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# Amyloid protofilament formation of hen egg lysozyme in highly concentrated ethanol solution

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**Abstract**

Mutant human lysozymes (Ile56Thr & Asp67His) have been reported to form amyloid deposits in the viscera. From the standpoint of understanding the mechanism of amyloid formation, we searched for conditions of amyloid formation in vitro using hen egg lysozyme, which has been extensively studied from a physicochemical standpoint. It was found that the circular dichroism spectra in the far-ultraviolet region of the hen egg lysozyme changed to those characteristic of a $\beta$-structure from the native $\alpha$-helix rich spectrum in 90% ethanol solution. When the concentration of protein was increased to 10 mg/mL, the protein solution formed a gel in the presence of 90% ethanol, and precipitated on further addition of 10 mM NaCl. The precipitates were examined by electron microscopy, their ability to bind Congo red, and X-ray diffraction to determine whether amyloid fibrils were formed in the precipitates. Electron micrographs displayed unbranched protofilament with a diameter of ~70 Å. The peak point of the difference spectrum for the Congo red binding assay was 541 nm, which is characteristic of amyloid fibrils. The X-ray diffraction pattern showed a sharp and intense diffraction ring at 4.7 Å, a reflection that arises from the interstrand spacing in $\beta$-sheets. These results indicate that the precipitates of hen egg lysozyme are amyloid protofilament, and that the amyloid protofilament formation of hen egg lysozyme closely follows upon the destruction of the helical and tertiary structures.

**Keywords:** aggregation; amyloid; $\beta$-sheet; ethanol; lysozyme; misfolding

Recently, the diseases caused by protein misfolding, such as Alzheimer's disease, late onset diabetes, prion-related transmissible spongiform encephalopathies, and amyloidosis, have attracted considerable attention as conformational diseases (Carrell & Lomas, 1997). The extracellular insoluble deposits (amyloid fibrils), resulting from protein misfolding, damage tissues leading to disease (Tan & Pepys, 1994; Kelly, 1996; Pepys, 1996). The amyloid fibrils, independent of the amino acid sequence, native protein structure, and function of the constituent protein (Sunde et al., 1997), are of indeterminate length, are unbranched with diameters of about 100 Å, and display pathognomonic green birefringence when viewed in polarized light after staining with Congo red (CR) (Pepys, 1996). X-ray diffraction patterns of amyloid fibrils show simple patterns with 4.7 Å meridional reflection and 10 Å equatorial reflection (Sunde et al., 1997). Sunde et al. (1997) have proposed that amyloid fibrils consist of a common core structure that is a cross-$\beta$-fiber structure, with $\beta$-strands perpendicular and $\beta$-sheets parallel to the fiber axis.

The mutant human lysozymes, Ile56Thr and Asp67His, have been identified as fibril proteins in amyloid deposits that cause hereditary systemic amyloidosis (Pepys et al., 1993). The native form of the Ile56Thr molecule is similar to the wild-type structure (Funahashi et al., 1996; Booth et al., 1997). However, the equilibrium and kinetic stabilities of the mutant protein are remarkably decreased due to the introduction of a polar residue (Thr) in the interior of the molecule. It has been reported that the amyloid formation of the mutant human lysozyme is due to a tendency to favor (partly or/and completely) denatured structures (Funahashi et al., 1996). However, the mechanism of the amyloid formation is still not clear.

Fortunately, lysozymes have been studied extensively and their physiochemical properties have been examined in detail (Artymiuk & Blake, 1981; Redfield & Dobson, 1990; Radford et al., 1992; Hooke et al., 1994). Therefore, lysozymes are suitable as a model protein to elucidate the mechanism of amyloid fibril formation. Hen egg lysozyme is known to increase in helical content in ethanol (alcohol) solution (Hamaguchi & Kurono, 1963;

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*Abbreviations:* CD, circular dichroism; CR, Congo red.

Kurono & Hamaguchi, 1964; Ikeda & Hamaguchi, 1970; Kamatari et al., 1998). During re-examinations of ethanol effects on the conformational stability of hen egg lysozyme, we found that in higher concentrations of ethanol (>80%), hen egg lysozyme molecules associate and form a $\beta$-structure. Furthermore, white precipitates appeared when 10 mM NaCl was added to the lysozyme solution in which the lysozyme is in a $\beta$-rich conformation in 90% ethanol. Congo red staining, electron microscopy, and X-ray diffraction all indicate that the precipitates were amyloid protofilament.

Recently, it has been reported that non-disease–related protein can also form amyloid fibrils in extreme environments, for example, in alcohol or acidic pH (Guijarro et al., 1998; Litvinovich et al., 1998; Chiti et al., 1999). It is important to elucidate whether amyloid formation is a property common to many proteins and how amyloid fibrils form to understand the mechanism of the misfolding of proteins. In this paper, the mechanism of amyloid protofilament formation from hen egg lysozyme in highly concentrated ethanol solution will be discussed.

## Results

### *Characterization of hen egg lysozyme in a highly concentrated ethanol solution*

CD spectra of hen lysozyme were measured in various concentrations of ethanol. Protein solutions (5 mg/mL) were incubated at 25 °C for 24 h for CD measurements. Figure 1 shows the far-ultraviolet (UV) CD spectra of hen egg lysozyme in various concentrations of ethanol. The values of negative peaks at $[\theta]$ 208 and 222 nm, which reflect the characteristic of the $\alpha$-helix structure were increased with an increase in ethanol concentration up to 80%. Under these conditions, all solutions were clear and no precipitation was observed. These ethanol effects on the lysozyme structure are well known (Hamaguchi & Kurono, 1963; Kurono & Hamaguchi, 1964; Ikeda & Hamaguchi, 1970). Whereas CD values at 208 and 222 nm of samples containing 85% ethanol were decreased, the spectra showed a single negative peak around 215 nm (curve 5 in Fig. 1). To analyze the features of the structural changes, secondary structure compositions of the protein were calculated from the CD spectra by the method of Provencher (1982). As shown in Table 1, the $\alpha$-helical content increased with increasing ethanol concentration up to 80%, but at 90% it decreased and the content of $\beta$-structure increased. These results suggest that the conformation of lysozyme transforms from an $\alpha$-helical structure to a $\beta$-sheet structure in highly concentrated ethanol solution.

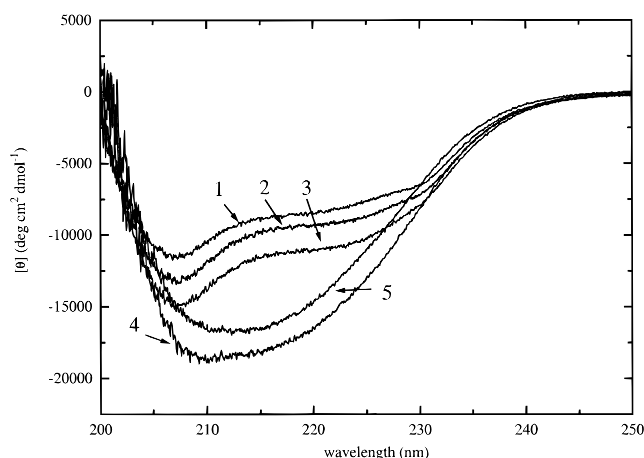


**Fig. 1.** Far-UV CD spectra of hen egg lysozyme (5 mg/mL) in various concentrations of ethanol incubated for 24 h at 25 °C. 1, 0%; 2, 50%; 3, 70%; 4, 80%; 5, 85% ethanol solution.

To examine the changes in the tertiary structure, CD spectra in the near-UV region were measured under the same conditions as those in the far-UV region (Fig. 2). The positive peak around 288 nm slightly increased at 50% ethanol. At ethanol concentration higher than 80%, a broad negative peak around 280 nm appeared. The spectra were similar to that of the denatured state in 5 M urea (Kamatari et al., 1998). These results indicate that the tertiary structure collapses in high concentration ethanol solution and that the transition point is between 70 and 80%. One-dimensional NMR spectra of hen egg lysozyme at the same concentration as the sample for CD were also measured in the 0, 40, and 60% ethanol-$d_5$ solutions (in $H_2O$). The spectrum essentially did not change up to 40% ethanol-$d_5$ except for the broadening of the signals. At 60% ethanol, however, a significant broadening was observed in the amide proton region (data not shown). This fact suggests that the exchange of the amide protons is significantly enhanced at 60% ethanol. NMR results were consistent with those of the CD spectra in the near-UV region.

To examine the protein concentration dependence of the $\alpha$- to $\beta$-structure transition, CD spectra in the far-UV region were measured at various protein concentrations (Fig. 3). At the lower protein concentration, the structure transition was not observed after incubation for 24 h at 25 °C in 85% ethanol solution. The spectrum still showed a helix-rich character at a protein concentration of 1 mg/mL, but at 3 mg/mL, it changed to a $\beta$-rich structure. The extent of association was examined by analytical ultracentrifugation. At less than 80% concentration of ethanol, the apparent molecular weight was that of a monomer. In 85% ethanol, precipitates appeared when the solution contained 10 mM NaCl. Without salt in the 85% ethanol solution, the apparent molecular weight was calculated to be 260,000. These results indicate that lysozyme molecules associate in 85% ethanol solution and that the $\alpha$- to $\beta$-structure transition proceeds with molecular association, suggesting the formation of an intermolecular $\beta$-structure. These phenomena are similar to those of amyloid fibrils, intermolecular $\beta$-structures, and protein concentration dependence (Jarrett & Lansbury, 1993; Harper & Lansbury, 1997; Lashuel et al., 1998).

To examine the effect of salt concentration, solutions containing 90% ethanol and 10 mg/mL lysozyme were prepared at 25 °C. The solution without salt was clear just after it was prepared, but after one week the solution changed to a gel form. When the solution contained 0.1 mM NaCl, a gel formed within 1 h. In 0.5 mM NaCl solution, white precipitates appeared at once, and after one week the solution changed to a gel containing white precipitates. In 1 mM NaCl solution, white precipitates appeared and no gel formed. These results indicate that salts promote the association of proteins. Whether the precipitates are dissolved and recover to the native structure when ethanol concentration is diluted was examined. The protein solution of 7 mg/mL lysozyme containing 90% ethanol and 10 mM NaCl was incubated at 25 °C for three days. The precipitates collected by centrifugation at 13,000 *g* could be easily dissolved in water. As shown in Figure 4, the CD spectrum of the dissolved sample was almost identical to that of the native state. The enzymatic activity also recovered to 50% of that of the

**Table 1.** *CD values at 208 nm, 222 nm, and secondary structure compositions*[a]

| Ethanol concentration (%) | [$\theta$] 208 nm | [$\theta$] 222 nm | $\alpha$-helix (%) | $\beta$-structure (%) | [$\theta$] 288 nm |
|---|---|---|---|---|---|
| 0 | −11,397 | −8,208 | 31 | 6 | 98.42 |
| 70 | −14,782 | −10,860 | 52 | 6 | 68.08 |
| 80 | −18,090 | −15,361 | 54 | 15 | −123.9 |
| 85 | −15,592 | −13,409 | 48 | 16 | −118.6 |
| 90 | −15,087 | −12,874 | 44 | 26 | −116.8 |

[a]Conditions were the same as those of Figure 1 in various ethanol concentrations.

native enzyme. These results indicate that the $\alpha$- to $\beta$-structure transition is reversible.

*Hen egg lysozyme fibril characterization*

Congo red staining, electron microscopy, and X-ray diffraction were used to determine whether the precipitates are amyloid fibrils. A sample solution containing 10 mg/mL of lysozyme, 90% ethanol, and 10 mM NaCl was incubated at 25 °C for a week, and the precipitates that appeared were used for the following experiments.

Figure 5A shows the absorption spectra of the mixture of CR and lysozyme precipitates, CR alone, and lysozyme precipitates alone. The difference spectrum was obtained by subtraction of the scattering due to precipitates (Fig. 5B). The maximum point of the difference spectrum was 541 nm, which is one of the characteristic features of amyloid fibrils. The stained precipitates were examined by optical microscope under cross-polarized light. The precipitates displayed pathognomonic green birefringence and filamenteous aggregations (data not shown).

All known amyloid fibrils, regardless of the nature of the main protein component or the source of the fibrils, are about 100 Å wide, and have no branch points (Sunde & Blake, 1997). In the electron micrographs, the lysozyme precipitates displayed a typical amyloid protofilament form, having a diameter of ~70 Å with no branching (Fig. 6). The lengths of the hen egg lysozyme fibrils were shorter by about 1,000 to 2,000 Å than that of transthyretin fibrils already reported (Sunde & Blake, 1997).

The X-ray diffraction pattern of the lysozyme precipitates showed one sharp reflection, which appeared as a ring because of the lack of relative fibril orientation within the sample. Figure 7 shows the radial intensity profile of the circular average after subtraction of the solvent scattering (Vonderviszt et al., 1992). The reflection at 4.7 Å, which arises from the interstrand spacing in the $\beta$-sheets, was observed as shown in Figure 7B. A broad intensity maxima should appear around 10 Å in the case of multilayer $\beta$-sheet fibrils. In the present case, the peak around 10 Å might be hidden behind a strong background in the central region of the diffraction pattern. As described, three hallmarks of amyloid fibrils indicate that the lysozyme precipitates in highly concentrated ethanol have the characteristics of amyloid protofilament.

## Discussion

*Structural changes in lysozyme in ethanol solution*

The effects of alcohol on protein structures have been extensively studied (Thomas & Dill, 1993; Shiraki et al., 1995). Increase in the helical content of proteins including lysozymes have been ob-

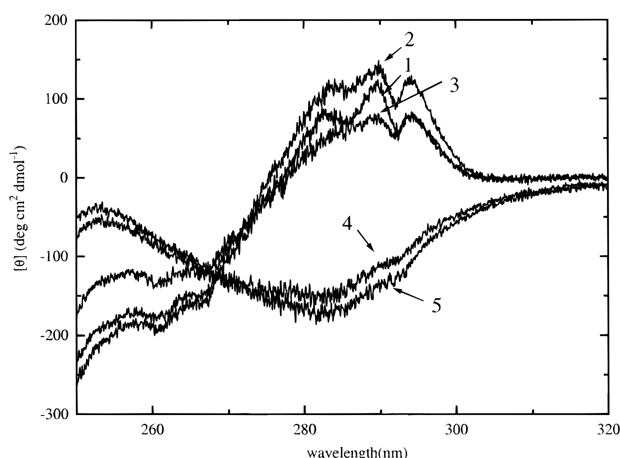


**Fig. 2.** Near-UV CD spectra of hen egg lysozyme (5 mg/mL) in various concentrations of ethanol incubated for 24 h at 25 °C. 1, 0%; 2, 50%; 3, 70%; 4, 80%; 5, 85% ethanol solution.

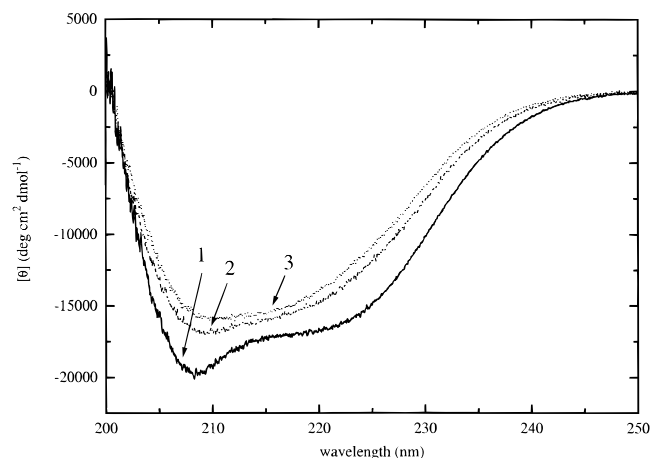


**Fig. 3.** Protein concentration dependence of CD spectra in the far-UV region. All solutions were incubated at 25 °C for 24 h in 85% ethanol solution. 1, 1 mg/mL; 2, 2 mg/mL; 3, 3 mg/mL hen egg lysozyme.

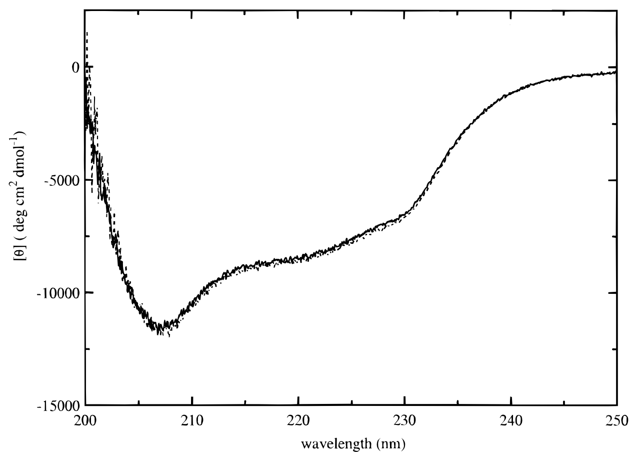


**Fig. 4.** Far-UV CD spectra of native and refolded forms from precipitant hen egg lysozyme. Protein concentration was 7 mg/mL. Solid line, native fold; dashed line, refolded from precipitant in 90% ethanol containing 10 mM NaCl solution.

served in high concentrations of ethanol (Hamaguchi & Kurono, 1963; Kurono & Hamaguchi, 1964; Ikeda & Hamaguchi, 1970). Alcohol also contributes to the stabilization of folding intermediates (Thomas & Dill, 1993). Recent NMR experiments have reported that in high concentration of alcohol, the helical content of hen egg lysozyme increases, its tertiary structure collapses and it is not as compact as the molten-globule state (Kamatari et al., 1998). In many cases, alcohol-induced denaturation results in stabilization

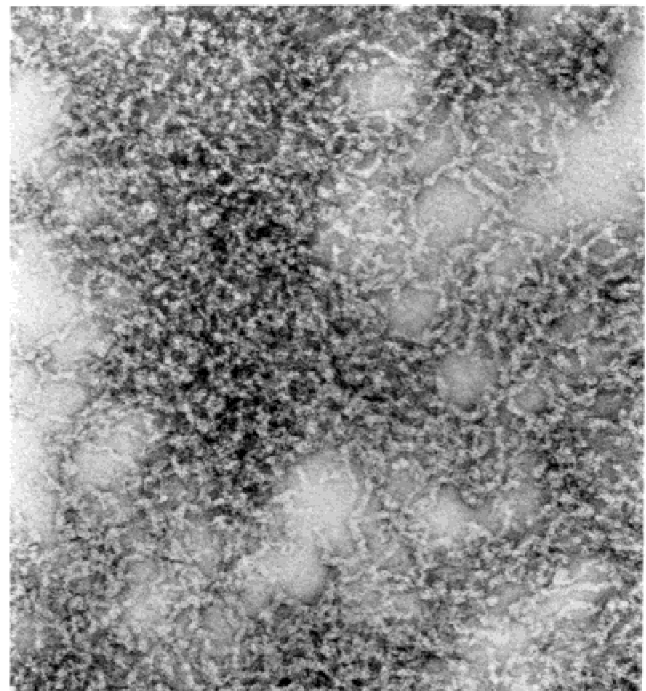

**Fig. 6.** Electron micrograph of a negatively stained preparation of 10 mg/mL hen egg lysozyme in 90% ethanol solution incubated at 25 °C for a week (bar = 100 nm).

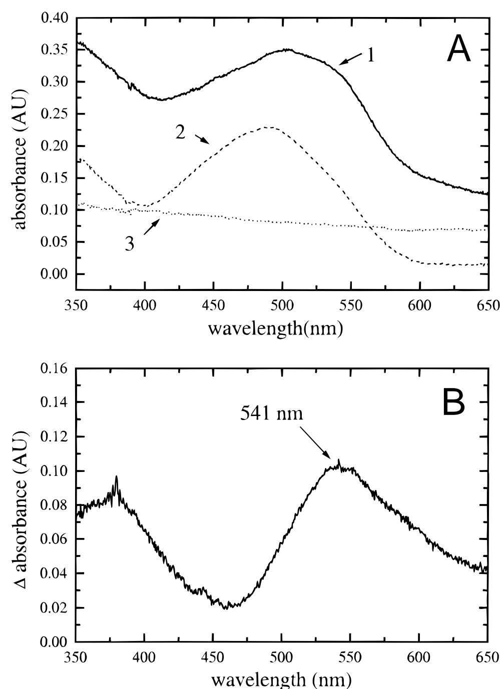


**Fig. 5. A:** Absorbance spectra of CR solution in the presence (1) and absence (2) of hen egg lysozyme fibers and lysozyme fibers alone (3). **B:** Difference spectra obtained by subtracting the spectra of CR alone and lysozyme alone from the spectrum of lysozyme + CR. The maximal point of difference spectrum is around 541 nm.

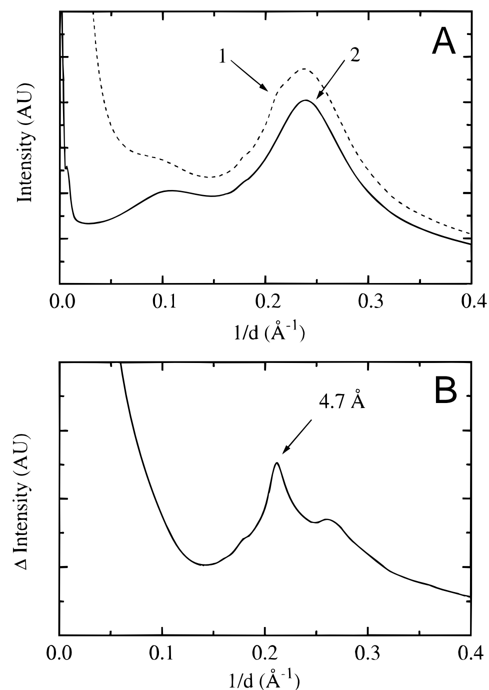


**Fig. 7. A:** Radial intensity distribution profile of X-ray diffraction pattern from hen egg lysozyme fibrils. The X-ray diffraction patterns were mostly circular symmetric with a little tendency of orientation. Therefore, circular averaging was done to make the plot of the radial intensity distribution. The peak is sharp on the broad background scattering from 90% ethanol solution. **B:** Difference intensity was obtained by subtracting 90% ethanol solution from hen egg lysozyme fibrils. The peak (4.7 Å) indicated by an arrow is of the interstrand spacing and is typical of amyloid fibrils.

of extended helical rods in which the hydrophobic side chains are exposed, whereas polar amide groups are shielded from the solvent (Thomas & Dill, 1993; Shiraki et al., 1995).

The present results also indicated that the helical content of lysozyme gradually increases up to an ethanol concentration of 80%. However, over 80%, we found that the helical content decreases and the $\beta$-structure increases. The tertiary structure was destroyed as reported (Parodi et al., 1973; Velicelebi & Sturtevant, 1979; Kamatari et al., 1998). In this high ethanol environment, hen egg lysozyme with a $\beta$-rich structure associated into amyloid protofilament. Recently, it has been reported that acylphosphatase, which does not cause amyloidosis, can form amyloid fibrils in a solution containing moderate concentrations of trifluoroethanol (Chiti et al., 1999). In the process of amyloid formation of acylphosphatase, the CD spectra in the far-UV region show a slow two-state transition between two conformations containing significant amounts of $\alpha$-helical and $\beta$-sheet structures. These observations suggest that the transformation from $\alpha$- to $\beta$-conformation is a common property for kinds of proteins under appropriate concentrations of alcohol (Chiti et al., 1999).

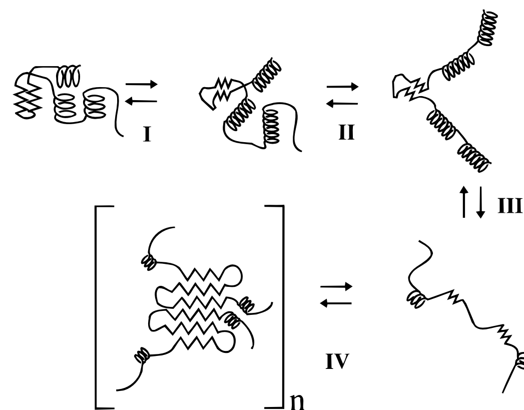


**Fig. 8.** Proposed mechanism for hen egg lysozyme amyloid fibril formation in ethanol solution. Helices and sheets are shown by spirals and zigzag lines, respectively. First, the helical content increases with perturbation of the tertiary structure (steps I and II). In highly concentrated ethanol solution, the helical structures are partly destroyed (step III). Finally, the lysozyme assembles to form amyloid fibrils (step IV).

### *Amyloid formation of hen egg lysozyme*

The examinations by CR staining, electron microscopy, and X-ray diffraction support that the precipitates of hen egg lysozyme are in an amyloid protofilament form. In electron micrographs, the diameter of the fibrils was 70 Å, and the length was from 1,000 to 2,000 Å. It has been reported that transthyretin, which causes amyloidosis, consists of four protofilaments (Serpell et al., 1995), electron micrographs of transthyretin amyloid protofilaments show a diameter of 40–50 Å, and the length is also around 1,000 Å (Lashuel et al., 1998). These features are similar to those of lysozyme protofilament in the present study. This means that hen egg lysozyme form protofilament (precursor of amyloid fibrils) in highly concentrated ethanol solution.

Generally, amyloid fibrils in vitro are stable and hardly susceptible to protease (Badman et al., 1998). In the case of amyloid fibrils of mutant human lysozyme (Asp67His), however, the fibrils can be dissolved in strong denaturant (6 M GuHCl) and the protein refolds to the native conformation on dilution, with its enzymatic activity also recovered (Booth et al., 1997). On the other hands, insulin fibrils do not cause amyloidosis but possess the three hallmarks of amyloid fibrils (Burke & Rougvie, 1972). The polymerization process of insulin fibrils has been reported to be reversible (Waugh, 1957). The protofilaments of transthyretin amyloid also recover to a monomer (Lashuel et al., 1998), suggesting that the transformation to amyloid protofilaments is reversible. The precipitates of hen egg lysozyme in the present study, which look very similar to the protofilaments as judged by an electron micrograph, could recover the native conformation with its enzymatic activity by removal of ethanol. These results indicate that amyloid fibrils, especially the protofilaments, can recover the original monomer conformation under appropriate conditions.

### *The mechanism of amyloid fibril formation by hen egg lysozyme*

Dobson's group has proposed that amyloid formation is a common property of globular proteins under appropriate conditions (Guijarro et al., 1998; Chiti et al., 1999). For example, the SH3 domain of the p85$\alpha$ subunit (Guijarro et al., 1998) and acylphosphatase (Chiti et al., 1999) form amyloid fibrils in an acid pH region and in the presence of trifluoroethanol, respectively, which are environments far from physiological conditions. Recently, our group also discovered that even the extremely stable methionine aminopeptidase (MAP) from a hyperthermophile, *Pyrococcus furiosus*, forms amyloid-like fibrils in the presence of guanidine hydrochloride (about 3 M) in an acidic region. In the case of MAP, it is clearly demonstrated that the amyloid-like form appears just after the protein is almost completely denatured (Yutani et al., 2000). This means that the $\beta$-structure of amyloid fibrils forms after destruction of the $\alpha$-helical structure. This mechanism was confirmed by the present results. On the other hand, proteins that known to cause amyloidosis were independent of the amino acid sequence, native protein structure, and function of the constituent protein (Sunde et al., 1997). These results indicate that the amyloid fibrils formation is not caused by a specific sequence but proteins generally form amyloid fibril under conditions in which population of partially (or completely) denatured state is increased.

The process of amyloid formation by hen egg lysozyme in ethanol solutions is summarized in Figure 8. In the first step, the helical content of the lysozyme increases with an increase in the concentration of ethanol. In the second step at higher concentrations of ethanol, the helical content further increases but the tertiary structure is destroyed. In the third step in highly concentrated ethanol solution, the helical structures are partly destroyed. Finally, in lysozyme the $\beta$-sheets associate with one another and form the protofilament of amyloid fibrils (step IV). The rate of the formation is highly dependent on protein concentration and the concentration of salts.

In this paper, we could find the conditions for amyloid protofilament formation by hen egg lysozyme, and demonstrate that amyloid protofilament formation of hen egg lysozyme occurs after the destruction of the helical and tertiary structures.

## Materials and methods

### *Protein preparation*

Hen egg lysozyme (6x Crystallization) was purchased from Seikagaku Kogyo (Tokyo, Japan). Further purification was carried out

using cation-exchange column chromatography, SP-sepharose (Pharmacia, Uppsala, Sweden), in 50 mM sodium acetate (pH 4.0) with a gradient from 0.3 to 0.5 M NaCl.

### *Circular dichroism*

Circular dichroism (CD) spectra in the near-UV and far-UV regions were obtained using a Jasco J-720 spectropolarimeter equipped with a water bath to control the temperature at 25 °C. The concentration of hen egg lysozyme was 5 mg/mL for the near- and far-UV experiments. Sixteen scans were averaged to obtain each spectrum. Cells with path lengths of 1 and 0.1 mm were used for near- and far-UV data acquisition, respectively.

### *Ultracentrifugation*

Sedimentation equilibrium data were collected on a temperature-controlled Beckman XL-A analytical ultracentrifuge equipped with an An60Ti rotor and a photoelectric scanner. Sedimentation equilibrium runs were performed on 29 $\mu$L samples at 20,000–25,000 rpm using a double sector cell equipped with a 3 mm charcoal-filled Epon centerpiece and quartz windows. The partial specific volume of hen egg lysozyme (0.703 $cm^3/g$) reported previously was used (Sophianopoulos et al., 1964). For the measurements, the protein solution contained 150 mM KCl in various concentrations of ethanol less than 80%, but not in 85% ethanol. The concentration of the protein solution was 10 mg/mL.

### *Congo red staining*

Samples were tested for amyloid-specific CR binding by the spectroscopic assay as described (Klunk et al., 1999). An aliquot of a 10 mg/mL protein sample (35 $\mu$L) was diluted in the reaction buffer (10 mM phosphate buffer, 2.7 mM KCl, and 137 mM NaCl, pH 7.4 and 10% ethanol), which contained 5 $\mu$M Congo red (1 mL final reaction volume). The CR solution was freshly prepared and filtered three times through a 0.2 $\mu$m filter before use. The reaction samples were thoroughly mixed and incubated at room temperature for at least 30 min before recording the absorbance spectrum.

### *Electron microscopy*

Suspensions of hen egg lysozyme in concentrated ethanol solution were applied to carbon-coated copper grids, blotted, washed, negatively stained with 2% uranyl acetate (wt/vol), air dried, and then examined with a JEOL JEM1010 transmission electron microscope operating at an accelerating voltage of 100 kV.

### *X-ray diffraction*

X-ray diffraction experiments were carried out using a RIGAKU FRD rotating anode X-ray generator, operated at 50 kV and 70 mA. Double mirror optics (RIGAKU-MSC/Yale) were used to produce a well-collimated beam of CuK$\alpha$ radiation ($\lambda$ = 1.5418 Å). Precipitates were put into a capillary with a diameter of 0.5 mm. The specimen-to-film distance and the exposure time were 275 mm and 10 h, respectively. X-ray diffraction patterns were recorded with a RIGAKU imaging plate detector R-AXIS IV.

### *NMR spectroscopy*

$^1$H-NMR spectra at 600 MHz were recorded with a Bruker DRX600 NMR spectrometer at 25 °C. The concentration of hen egg lysozyme was 5.0 mg/mL. The mixture of $H_2O$ and $C_2{}^2H_5OH$ ($^2$H 98%, Cambridge Isotope Laboratories, Inc., Woburn, Massachusetts) was used as a solvent. Ten percent $^2H_2O$ was used for the 0% ethanol solution.

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