J. Biol. Macromol., **8** (1), 11-18 (2008)

Article

Efficient conversion of intact hen lysozyme into amyloid fibrils by seeding

Ken Sasaki, Ken Nakatsuka, Itsuho Hayashi, Buddha R. Shah, Koichi Morimoto, and Kazuyuki Akasaka

Department of Biotechnological Science, School of Biology-Oriented Science and Technology, Kinki University, 930 Nishimitani, Kinokawa-shi, Wakayama 649-6493, Japan.

Received December 26, 2007; Accepted January 21, 2008

Clarifying the mechanism how intact protein is transformed into amyloid fibrils has central importance in the study of amyloidosis. When hen lysozyme is kept in an acidic solution (pH 2.2, 80 mM NaCl) at a temperature close to its transition temperature (e.g., 57 ºC), amyloid fibrils are formed after a lag-time of several to 11 days. These fibrils are mainly composed of peptide fragments produced by acid-degradation of the protein at selective Asp-Gly sites (Mishra *et al.*, *J. Mol. Biol.* 366, 1029-1044, 2007). We found that, when these fibrils are sonicated into small particles and a fraction is mixed into a solution of fresh intact hen lysozyme (pH 2.2, 80 mM NaCl), the second-generation amyloid fibrils are rapidly formed from the intact protein in an apparent two-state manner. The rate of the fibril formation depends critically on temperature in the transition region, showing that the fibrillation formation initiates by the interaction of the seed with heat-denatured protein. The efficient conversion of α-rich monomer proteins into β-rich amyloid fibrils by seeding with preformed fibrils could be part of a general mechanism of amyloid fibril formation and infection *in vivo*.

Keywords: hen lysozyme, degraded peptides, amyloid fibrils, sonication, seed-induced fibril formation, circular dichroism, atomic force microscopy

Introduction

The amyloid fibril formation has been linked to tissue or organ dysfunction such as neurodegeneration, i.e., Alzheimer's disease in the case of amyloid-β peptide, Parkinson's disease (α-synuclein) and Creutzfeldt-Jakob disease (prion protein) [1]. Various globular proteins undergo conformational changes *in vivo* and *in vitro* into β-sheet-rich structure forming fibrillar aggregates, generally known as amyloid fibrils [2, 3]. The mechanism of transformation of intact protein into amyloid fibrils has central importance in the study of amyloidosis. In our recent studies using disulfide-deficient mutant (0SS) of hen lysozyme, we have shown that the protein exists as fully denatured protein as monomer, but that it forms amyloid protofibrils spontaneously when certain ionic concentration is present [4]. Furthermore, we have also shown that the growth of 0SS takes place by the "linear polymerization mechanism" in which a monomer is added successively to a growing end of the protofibril [5, 6]. Here the average rate of growth of the protofibril will be proportionate to the concentration of the growing end of the protofibril rather than to the total concentration of the protein.

In the present work, we use wild-type hen lysozyme (4 S-S) (Fig. 1A) as a model to human lysozyme whose specific mutants are known to cause hereditary systemic amylodosis [7]. Previous studies reported that hen lysozyme is slowly degraded into fragments when kept in acidic solution at elevated temperatures, from which the amyloid fibrils are formed [8, 9]. In addition, acceleration of the fibril formation by the addition of preformed amyloid fibrils (without sonication or other treatments) was reported [10], but no detailed study has been made.

Correspondence to Kazuyuki Akasaka.
Tel: +81-736-77-0345 ext. 4110
Fax: +81-736-77-4754
E-mail: akasaka8@spring8.or.jp

The abbreviations used are: CD, circular dichroism; AFM, atomic force microscopy.

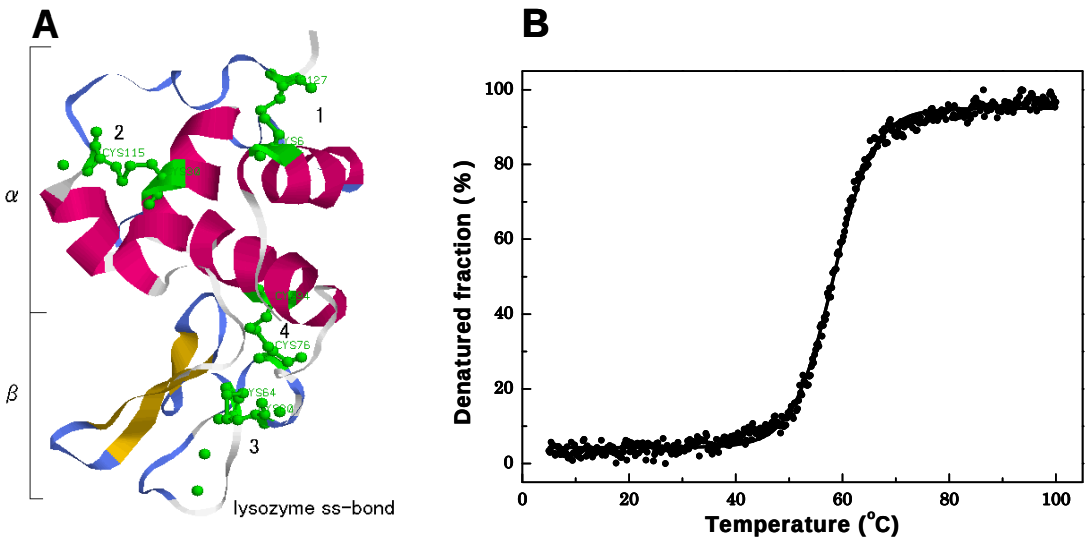

Figure 1. A. Structure of hen lysozyme (ribbon model). The α and β domains are shown with locations of the four disulfide bonds. B. Thermal transition of hen lysozyme (1.0 mg ml^{-1} in 80 mM NaCl, pH 2.2) as monitored by CD absorption at 222 nm. The mid point of transition is at ~ 57 ºC.

In the present work, using circular dichroism (CD) and atomic force microscopy (AFM) along with SDS-PAGE analysis, we demonstrate that the α-rich intact hen lysozyme (in 80 mM NaCl, pH 2.2) is rapidly and efficiently converted into the β-rich fibrils by seeding with particles produced from the first-generation amyloid fibrils by extensive sonication. We will show that the efficient conversion takes place without degradation of intact hen lysozyme only in the temperature range in which thermal denaturation of hen lysozyme is significant, showing that the fibril forming reaction is caused by the interaction of the seed with heat-denatured and non-degraded hen lysozyme.

Materials and Methods

Preparation of the first-generation amyloid fibrils

Lyophilized hen lysozyme obtained from Sigma was first dissolved in pure water. The protein concentration was determined by MultiSpec-1500 spectrophotometer (Shimadzu, Kyoto) at λ = 280 nm, using an extinction coefficient of 2.57 (mg)$^{-1}$ cm^2. The solution was mixed with sodium chloride to give a final protein concentration of 4.0 mg ml^{-1} in 80 mM NaCl at pH 2.2 (pH adjusted with HCl). The solution (~ 1.5 ml) was then incubated in plastic tubes (Eppendorf) at 57 ºC for as long as 11 days. For the study of temperature dependence of the fibril formation, a protein concentration of 1.0 mg ml^{-1} was chosen for the easier measurement of CD spectra in an optical cell of 0.1 cm light path. The fibril forming reaction was qualitatively the same for the two concentrations.

Preparation of the seeds

The fibrils in the tube (~ 1.5 ml) were subjected to extensive sonication on ice using a BRANSON SONIFIER model S-250A, which operated continuously for 60 min at a frequency of 20 kHz at a maximum power of 20 watts. The sonicated (first-generation) fibrils were used as "seeds" for producing the second-generation fibrils without separating non-fibril peptides.

Preparation of the second-generation amyloid fibrils by seeding

A quantity (150 µl) of the seed solution was mixed with 1350 µl of the hen lysozyme solution (1.0 or 4.0 mg ml^{-1}) in 80 mM NaCl, pH 2.2, the seed-to-protein ratio being 1/9 by weight. The mixture solution was then incubated at 57 ºC or at other temperatures for typically 10 h to produce the second-generation fibrils.

AFM measurements

The first and second generation fibrils were examined by AFM on an SPI-3800 (Seiko Instruments Inc.) at 25 ºC. An aliquot (2 µl) each of

the fibril solution was dropped on a fresh surface of mica and washed with 6 ml distilled water, before AFM images were recorded with the cyclic contact mode at a frequency of 119 Hz.

CD measurements

The time-dependent change of the CD spectrum upon the protofibril formation was recorded on a J-820 spectropolarimeter (JASCO, Tokyo) using cylindrical quartz cells of 0.01 cm path length for 4.0 mg ml^{-1} and 0.1 cm path length for 1.0 mg ml^{-1}, both cells being water-jacketed for temperature control.

SDS-PAGE

The SDS gel electrophoresis was performed on second-generation fibrils using an SDS treating buffer containing 8 M Urea, 1% β-mercaptoethanol and 1% SDS. The regular boiling treatment of the solution was not employed for feat of degrading the protein molecule. Instead, the solutions were kept at room temperature for 1 h before applying them on gels made of 15% SDS polyacrylamide. Reference markers obtained from APRO Science, Inc. were used.

Results

Formation of the first-generation fibrils

The solution of hen lysozyme (4.0 mg ml^{-1}) containing 80 mM NaCl, pH 2.2 was kept at 57 ºC, close to the transition temperature of hen lysozyme (~ 57 ºC) (Fig. 1B), similarly to the procedure in the literature [8, 9]. The change in mean residue ellipticity at 222 nm started after a lag time of ~ 5 days (data not shown), when protofibrils, consisting of long straight fibers of 2.0 ~ 3.5 nm in height, started to form on AFM. Finally, after ~ 11 days, some of the protofibrils formed a bundle omonomeric hen lysozyme into β-amyloid fibrils at 57 ºC. A. CD spectral changes with time of incubation of hen lysozyme solution (4.0 mg ml^{-1} in 80 mM NaCl, pH 2.2) in the presencf fibrils of 8 ~ 12 nm in height, suggesting the formation of matured amyloid fibrils (Fig. 2A). Examination by SDS-PAGE showed that the protein was degraded into fragments with time, which nearly completed in ~ 4 days (data not shown), in close agreement with the literature [8]. The time required for the degradation nearly coincided with the start of the fibril formation, suggesting that the degraded fragments, and not the intact protein, are responsible for the formation of the fibrils. Recently, Mishra *et al*. showed in their detailed analysis that the fibrils (hereafter called the first-generation fibrils) mainly consist of fragmented peptides (residues 49-101 and 53-101) specifically cleaved in Asp-Gly bonds

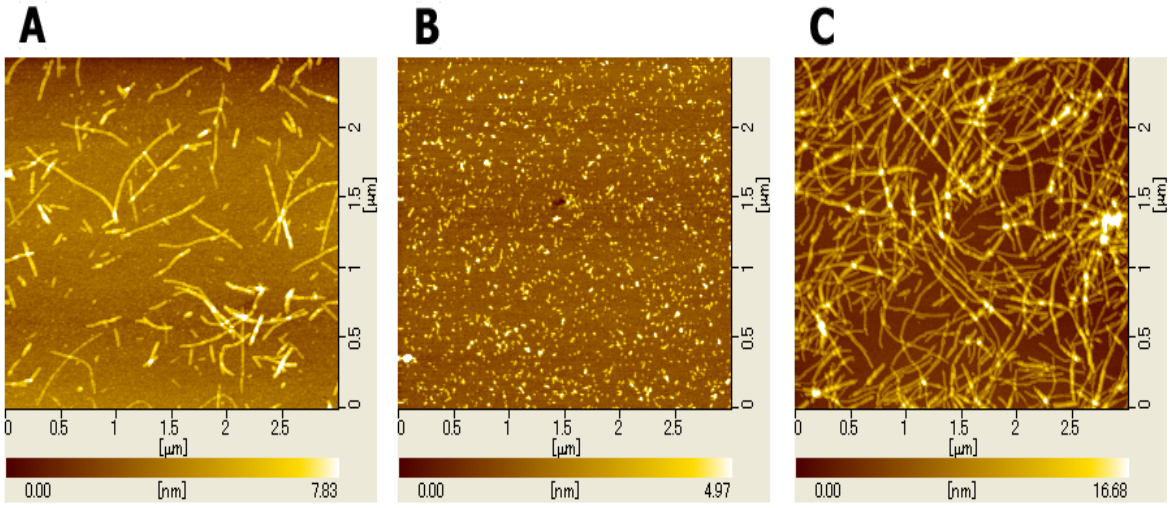

Figure 2. Amyloid fibrils from hen lysozyme monitored by AFM. A. The first-generation amyloid fibrils formed by incubating the acidic solution of hen lysozyme (4.0 mg ml^{-1} in 80 mM NaCl, pH 2.2) at 57 ºC for 11 days. B. Fragmented first-generation amyloid fibrils formed by extensive sonication (used as *seeds* to produce the second-generation amyloid fibrils). C. The second-generation amyloid fibrils formed by incubation of hen lysozyme solution (4.0 mg ml^{-1} in 80 mM NaCl, pH 2.2) in the presence of 10% (w/w) seeds at 57 ºC for 24 h.

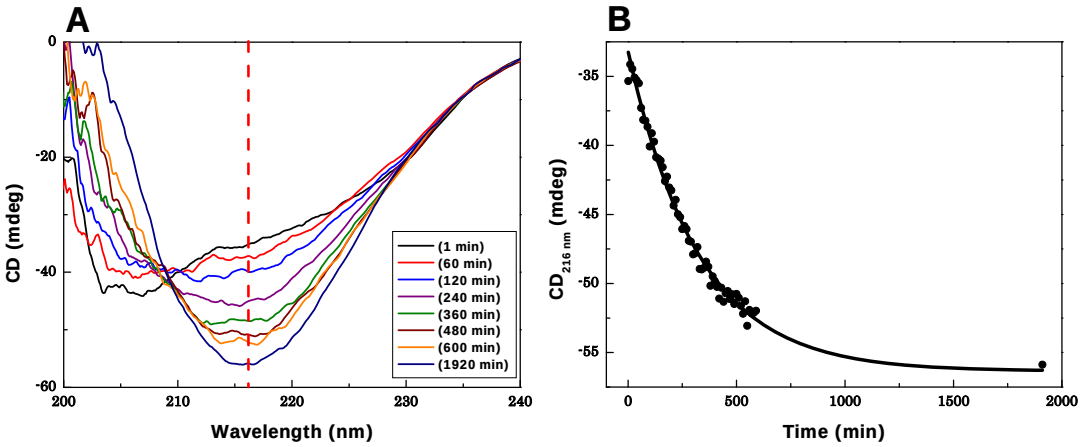

Figure 3. Seed-induced transition of intact e of 10% (w/w) seeds. B. Plot of ellipticity at 218 nm against time of incubation. The solid curve is an exponentially decaying function of time best-fitted to the experimental points.

besides some non-fibril-forming peptides [9].

Preparation of seeds from the first-generation fibrils

The solution, containing the first-generation fibrils as main component, was extensively sonicated according to the procedure described in Materials and Methods. The AFM image showed that the fibrils were degraded into much smaller round-shaped particles (Fig. 2B), which were used as seeds for the formation of the second-generation fibrils.

Preparation of the second-generation fibrils using seeds

The extensively sonicated solution (termed here as "seeds"), which contains the first-generation fibrils as main component, was added to the solution of intact hen lysozyme (4.0 mg ml^{-1}, 80 mM NaCl, pH 2.2) at 57 ºC to the ratio of 1/9 (10% seed against 90% hen lysozyme, weight by weight). Fig. 3A shows CD spectral changes during the formation of the second-generation fibrils at 57 ºC. The spectral changes depict a transition of the α-rich hen lysozyme (constituting 90% of the protein materials in the solution) into a β-rich structure without a lag time, almost like a two-state transition with an effective isodichroic point at 209 nm. The plot of the mean residue ellipticity at 216 nm with time (Fig. 3B), considered to represent the growth of the β-sheet, shows that the growth of the β-sheet is largely (~ 90%) complete within 10 h. Examination by AFM indicates that by this reaction protofibrils are formed (Fig. 2C). Thus the seeded reaction, forming the β-sheet structure (Fig. 3),

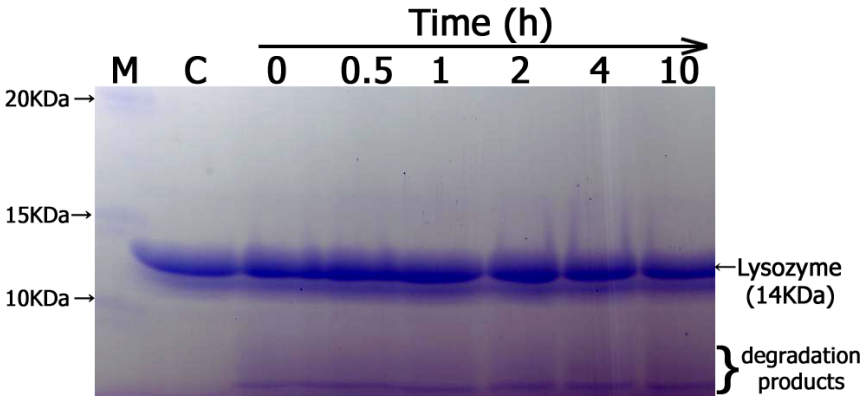

Figure 4. The SDS PAGE analysis of the second-generation amyloid fibrils produced by incubation of hen lysozyme solution (4.0 mg ml^{-1}) in 80 mM NaCl, pH 2.2 in the presence of 10% (w/w) seeds for different incubation times. See Materials and Methods for experimental details. Lane M: molecular weight markers, Lane C: intact hen lysozyme, Lanes 0 to 10: the second-generation amyloid fibrils with increasing time of incubation (0-10 h).

represents the monomer to protofibril transition, i.e., the formation of the second-generation amyloid fibril. It is clear that the growth of the second-generation fibril is much faster than that of the first-generation fibrils requires ~ 11 days under the same solution condition and temperature.

To examine whether or not the degradation of intact hen lysozyme occurs similarly in the first-generation fibrils, the SDS-PAGE analysis (Fig. 4) was performed on the second-generation fibrils. The result confirms clearly the presence of a main band at 14 kDa attributable to intact, non-degraded hen lysozyme and diffuse bands below 14 kDa, attributable to the fragmented peptides in seeds from the first-generation fibrils. The origin of the relatively sharp band below the main 14 kDa band, found also in the control lane, is not known. The fact that the SDS-PAGE pattern is nearly invariant over 10 h of incubation, along with the AFM and CD results in Figs. 2 and 3, gives clear evidence that the second-generation fibril is formed before the acid degradation of the protein takes place, namely a successful conversion of intact hen lysozyme into amyloid fibrils.

The extremely efficient conversion of intact hen lysozyme rich in α-helix (the major component of the reactants) in the monomeric state into the amyloid fibril state rich in β-structure is quite interesting in view of the fact that a similar conversion from α-monomer into β-oligomer is considered as a key event governing the infection of prion disease. Such a clear two-state like conversion of α-rich structure into β-rich structure as observed here (Fig. 3A) may shed new light on the infection mechanism of prion diseases and, furthermore, suggests the possibility of infection in amyloidosis.

In an effort to understand the mechanism of the conversion, we examined the fibril forming reaction as a function of temperature. Figure 5A-D shows the time-dependent CD spectral changes at different temperatures of hen lysozyme solutions (1.0 mg ml^{-1} in 80 mM NaCl, pH 2.2) with a seed-to-protein ratio of 1/9 by weight. Figure 5E gives the plot of ellipticity at 215.5 nm as a function of time at respective temperatures. Figure 6 shows the AFM images of fibrils from the above reaction after 24 h. We notice that the development of the β-oligomer (Fig. 5) and the formation of fibrils (Fig. 6) barely proceed at 40 ºC, i.e., at a temperature below the onset of thermal transition of hen lysozyme (cf. Fig. 1B). However, the rate of the reaction increases dramatically at higher temperatures (Fig. 5E) at which thermal transition begins and denaturation becomes prominent. The results indicate that the conversion of the α-rich structure to the β-rich oligomer takes place only when the thermally denatured fraction of hen lysozyme is significantly present in the system. This suggests that the β-oligomer formation is initiated by the interaction of the seed with denatured hen lysozyme. Namely,

$$\text{Native lysozyme} \rightleftarrows \text{Denatured lysozyme} + \text{seed} \Rightarrow \beta\text{-oligomer (Scheme 1).}$$

Once the first β-oligomer is formed, it will grow into a protofibril (as observed in Fig. 2C) by attaching another denatured intact hen lysozyme to its end repeatedly according to the linear polymerization mechanism [6, 11].

$$\text{Native lysozyme} \rightleftarrows \text{Denatured lysozyme} + \beta\text{-oligomer} \Rightarrow \cdots \Rightarrow \text{protofibril (Scheme 2).}$$

Scheme 1 and 2 correspond to the initiation and elongation reactions, respectively. As “seed” here actually consists of a mixture of the fragmented fibrils and non-fibril forming peptides, only the latter would participate in the proposed reactions above. Further studies on details of the above schemes along with the structural information on “seeds” themselves may shed new light on the general mechanism of amyloidosis and prion disease.

Discussion

We have confirmed that amyloid fibrils are formed from hen lysozyme after ~ 11 days of incubation at pH 2.2 at 57 ºC, which consist of thin filaments (protofibrils) and bundles of thin filaments (matured amyloid fibrils) and further that these fibrils consist of peptide fragments specifically cleaved at Asp-Gly bonds. Although the first-generation amyloid fibrils (without further treatments) were reported to accelerate the amyloid fibril formation of the second generation [10], the

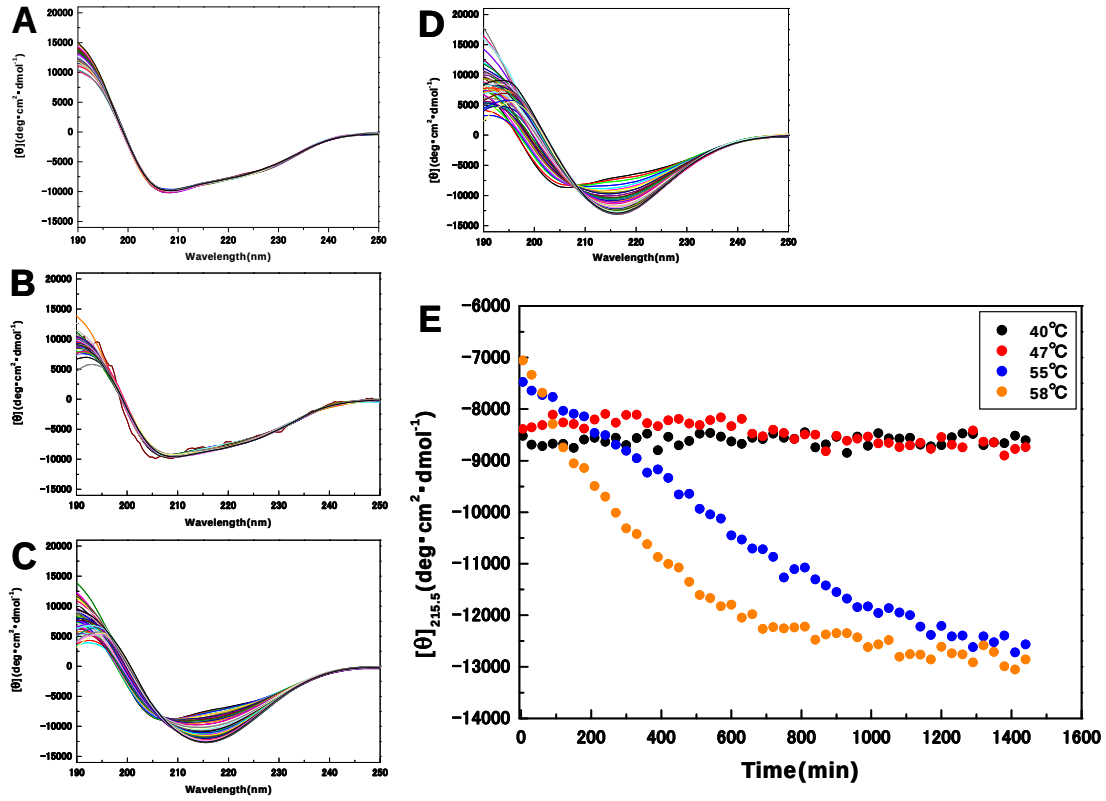

Figure 5. A-D: Seed-induced changes of the CD spectrum of hen lysozyme at various temperatures (A: 40 ºC, B: 47 ºC, C: 55 ºC, D: 58 ºC). Hen lysozyme solution (1.0 mg ml^{-1}) was incubated in 80 mM NaCl, pH 2.2 in the presence of 10% (w/w) seeds. E: Plot of ellipticity at 215.5 nm against time of incubation.

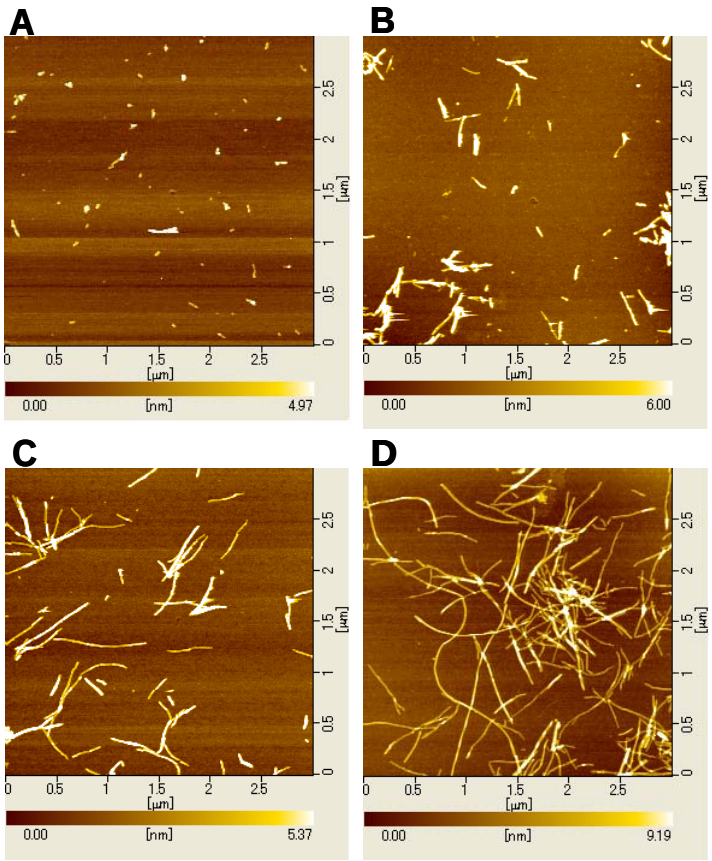

Figure 6. AFM images of fibrils produced in hen lysozyme solution (1.0 mg ml^{-1}) by incubation in 80 mM NaCl, pH 2.2 in the presence of 10% (w/w) seeds at various temperatures (A: 40 ºC, B: 47 ºC, C: 55 ºC, D: 58 ºC).

phenomenon has not been examined in detail. We found that when the first-generation fibrils are made into small particles by extensive sonication and an aliquot is added to the solution of intact hen lysozyme at 57 ºC or other temperatures above 40 ºC, β-oligomers start to form immediately without a lag time as monitored by CD and grow into protofibrils as monitored by AFM. The CD spectral changes without a lag time suggest that intact hen lysozyme can be transformed into β-rich protofibrils by interacting with the seed before it is fragmented into peptides by acid degradation. This expectation is confirmed by the SDS-PAGE analysis of Fig. 4. The efficient conversion, almost like a two-state transition from the intact protein into amyloid fibrils is a process in which the intact protein is "infected" in shape by the sonicated amyloid fibril.

We have shown that the extent and the rate of the conversion, as observed by CD, is critically dependent on temperature. The reaction occurs only in the temperature range for thermal transition, in which a significant fraction of denatured protein is present in solution (Fig. 1B). This observation indicates that the presence of denatured fraction of the protein is essential for the reaction, meaning that the transformation of intact hen lysozyme into the β-oligomer will take place only by the interaction of the seed with the denatured hen lysozyme (Scheme 1). Once the initial β-oligomer is formed, its growing end will react further with another denatured protein and the reaction will be repeated to form longer oligomers or protofibrils according to the linear polymerization mechanism [6, 11] (Scheme 2). After a long run, the protofibrils may eventually grow into matured fibrils with some bundle structures.

The present result, showing a clear conversion of the wild-type protein conformation from one to another by contacting with a preformed seed, may have general implication for amyloid disease. If such seeds are produced continuously in living cells by the degradation of pre-formed amyloid fibrils by some stress or environmental effects, it may result in an efficient conversion of intact proteins into the second-generation amyloid fibrils. The cycle, represented by Scheme 2, may continue auto-catalytically to finally convert a large quantity of intact proteins into amyloid fibrils, leading to amyloidosis. The growth mechanism of amyloid fibrils studied *in vitro* will continue to give clues to the mechanisms of growth of amyloid fibrils *in vivo*.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research No. 16370054 from Ministry of Education, Culture, Sports, Science and Technology and by the Bovine Spongiform Encephalopathy Control Project from Ministry of Agriculture, Forest and Fishery. Buddha R. Shah is a recipient of the JSPS RONPAKU fellowship.

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Communicated by Masayuki Oda