J. Biochem. **132**, 655–661 (2002)

Elongation in a β-Structure Promotes Amyloid-Like Fibril Formation of Human Lysozyme[1]

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Received June 11, 2002; accepted August 4, 2002

To understand the mechanism of amyloid fibril formation of a protein, we examined wild-type and three mutant human lysozymes containing both amyloidogenic and non-amyloidogenic proteins: I56T (amyloidogenic); EAEA, which has four additional residues (Glu-Ala-Glu-Ala-) at the N-terminus located on a β-structure; and EAEA-I56T, which is an I56T mutant of EAEA. All formed amyloid-like fibrils through an in the increase contents of α-helix with increasing concentration of ethanol. The order of propensity for amyloid-like fibril formation in highly concentrated ethanol solution is EAEA-I56T > EAEA > I56T > wild-type. This order is almost the reverse of the order of conformational stability of these proteins, wild-type > EAEA > I56T > EAEA-I56T. The important views in this work are as follows. (i) Artificially modified proteins formed amyloid fibrils *in vitro*. This means that amyloid formation is a generic property of polypeptide chains. (ii) The amyloidogenic mutation Ile56 to Thr caused the destabilization and promoted fibril formation in the wild-type and EAEA human lysozymes, indicating that instability facilitates amyloid formation. (iii) The mutant protein EAEA human lysozyme had higher propensity for fibril formation than the amyloidogenic mutant protein, indicating that amyloid formation is controlled not only by stability but also by other factors. In this case, appending polypeptide chains to a β-structure accelerated amyloid formation.

Key words: amyloid formation, amyloidogenicity, conformational stability, mutant human lysozyme.

Human diseases such as Alzheimer s disease, late onset diabetes, prion-related transmissible spongiform encephalophathies and amyloidosis are caused by the deposition of protein aggregates known as amyloid fibrils in tissue (*1–5*). Under some pathological conditions, the proteins that normally fold to native (soluble and functional) structures fold incorrectly and generate stable insoluble fibrils. Amyloid fibrils are usually formed by mutant proteins that have a lower stability than wild-type proteins. Recently, several studies have reported a correlation between instability and propensity for *in vitro* fibril formation in mutants from some proteins (*6–9*). This raises the question of whether protein stability is the only determinant for amyloid fibril formation.

The mutant human lysozymes I56T and D67H have been reported to form amyloid deposits in the viscera, causing hereditary non-neuropathic systemic amyloidosis (*10*). Recently, another amyloidogenic variant, W64R, has been also reported (*11*). Previous studies have shown that both I56T and D67H decrease Gibbs energy changes of unfolding not only between the native and denatured states but also between the native and folding intermediate states, resulting in amyloid formation (*12, 13*).

In this study, to further understand the mechanism of amyloid fibril formation of human lysozyme, the relation between the stability and potential to generate amyloid-like fibrils of the wild-type and three mutant human lysozymes was investigated. The three mutant proteins selected were I56T (amyloidogenic), EAEA, which has four additional residues (Glu-Ala-Glu-Ala-) at the N-terminus located on a β-sheet, and EAEA-I56T, which is an I56T mutant of EAEA. The stability and structure of EAEA have

[1]This work was supported in part by a Grant-in-Aid for REIMEI Research from the Japan Atomic Energy Research Institute (S.G. and K.Y.), by Fellowships from the Japan Society for the Promotion of Science for Young Scientists (K.T.), by a Grant-in-Aid for Scientific Research on Priority Areas (C) Genome Information Science from the Ministry of Education, Science, Sports and Culture of Japan (K.Y.), and by the Structural Biology Sakabe Project (K.Y. and Y.Y.). The coordinate of EAEA-I56T human lysozyme has been deposited in the Protein Data Bank, accession number 1IOC.

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been already well characterized (*14*): (i) EAEA is less stable than the wild-type but more stable than I56T, (ii) the refolding-unfolding reaction of EAEA is similar to that of I56T, (iii) the additional residues do not form a secondary structure but are located near β-sheets in the native structure. We determined the crystal structures and thermal stability of these mutant proteins and examined their fibril formation rate and conformational change in highly concentrated ethanol solution. Hen egg white lysozyme has been shown to form amyloid-like fibrils in the presence of high concentrations of ethanol (*15*). We here report that all three mutant proteins are less stable and form the amyloid-like fibrils faster than the wild-type protein. In pasticular, EAEA human lysozyme has a higher stability and a higher propensity for fibril formation than the amyloidogenic mutant protein I56T. Findings are discussed in terms of the amyloidogenicity of human lysozyme and the determinants for its amyloid fibril formation.

EXPERIMENTAL PROCEDURES

Materials—Mutagenesis, expression (*Pichia pastoris* system) and purification of human lysozymes were performed as described (*14*). All other chemicals were of reagent grade. The concentration of human lysozyme solutions was determined spectrophotometrically using $E^{1\%}$ (1 cm) = 25.65 at 280 nm (*16*).

X-Ray Crystallography—The mutant human lysozyme, EAEA-I56T, was crystallized as described previously (*14*). The crystal belongs to the space group $P6_122$ with a crystal form identical to that of EAEA (*14*). The crystal structures of I56T and EAEA have already been determined (*12, 14*).

The intensity data set of the EAEA-I56T human lysozyme was collected with a Weissenberg camera (*17*) on beam line 18B at the Photon Factory (Tsukuba, Japan; Proposal No. 99G309). The data was processed with the program DENZO (*18*). The structure was solved by the isomorphous method and refined with the program X-PLOR (*19*) as described previously (*20, 21*).

Differential Scanning Calorimetry (DSC)—Calorimetric measurements were carried out with a DASM4 microcalorimeter. The buffer solution used was 0.05 M Gly-HCl. EAEA-I56T was measured five times at different pH points between pH 2.6 and 3.3. The DSC measurements of I56T and EAEA have already been carried out (*12, 14*). DSC data were analyzed using the Origin software (MicroCal, MA), as described previously (*20*). The thermodynamic parameters for denaturation as a function of temperature were calculated using the following equations (*22*),

$$\Delta H(T) = \Delta H(T_d) - \Delta C_p (T_d - T) \quad (1)$$

$$\Delta S(T) = \Delta H(T_d)/T_d - \Delta C_p \ln(T_d/T) \quad (2)$$

$$\Delta G(T) = \Delta H(T) - T\,\Delta S(T) \quad (3)$$

assuming that ΔC_p does not depend on temperature.

Congo Red Staining—For Congo red birefringence experiments, aliquots of 10 mg/ml protein samples in concentrated ethanol solution were air-dried onto glass slides. The resulting films were stained with the reaction solution as described previously (*15*). The stained slides were examined with an optical microscope between crossed polarizers. The Congo red solution was freshly prepared and filtered three times through a 0.2 μm filter before use.

For quantitative Congo red experiments, the samples (0.1 mg/ml) were tested by the spectroscopic assay as described (*23*). Amyloid fibrils in 80 % ethanol solution were collected by centrifugation (30 min at 13,000 ×*g*) and resuspended in the same solution as that used in the birefringence experiments. The reaction samples were thoroughly mixed and incubated at room temperature for at least 30 min before measurement. Amounts of amyloid fibrils could be quantified from the maximum point of the difference spectra of the Congo red solution between the presence and absence of amyloid fibrils (*23*).

Electron Microscopy—Electron micrographs were acquired using a JEOL JEM1010 transmission electron microscope at 100 kV acceleration voltage as described previously (*15*). Suspensions of the wild-type and mutant human lysozymes (10 mg/ml) in concentrated ethanol solution were applied to carbon-coated copper grids, blotted, washed, negatively stained with 2% uranyl acetate (w/v), air-dried, and then examined with a JEOL JEM 1010 transmission electron microscope operating at an accelerating voltage 100 kV.

X-ray Diffraction—X-ray diffraction experiments were carried out using a Rigaku rotating anode X-ray generator, RU-300, operated at 40 kV and 200 mA (λ = 1.5418 Å), equipped with a Rigaku imaging plate detector R-AXIS IIc as described previously (*15*). Precipitates were put into a capillary with a diameter of 0.5 mm. The specimen-to-film distance and the exposure time were 100 mm and 10 h, respectively.

RESULTS AND DISCUSSION

Crystal Structures of Mutant Human Lysozymes—To investigate the structural changes due to mutation, we determined the crystal structures of the mutant human lysozymes by X-ray analysis. The structures of the wild-type, I56T and EAEA have been already analyzed (*12, 14, 20*). Data collection and refinement statistics of EAEA-I56T are summarized in Table I. The structure of EAEA-I56T was essentially identical to that of EAEA at the N-terminal region and that of I56T in the vicinity of position 56. The overall structures of the mutant proteins, I56T, EAEA and EAEA-I56T, are similar to that of the wild-type protein, as shown in Fig. 1, except for the mutation sites. This result

TABLE I. **X-ray data collection and refinement statistics of EAEA-I56T human lysozyme.**

	EAEA-I56T
Data collection	
Crystal system	Hexagonal
Space group	$P6_122$
a, *c* (Å)	90.117, 92 641
Resolution (Å)	2.4
No. of measured reflections	72,930
No. of ind. reflections	9,168
Completeness of data (%)	99.8
R_{merge}[a] (%)	11.8
Refinement	
No. of protein atoms	1,047
No. of solvent atoms	91
Resolution (Å)	6–2.4
No. of used reflections	7,612
Completeness (%)	89.7
R-factor[b]	0.21

[a] $R_{merge} = 100\ \Sigma|I-\langle I\rangle|/\Sigma\langle I\rangle$. [b] *R*-factor = $\Sigma||F_o|-|F_c||/\Sigma|F_o|$.

indicates that these mutations do not seriously affect the native structure of human lysozyme.

Conformational Stability of Mutant Human Lysozymes—To measure the changes in conformational stability of the mutant human lysozymes, we examined their heat denaturation by DSC. The stability of the wild-type, I56T and EAEA has been already analyzed (*12, 14, 20*). The DSC measurements were carried out in the acidic pH region, where the heat denaturation of the human lysozyme is highly reversible. Figure 2 shows typical DSC curves of the human lysozymes. Table II represents the denaturation temperatures (T_d), the calorimetric enthalpies (ΔH_{cal}), and the van't Hoff enthalpies (ΔH_{vH}) of each measurement for EAEA-I56T. Using these data and Eqs. 1 to 3, the thermodynamic parameters for the denaturation of the mutant and wild-type proteins at the same temperature, 64.9°C, which is the denaturation temperature of the wild-type protein at pH 2.7 (*19*), were calculated as shown in Table III. The denaturation Gibbs energy changes (ΔG) of all mutant proteins were smaller than that of the wild-type protein, indicating the equilibrium destabilization due to substitution. The order of the stability of the human lysozymes was as follows: wild-type > EAEA > I56T > EAEA-I56T. The changes in ΔH and ΔG of EAEA-I56T were similar to the sum of each of the changes in I56T and EAEA. This result shows that the effects of the mutations at the N-terminal and position 56 on the stability are independent of each other.

Amyloid Fibril Formation of Human Lysozymes in Ethanol—Goda *et al.* (*15*) have reported that hen egg white lysozyme forms amyloid-like fibrils in highly concentrated ethanol. The amyloid-like fibrils are formed through an increase in the contents of α-helix with increasing concentration of ethanol (*15*). In this paper, we used ethanol to promote the amyloid formation of human lysozymes, be-

TABLE II. **Thermodynamic parameters for denaturation of EAEA-I56T human lysozyme at different pH values.**

	T_d (°C)	ΔH_{cal} (kJ mol^{-1})	ΔH_{vH} (kJ mol^{-1})	$\Delta H_{cal}/\Delta H_{vH}$
2.69	40.5	218	241	0.90
2.80	41.7	226	246	0.92
2.96	47.8	277	300	0.92
3.08	50.1	288	310	0.93
3.22	52.7	301	302	1.00

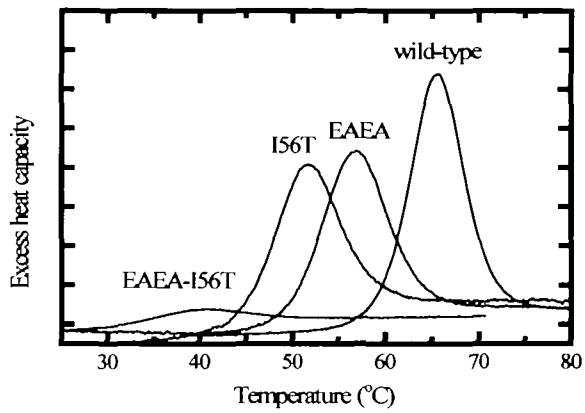


Fig. 2. **Typical excess heat capacity curves of the wild-type and I56T, EAEA, and EAEA-I56T mutants of human lysozyme.** The increments in excess heat capacity are 10 kJ mol^{-1} K^{-1}.

Fig. 1. **Overall structures (backbone) of the wild-type and I56T, EAEA, and EAEA-I56T mutants of human lysozyme.** The four structures are superimposed. Position 56 and the N-terminal are labeled.

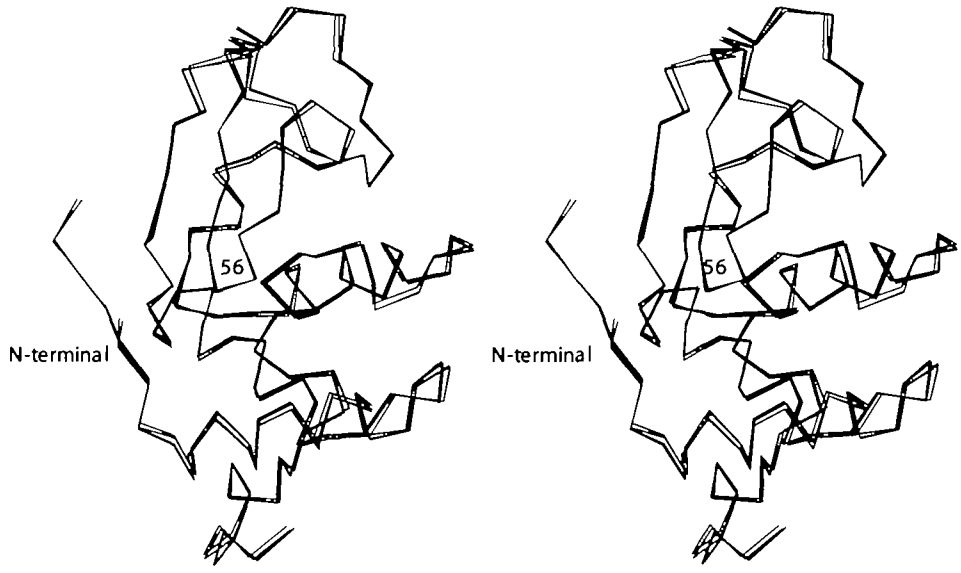


TABLE III. **Thermodynamic parameters for denaturation of the wild-type and mutant human lysozymes at the denaturation temperature (64.9°C) of the wild-type protein at pH 2.7.**

	T_d (°C)	ΔT_d (°C)	ΔC_p[a] (kJ mol^{-1} K^{-1})	ΔH (kJ mol^{-1})	$\Delta\Delta H$ (kJ mol^{-1})	$\Delta\Delta G$ (kJ mol^{-1})
Wild-type[b]	64.9	(0)	6.6	477	(0)	(0)
EAEA[c]	57.3	−7.6	6.7	446	−31	−9.6
I56T[d]	52.4	−12.5	5.2	425	−52	−15.2
EAEA-I56T	40.4	−24.5	7.0	390	−87	−23.7

[a]ΔC_p was obtained from the slope of ΔH_{cal} against T_d. [b]From Takano *et al.* (*20*). [c]From Goda *et al.* (*14*). [d]From Funahashi *et al.* (*12*).

cause it has been shown that there is no difference in amyloidogenicity upon mutation between the absence and presence of alcohol (*8*). In the case of human lysozyme, the wild-type protein (0.1 mg/ml) precipitated in 85% ethanol, and the three mutant proteins (0.1 mg/ml) precipitated in 75–80% ethanol. Congo red staining, electron microscopy and X-ray diffraction were used to determine whether the precipitates in highly concentrated ethanol of human lysozyme are amyloid fibrils. Sample solution containing 10 mg/ml of the human lysozymes was incubated at 25°C for one week in 80% (for wild-type) or 70% (for three mutants) ethanol.

Optical microscope images of all the precipitates of the human lysozymes (wild-type and three mutants) stained by Congo red displayed pathognomonic green birefringence under cross-polarized light. Figure 3 shows an optical microscopic image of the EAEA human lysozyme. The photograph shows the blots of green birefringence coming from regions rich in amyloid fibrils. The electron micrograph of the EAEA human lysozyme showed a typical amyloid form

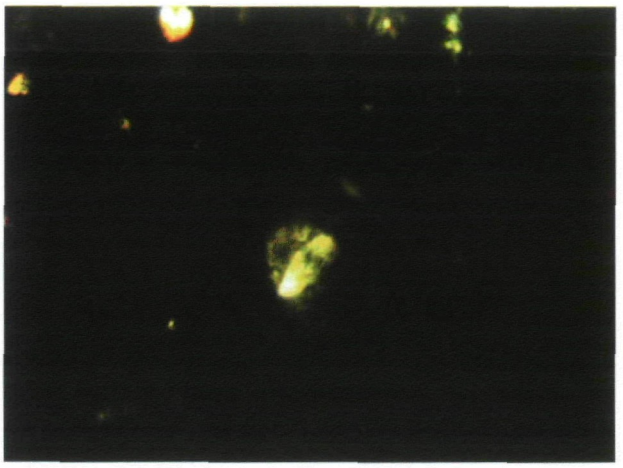


Fig. 3. **Optical microscopy image of EAEA human lysozyme obtained under cross-polarized light.** The precipitate of the EAEA human lysozyme (10 mg/ml) was obtained after 1 week of incubation in 70% ethanol solution and was stained with Congo red. The photograph shows the blots of green birefringence coming from regions rich in amyloid fibrils.

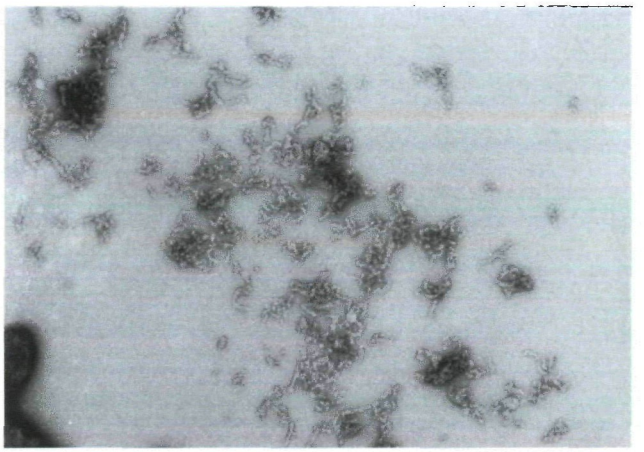
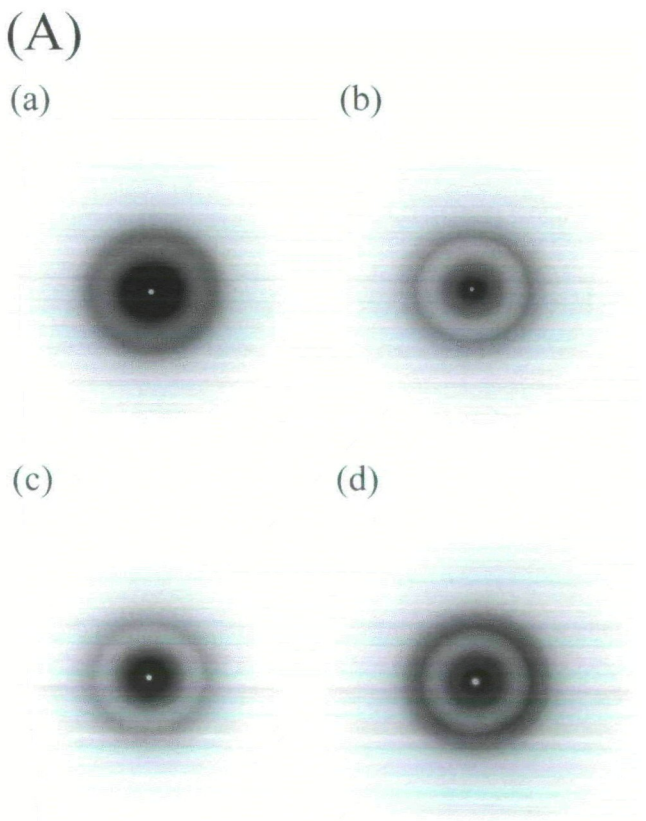


Fig. 4. **Electron micrograph of a negatively stained preparation of EAEA of human lysozyme (10 mg/ml) in 70% ethanol solution incubated at 25°C for 1 week (bar = 100 nm).**



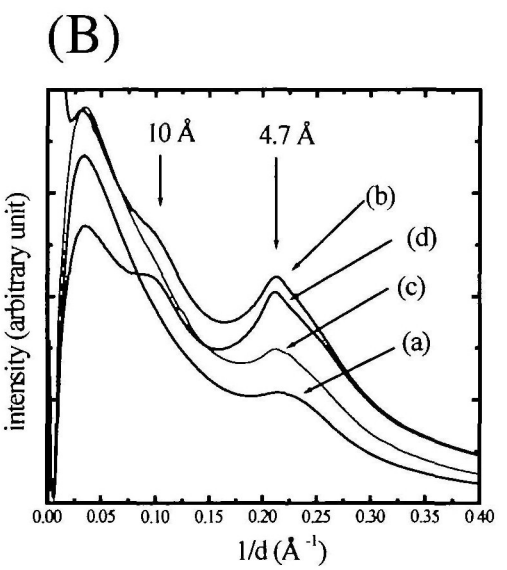


Fig. 5. **X-ray diffraction patterns (A) and radial intensity distribution profiles of the X-ray diffraction patterns (B) of the wild-type and EAEA, I56T, and EAEA-I56T mutants of human lysozyme.** Wild-type (a), EAEA (b), I56T (c), and EAEA-I56T (d). The reflections generated by the interstrand spacing (4.7 Å) and the intersheet spacing (about 10 Å) typical of amyloid fibrils are marked by arrows (B).

with a diameter of approximately 70 Å (Fig. 4). The lengths of the fibrils were about 1,000 to 2,000 Å. These features are similar to those of transthyretin amyloid protofilaments (*24*). The X-ray diffraction pattern of the precipitates of the human lysozymes (wild-type and three mutants) showed one sharp and one broad reflection. Figure 5a shows the diffraction patterns, and Fig. 5b shows the radial intensity profiles for the circular average (*25*) of the human lysozymes. The sharp reflection at 4.7 Å arises from the interstrand spacing in the β-sheets, and the broad reflection around 10 Å arises from the intersheet spacing between one β-sheet and the next β-sheet. These results indicate that the precipitates in highly concentrated ethanol of the wild-type and three mutant human lysozymes have the characteristics of amyloid-like fibrils, similar to those of hen egg white lysozyme (*15*).

As described above, not only the amyloidogenic protein, I56T human lysozyme, but also the wild-type and mutant human lysozymes formed amyloid-like fibrils in highly concentrated ethanol solution. Morozova-Roche *et al.* (*26*) have also shown that the amyloid fibrils of the wild-type human lysozyme are formed upon incubation at low pH at high temperature for several days. Recently, amyloid fibrils formed by a number of proteins unlinked to disease have been reported (*27–31*). For example, methionine aminopeptidase from a hyperthermophile forms amyloid-like fibrils (*31*). Dobson (*1*) has described that amyloid formation is not restricted to the small number of protein sequences associated with diseases. The present results confirm that amyloid formation is a property common to polypeptide chains.

Amyloidogenicity of Human Lysozymes—To determine the amount of fibrils formed at various ethanol concentrations for each human lysozyme (0.1 mg/ml), a decrease in the absorbance of the supernatant was measured at 280 nm after the precipitates were separated by centrifugation (for 30 min at 13,000 ×*g*). Figure 6 shows the relation between the ethanol concentration and the decrease in absorbance of the supernatants of the wild-type and mutant human lysozymes. The decrease in absorbance means that highly associated precipitates due to formation of fibrils appear in the solution. In 80% ethanol, the amount of fibrils formed was different among the proteins.

To elucidate the propensity for amyloid fibril formation of human lysozyme, the kinetic measurements of amyloid fibril formation were carried out in 80% ethanol solution. The amount of fibrils was evaluated by the quantitative Congo red stain method (*23*). Figure 7 shows the time course of amyloid fibril formation of the human lysozymes. The EAEA-I56T and EAEA human lysozymes formed amyloid fibrils within 1 h, and the amyloidogenic mutant human lysozyme, I56T, formed fibrils in up to 8 h. The wild-type protein, however, did not form fibrils after 30 h under these conditions. The order of the fibril formation rate of the human lysozymes was EAEA-I56T > EAEA > I56T > wild-type. This result indicates that the elongation of four residues at the N-terminal of human lysozyme remarkably promotes the formation of amyloid fibrils.

Why do the four extra N-terminal residues of human lysozyme facilitate the amyloid formation? The N-terminal region of the wild-type human lysozyme consists of a β-sheet. Crystal structural analysis revealed that the four extra residues in EAEA or EAEA-I56T do not form secondary structures but are near another larger β-sheet in the β-domain of the molecule. Thus, the four residues might actively participate in the association of β-structures in amyloid fibrils. Krebs *et al.* (*32*) have reported that the peptides derived from the β-sheet region of hen egg white lysozyme, which is homologous to human lysozyme, form fibrils very readily, suggesting that the β-domain is of particular significance in the fibril formation. Hydrogen exchange experiments have also shown that an amyloidogenic D67H mutant human lysozyme reduces the stability of the β-domain in the native structure (*33*).

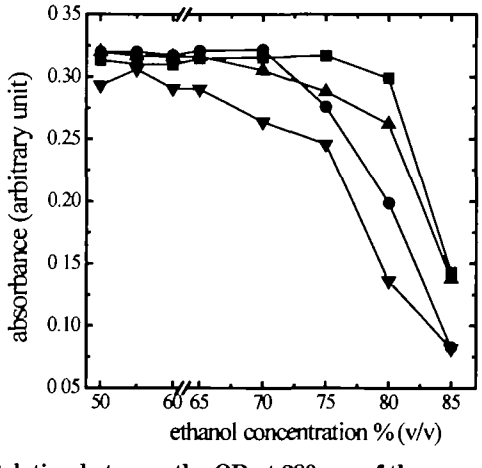


Fig. 6. **Relation between the OD at 280 nm of the supernatants and ethanol concentration of the wild-type and EAEA, I56T, and EAEA-I56T mutants of human lysozyme.** Wild-type (squares), EAEA (circles), I56T (up-triangles), and EAEA-I56T (down-triangles). The concentration of human lysozymes was 0.1 mg/ml. After incubation with various concentrations of ethanol at 25°C for 15 h, centrifugation was carried out for 13,000 ×*g* for 30 min. The changes in OD at 280 nm of the supernatant of the wild-type and mutant human lysozyme solutions in the various concentrations of ethanol were measured.

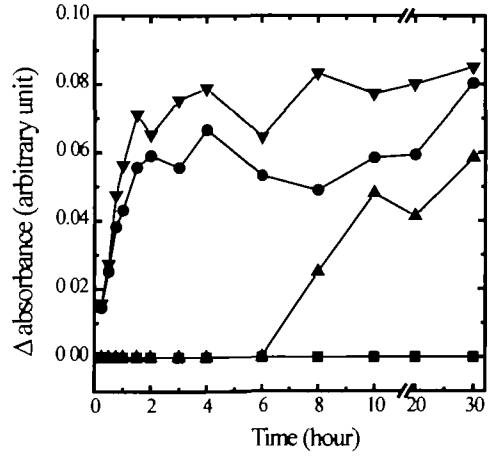


Fig. 7. **Time course of amyloid fibril formation of the wild-type and EAEA, I56T, and EAEA-I56T mutants of human lysozyme (0.1 mg/ml) in 80% ethanol solution.** Wild-type (squares), EAEA (circles), I56T (up-triangles), and EAEA-I56T (down-triangles). After incubation at 25°C, centrifugation was carried out for 13,000 ×*g* for 30 min. The amounts of amyloid fibrils were quantified by a Congo red staining method.

Relation between Stability and Potential to Form Amyloid Fibrils of Human Lysozymes—Recently, several studies have reported a correlation between instability and the propensity for *in vitro* fibril formation in mutants of some proteins (*6–9*). This indicates that fibril formation is predominantly controlled by thermodynamic stability. In the case of human lysozyme presented here, the amyloidogenic mutation Ile56 to Thr, which causes the destabilization in both the wild-type and EAEA human lysozymes, also promotes the fibril formation in both proteins. This indicates that instability is also important in the amyloid formation process in the EAEA human lysozyme. However, there was not a linear correlation between the level of instability and amyloidogenic potential in the wild-type and all mutant proteins: the order of the conformational stability was wild-type > EAEA > I56T > EAEA-I56T, but the order of the propensity for amyloid fibril formation was EAEA-I56T > EAEA > I56T > wild-type. It has also been reported that the order of stability of wild-type and mutant transthyretins does not correlate with their known amyloidogenic potential (*34*), and a highly stable protein, methionine aminopeptidase from a hyperthermophile, can form amyloid-like conformation (*31*). These results suggest that mutant-induced thermodynamic destabilization is insufficient to explain amyloid formation. Amyloid fibril formation must also be controlled by other factors. This is also supported by evidence that not every unstable natural protein forms amyloid fibrils *in vivo*.

What factors other than stability are related to amyloid formation? It is known that formation of amyloid by natural proteins not related to disease is promoted *in vitro* by partially denaturing conditions such as low pH (*26, 35*), high temperature (*36*) or the presence of alcohols (*15, 28*). These findings suggest the importance of the partly denatured structure for amyloid formation. Takano *et al.* (*13*) have also have shown that the intermediate state tends to facilitate amyloid formation by the mutant human lysozymes more than equilibrium destabilization between the native and completely denatured states.

Previous reports have also proposed that the mechanism of amyloid formation consists of two steps: (i) a monomer molecule misfolds and transforms into a precursor, and (ii) the precursors associate and elongate into fibrils (*6, 37–39*). The first step should be strongly related to the overall instability or tendency to favor the partially denatured structure of proteins. In contrast, the second step should be affected by the capacity for the association into fibrils. This would be related to the tendency to form the β-sheet structure, because amyloid fibrils consist of a number of β-sheets. Recently, it has been reported that the changes in amyloidogenicity upon mutation correlate with changes in the β-sheet propensity and hydrophobicity (*40*), and mutations at the edge of β-strands strongly affect amyloid fibril formation (*41, 42*). This means that amyloid fibril formation is controlled not only by stability but also by amino acid sequence. In the case of the EAEA human lysozymes, the four additional residues at the N-terminus β-structure might contribute to the association into fibrils as described above, resulting in a faster fibril formation of EAEA than I56T, although the stability of EAEA is higher than that of I56T. This also suggests that deletion of amino acid residues in β-structures in the native structure of a protein would inhibit amyloid fibril formation.

Conclusion—Here, we show that an artificially modified protein, EAEA human lysozyme, which has four additional residues at the N-terminal located on a β-structure, has a higher propensity for fibril formation than an amyloidogenic mutant human lysozyme. This indicates that amyloid formation is controlled not only by stability but also by other factors: in this case, elongation in a β-structure promotes amyloid fibril formation.

We thank Takeda Chemical Industries (Osaka) for providing the plasmid pGEL 125.

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