify the

molecular mechanism for the differential effect of certain disulfide bonds observed between the two fibrillation schemes.

What mechanism coupled with disulfide bond can promote the misfolding into amyloid fibrils? One possibility is that the formation of a disulfide bond such as SS3(C64-80) favors a development of β-structure over α-helix in a peptide region which has high propensity for both of the two secondary structures. The possible contribution of the introduction of disulfide bond SS3(C64-80) to the β-structure formation of this peptide region has been suggested from dynamic structural studies using NMR [23]. If this peptide segment does not have a proper β-structure counterpart strand within a protein molecule, or does not form a turn around its central residue positions and therefore does not by itself form an antiparallel β-structure, the exposed lonely β-segment is able to self-associate with the corresponding β-segments in other protein molecules, eventually forming an amyloid fibril. However, as for the other disulfide bond, SS4(C76-C94), which enhanced fibrillation too, Cys94 participates in α-helix "D" in the natively folded lysozyme structure, and does not conform to this mechanism.

Another possibility is that the *intra*molecular disulfide bond originally connected Cys residues within a monomeric protein is converted, coupled with the fibrillation process, to an *inter*molecularly-bridged disulfide bond . In connection with this possibility, the specifically low efficiency of 0SS as compared with 1SS-3 or 4 in seeded-fibrillation is worth noting: in spontaneous fibrillation, 0SS shows the similar level of high efficiency as 1SS-4. Also, the level of unfoldedness in conformation is similar between 0SS and 1SS-4 [8,19,24]. Thus, the presence or absence of Cys residues that could participate in *inter*molecular disulfide bridging may have critical importance in seeded-fibrillation. Another point to be noted is that for the disulfide exchange from *intra*molecular to *inter*molecular ones to take place, catalyzing amount of reducing material is needed for thiol-disulfide transfer reactions [13]: $S_xH+S_aS_b+S_aS_b+S_aS_b+\cdots \rightarrow S_xS_a+S_bH+S_aS_b+S_aS_b+\cdots \rightarrow S_xS_a+S_bS_a+S_bH+S_aS_b+\cdots \rightarrow S_xS_a+S_bS_a+S_bS_a+S_bH+\cdots \rightarrow$, where S_aS_b and S_bS_a indicate, respectively, an originally formed *intra*molecular disulfide bond and a newly formed *inter*molecular disulfide bond in a fibril. Whether such an exchange of disulfide actually occurs in the fibril or not remains to be clarified.

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和文抄録

天然ジスルフィド結合によるミスフォールディングとアミロイド線維形成の増進

橘秀樹[1,2]、河野良平[3]

天然状態の蛋白質に見られるジスルフィド結合は正しいフォールディングを助長し、ミスフォールディングを抑制すると一般に考えられている。ここに報告する研究では、一連のジスルフィド結合変異体を用いて、ニワトリリゾチームのアミロイド線維化に及ぼすジスルフィド結合の効果を調べた。これらの変異体は、全てのジスルフィド結合を欠損したもの (0SS)、１個のジスルフィド結合を保持したもの (1SS)、２個のジスルフィド結合を保持したもの (2SS)、３個のジスルフィド結合を保持したもの (3SS)、そして４個のジスルフィド結合を保持したもの（これは野生型蛋白に相当）である。これらの変異体は、単一分子状態での蛋白質コンフォメーションとして、全体的にアンフォールドした状態、色々な部位が局所的にフォールドした状態、そして全体がフォールドした状態を網羅する。自発的線維化反応が起こりにくい溶液条件下において、野生型リゾチームアミロイド線維を超音波処理によって断片化したものを種として上記の色々なジスルフィド結合変異体を付加・伸長させる「種由来線維化反応」を行い、チオフラヴィン T 蛍光、円二色性分光、走査探針顕微鏡によってモニターした。その結果、一般的な予測に反し、ジスルフィド結合 C64-C80 ならびに C76-C94 はアミロイド線維化反応を増進する、即ち、これらの天然のジスルフィド結合は規則的な分子間β構造へのミスフォールディング、そして線維形成を促進する役割を持つことがわかった。一方、他の２本の天然のジスルフィド結合 C6-C127 および C30-C115 は線維化反応を低下させることが示された。

キーワード：アミロイド線維化、ジスルフィド結合、蛋白質のミスフォールディング、リゾチーム変異体

1. 近畿大学生物理工学部生物工学科, 〒649-6493 和歌山県紀の川市西三谷 930
2. 近畿大学先端技術総合研究所高圧力蛋白質研究センター, 〒649-6493 和歌山県紀の川市西三谷 930
3. 和歌山県立医科大学機能性医薬食品探索講座, 〒641-0012 和歌山県紀三井寺 811-1