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# Investigation of a Peptide Responsible for Amyloid Fibril Formation of $\beta_2$ -Microglobulin by *Achromobacter* Protease I\*

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To obtain insight into the mechanism of amyloid fibril formation from  $\beta_2$ -microglobulin ( $\beta_2$ -m), we prepared a series of peptide fragments using a lysine-specific protease from *Achromobacter lyticus* and examined their ability to form amyloid fibrils at pH 2.5. Among the nine peptides prepared by the digestion, the peptide Ser<sup>20</sup>-Lys<sup>41</sup> (K3) spontaneously formed amyloid fibrils, confirmed by thioflavin T binding and electron microscopy. The fibrils composed of K3 peptide induced fibril formation of intact  $\beta_2$ -m with a lag phase, distinct from the extension reaction without a lag phase observed for intact  $\beta_2$ -m seeds. Fibril formation of K3 peptide with intact  $\beta_2$ -m seeds also exhibited a lag phase. On the other hand, the extension reaction of K3 peptide with the K3 seeds occurred without a lag phase. At neutral pH, the fibrils composed of either intact  $\beta_2$ -m or K3 peptide spontaneously depolymerized. Intriguingly, the depolymerization of K3 fibrils was faster than that of intact  $\beta_2$ -m fibrils. These results indicated that, although K3 peptide can form fibrils by itself more readily than intact  $\beta_2$ -m, the K3 fibrils are less stable than the intact  $\beta_2$ -m fibrils, suggesting a close relation between the free energy barrier of amyloid fibril formation and its stability.

Many proteins and peptides form amyloid fibrils (1–3). Although most are related to diseases, it has been shown that several proteins (4, 5) and peptides (6, 7) that are not related to disease can also form amyloid fibrils. Amyloid fibril formation is now recognized as a phenomenon common to many proteins. On the other hand, amyloid fibrils are homogeneous, and it is rarely possible to form chimeric fibrils composed of distinct amyloid proteins or peptides (8, 9). This high species barrier suggests that the amyloid fibrils are stabilized by specific interactions of amyloid proteins, which are governed by the characteristic primary and higher order structures of each amyloid protein. Thus, amyloid fibrils can be considered to be alternatively folded conformations of globular proteins, and under-

standing of their properties is essential to obtain further insight into the mechanism of protein folding.

$\beta_2$ -Microglobulin ( $\beta_2$ -m)<sup>1</sup>-related amyloidosis is a common and serious complication in patients on long term hemodialysis (10–12). Carpal tunnel syndrome and destructive arthropathy associated with cystic bone lesions are the major clinical manifestations of  $\beta_2$ -m-related amyloidosis (13). Although  $\beta_2$ -m, the light chain of the type I major histocompatibility complex (14, 15), was identified as a major structural component of amyloid fibrils deposited in the synovia of the carpal tunnel (10), the mechanism of amyloid fibril formation by  $\beta_2$ -m is still unknown (16, 17, 25, 26). Naiki and co-workers (18–20) have been studying the amyloid fibril formation of  $\beta_2$ -m as well as other amyloid fibrils (21–24). They established a kinetic experimental system to analyze amyloid fibril formation *in vitro*, in which the extension phase with the seed fibrils is quantitatively characterized by the fluorescence of thioflavin T (ThT) (20, 23). They have proposed that a nucleation-dependent polymerization model could explain the general mechanisms of amyloid fibril formation *in vitro*, applicable to various types of amyloidosis. This model consists of two phases, *i.e.* nucleation and extension phases. Nucleus formation requires a series of association steps of monomers, which are thermodynamically unfavorable, representing the rate-limiting step in amyloid fibril formation *in vitro*. Once the nucleus (*n*-mer) has been formed, further addition of monomers becomes thermodynamically favorable, resulting in rapid extension of amyloid fibrils *in vitro*.

Many amyloidogenic proteins carry key regions that are specific to each amyloid protein (7, 27–30). For an example, a 10-residue peptide of transthyretin can make fibrils that exhibit characteristics typical of the intact (127-amino acid residues) transthyretin (7). In the case of medin, responsible for aortic medial amyloid, the most common human amyloid, the C-terminal 8-residue peptide, can form the amyloid fibrils (27). Identifying such a key region provides a clue to understanding the mechanism of amyloid fibril formation.

In the present study, with the recombinant human  $\beta_2$ -m expressed, we investigated the possible key regions responsible for the amyloid formation of  $\beta_2$ -m. Using a series of peptides obtained with *Achromobacter* protease I, we first showed that a peptide of 37 amino acid residues linked by a disulfide bond, *i.e.* Ser<sup>20</sup>-Cys<sup>25</sup>-Lys<sup>41</sup> (K3) and Asp<sup>76</sup>-Cys<sup>80</sup>-Lys<sup>91</sup> (K7), has the potential to form amyloid fibrils. Among the two peptides produced by reduction of the disulfide bond, the N-terminal K3 peptide of 22 amino acid residues retained the potential to form

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<sup>1</sup> The abbreviations used are:  $\beta_2$ -m,  $\beta_2$ -microglobulin; ThT, thioflavin T.

amyloid fibrils, arguing that this peptide contains the minimal sequence. Kinetics of amyloid fibril formation and depolymerization suggested that, although the K3 peptide can form the amyloid fibrils by itself, the fibrils made of K3 are less stable than those of intact  $\beta_2$ -m. The results can be interpreted in terms of the change in free energy barriers of the nucleation and extension processes.

#### EXPERIMENTAL PROCEDURES

**Recombinant  $\beta_2$ -m**—cDNA encoding  $\beta_2$ -m was amplified by PCR using the primers 5'-aggtcgcgaatcgaatccagctactcc and 5'-cataccgagcggcgccactgtgctg. The amplified DNA fragment was digested with *Eco*1051 and *Not*I and cloned into the *Pichia pastoris* expression vector pPIC11 resulting in pPIC $\beta_2$ M. pPIC $\beta_2$ M was digested with *Aat*I and transformed into the *P. pastoris* GS115. The most efficient transformant was selected based on the expression efficiency in small scale test tube culture. High cell density fermentation of the selected strain was carried out in a 2-liter fermenter (31). The culture was continued for 2 days after the induction of protein expression by the addition of methanol. The supernatant of medium containing secreted  $\beta_2$ -m was first desalted by passing through a Sephadex G-25 (Amersham Biosciences, Inc.) column equilibrated with 10 mM sodium phosphate, pH 7.5. The sample was added to a calcium tartrate column (32) and eluted with a linear gradient of 40–300 mM sodium phosphate buffer. The  $\beta_2$ -m fraction was further purified by passing through a DEAE-Sepharose CL-6B (Amersham Biosciences, Inc.) column equilibrated with 20 mM Tris-HCl, pH 8.5, using a linear gradient of 0–200 mM NaCl. Three peaks of  $\beta_2$ -m species with different N termini were obtained. The three species had 6 (Glu-Ala-Glu-Ala-Tyr-Val-), 4 (Glu-Ala-Tyr-Val-), and 1 (Val-) additional amino acid residues, respectively, added to the N-terminal (Leu) of intact  $\beta_2$ -m. The second peak with 4 additional amino acid residues was the major peak, and this fraction was used in this study.

**Protease Digestion**— $\beta_2$ -m was digested with lysyl endopeptidase from *Achromobacter lyticus* (*Achromobacter* protease I, Wako Pure Chemical, Osaka, Japan) at a 1:50 enzyme to substrate ratio at pH 7.0 and 37 °C for 24 h. Digests were separated by reverse phase HPLC on a Cosmosil C18 column (4.6 x 250 mm) (Nacalai Tesque, Kyoto, Japan). The running conditions were a 65-min gradient from 0 to 80% acetonitrile in 0.05% trifluoroacetic acid at a flow rate of 0.5 ml min<sup>-1</sup>. Individual peaks were collected and identified by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry (PerSeptive Biosystems) and amino acid analysis.

**CD**—CD measurements were carried out with a Jasco spectropolarimeter, Model J-720, at 20 °C. The results are expressed as the mean residue ellipticity [ $\theta$ ]. Far-UV CD spectra were measured using a cell with a light path of 1 mm at a protein concentration of 0.3 mg ml<sup>-1</sup>.

**Polymerization Assay**—Amyloid fibril formation of intact  $\beta_2$ -m was carried out by the fibril extension method established by Naiki *et al.* (19, 20, 23, 24), in which the seed fibrils were extended by the monomeric  $\beta_2$ -m at pH 2.5 and 37 °C, and the reaction was monitored by fluorometric analysis with ThT. Hereafter, this extension reaction will be referred to as the standard extension reaction. First, a solution of monomeric  $\beta_2$ -m at 35  $\mu$ M in 50 mM citrate buffer, pH 2.5, and 100 mM KCl at 4 °C was prepared. Then,  $\beta_2$ -m seed fibrils originally taken from patients were added to the monomeric solution to yield a final concentration of 0.5  $\mu$ M. It should be noted that the concentration of seeds is expressed in terms of monomer concentration because the size of seeds was difficult to define. The reaction was started by increasing the temperature to 37 °C in a water bath. From each reaction tube, an aliquot of 7.5  $\mu$ l was taken and mixed with 1.5 ml of 5  $\mu$ M ThT in 50 mM glycine NaOH buffer (pH 8.5). The fluorescence of ThT was monitored at 485 nm with excitation at 445 nm with a Hitachi fluorescence spectrophotometer, F4500. The extension reaction of the recombinant  $\beta_2$ -m was the same as that obtained from patients, confirming that the recombinant  $\beta_2$ -m was indistinguishable from  $\beta_2$ -m obtained from patients with respect to amyloid fibril formation.

Fibril formation of fragments of  $\beta_2$ -m were examined under similar solvent conditions at pH 2.5 and 37 °C. First, the spontaneous fibril formation at various peptide concentrations was examined without seeds. The lyophilized peptide fragments were dissolved in 50% (v/v) acetonitrile, and the final concentration of acetonitrile was less than 5% (v/v). We confirmed that 5% (v/v) acetonitrile did not affect the standard extension reaction. The cross-reactions between the intact  $\beta_2$ -m and K3 peptide were examined at the same protein concentrations used for the standard extension reaction; *i.e.* the concentrations of seed and monomeric form were 0.5 and 35  $\mu$ M, respectively.

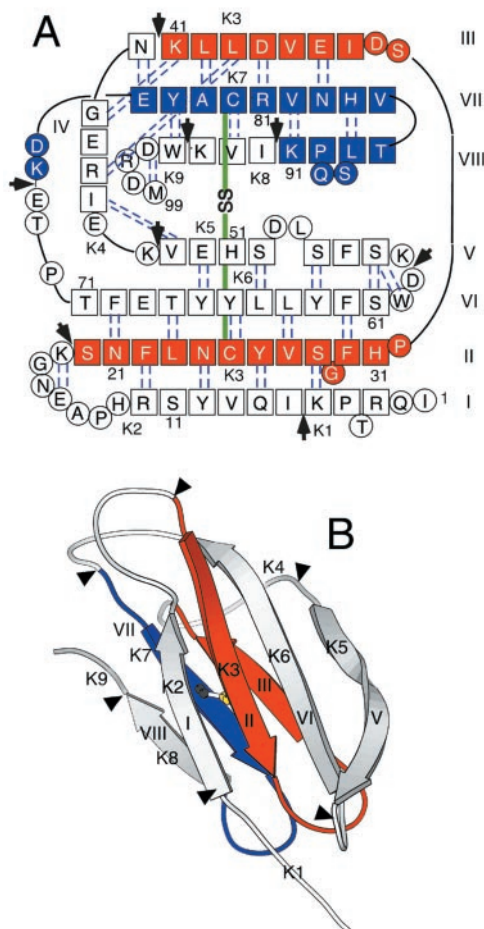


FIG. 1. Amino acid sequence (A) and structure (B) of  $\beta_2$ -m. A, the locations of cleavage sites by *Achromobacter* protease I are indicated by arrows. Secondary structures are indicated with hydrogen bonds and the numbering of  $\beta$ -strands. B, the locations of peptide fragments (K1–K9) are indicated by the number. The diagram was created by Molscript (34) with the structure reported by Bjorkman *et al.* (14).

**Electron Microscopy**—Reaction mixtures were spread on carbon-coated grids, negatively stained with 1% phosphotungstic acid (pH 7.0), and examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

#### RESULTS

**Protease Digestion**—Intact recombinant  $\beta_2$ -m at pH 7 was digested with *Achromobacter* protease I, producing nine peptides: K1 (Glu<sup>4</sup>-Lys<sup>6</sup>), K2 (Ile<sup>7</sup>-Lys<sup>19</sup>), K3 (Ser<sup>20</sup>-Lys<sup>41</sup>), K4 (Asn<sup>42</sup>-Lys<sup>48</sup>), K5 (Val<sup>49</sup>-Lys<sup>58</sup>), K6 (Asp<sup>59</sup>-Lys<sup>75</sup>), K7 (Asp<sup>76</sup>-Lys<sup>91</sup>), K8 (Ile<sup>92</sup>-Lys<sup>94</sup>), and K9 (Trp<sup>95</sup>-Met<sup>99</sup>), two of which, K3 and K7, were linked by a disulfide bond between Cys<sup>25</sup> and Cys<sup>80</sup> (Fig. 1). Eight peptides were separated by HPLC and were identified by mass and amino acid analysis (Fig. 2).

**Spontaneous Amyloid Fibril Formation**—The intact  $\beta_2$ -m did not form the amyloid fibrils spontaneously at least for several days at pH 2.5 and 37 °C, although the rapid extension reaction was observed with the seed fibrils, as established by Naiki *et al.* (18) (see below). Under the same conditions, we observed a significant increase in ThT fluorescence for K3-K7 peptide by incubation for 24 h at 67  $\mu$ M (Fig. 3). We separated K3 (22 residues) and K7 (16 residues) peptides by HPLC after reduction of the disulfide bond by 10 mM dithiothreitol at pH 8.0 and examined their amyloidogenic potential under the same conditions at pH 2.5. K3 peptide still exhibited ThT binding, although the fluorescence intensity was less than that of K3-K7 peptide.

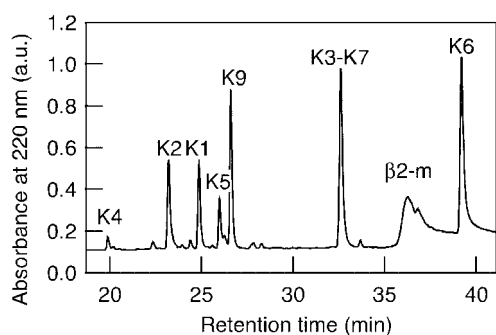


FIG. 2. Elution profiles by reverse-phase HPLC of the peptide fragments obtained by *Achromobacter* protease I digestion of  $\beta_2$ -m. The peptide fragments are assigned according to the numbering indicated in Fig. 1.

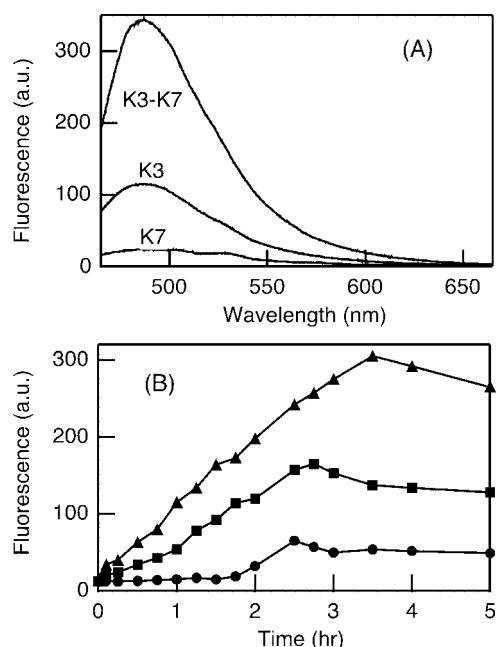


FIG. 3. Spontaneous amyloid fibril formation of the peptide fragments measured by ThT fluorescence. A, fluorescence spectra of ThT after incubation of  $67 \mu\text{M}$  K3-K7, K3, and K7 peptides for 24 h. B, kinetics of fibril formation of K3 peptide at various peptide concentrations:  $50 \mu\text{M}$  (●),  $150 \mu\text{M}$  (■), or  $300 \mu\text{M}$  (▲). ThT fluorescence intensity of intact  $\beta_2$ -m fibrils at  $67 \mu\text{M}$  was calculated to be about 800, much higher than those of the peptide fragments at the same molar concentration.

The kinetics of the increase in ThT fluorescence in the presence of K3 peptide was dependent on the peptide concentration (Fig. 3B). Although it showed a linear increase in fluorescence at  $300 \mu\text{M}$ , a lag phase was observed at  $50 \mu\text{M}$ . The lag time was about 2 h at  $50 \mu\text{M}$ , and it extended to 6 h at  $35 \mu\text{M}$  K3 (see Fig. 6C below). Although the final ThT fluorescence intensity was dependent on the peptide concentration, ThT fluorescence divided by the peptide concentration seemed to be independent of peptide concentration, suggesting the formation of a similar structure. ThT fluorescence of the K3 fibrils at the same molar concentration was much less than that of the intact  $\beta_2$ -m fibrils. Whereas ThT fluorescence intensity for the intact  $\beta_2$ -m fibrils at  $35 \mu\text{M}$  was about 400 under the experimental conditions, that of the K3 fibrils was about 50. However, ThT fluorescence normalized per weight was roughly similar among the intact  $\beta_2$ -m, K3-K7, and K3 fibrils. These results as well as the CD results described below suggested that, in the intact  $\beta_2$ -m fibrils, the regions other than K3 assumed the amyloid fibril conformation that can bind ThT.

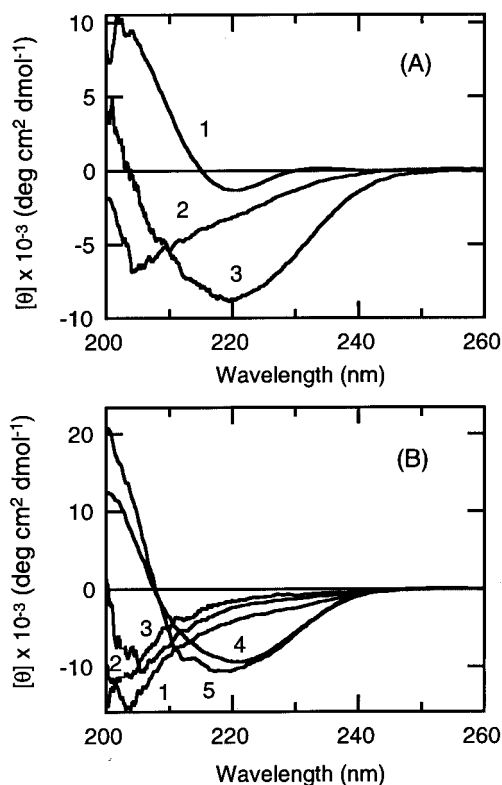


FIG. 4. The far-UV CD spectra of intact  $\beta_2$ -m (A) and its peptide fragments (B). A, native state at pH 7.0 (1), acid-unfolded state at pH 2.5 (2), and intact  $\beta_2$ -m fibrils at pH 2.5 (3). B, acid-unfolded state at pH 2.5 of K3-K7 (1), K3 (2), and K7 (3) and the fibril forms of K3-K7 (4) and K3 (5).

**Characterization of K3 Fibrils**—CD spectra showed that K3-K7 and K3 peptides were largely unfolded at pH 2.5 (Fig. 4B). Upon fibril formation detected by ThT fluorescence, the CD spectra became that of the  $\beta$ -sheet conformation with a minimum at around 218–220 nm. For comparison, the CD spectra of intact  $\beta_2$ -m in the native, acid-unfolded, and fibrillar forms are shown (Fig. 4A). The CD spectrum of K3 fibrils was similar to that of intact  $\beta_2$ -m amyloid fibrils. As the CD signal was expressed as mean residue ellipticity, this suggested that regions other than K3 of the intact  $\beta_2$ -m assume the  $\beta$ -sheet conformation in the fibrils, consistent with the results of ThT binding.

Formation of fibrils by K3-K7 and K3 peptides was confirmed by electron microscopy (Fig. 5, B and C). The newly formed straight fibrils, with a diameter of about 10–15 nm and a longitudinal periodicity, were similar to intact  $\beta_2$ -m amyloid fibrils (Fig. 5A). Polarized micrographs of fibrils after staining with Congo red showed orange-green birefringence, typical of amyloid fibrils (data not shown). These results confirmed that the K3-K7 and K3 peptides formed amyloid fibrils.

**Cross-reactions between  $\beta_2$ -m and K3 Peptide**—To gain insight into the mechanism of fibril formation, we examined cross-reactions between K3 peptide and  $\beta_2$ -m. Although intact  $\beta_2$ -m at  $35 \mu\text{M}$  does not form amyloid fibrils at least for several days at pH 2.5 and  $37^\circ\text{C}$ , the addition of seeds composed of intact  $\beta_2$ -m fibrils at  $0.5 \mu\text{M}$  induces amyloid fibril formation, which follows first-order kinetics (Fig. 6B). Intriguingly, the addition of monomeric K3 peptide at  $10 \mu\text{M}$  to the monomeric  $\beta_2$ -m at  $35 \mu\text{M}$  caused fibril formation with a lag time of about 40 h (Fig. 6A, curve 2). The fluorescence intensity at maximum (about 700) was evidently higher than that (about 400) of the standard extension reaction but slowly decreased with time to the value of the standard reaction. The lag time was signifi-



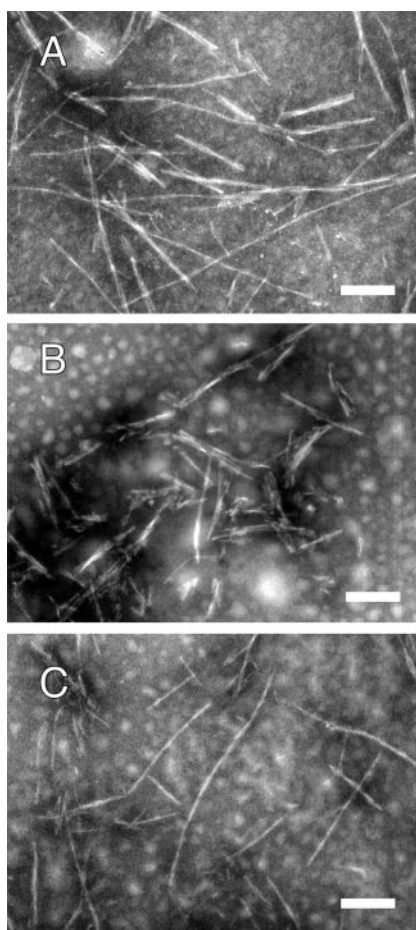


FIG. 5. Electron micrographs of amyloid fibrils of  $\beta_2$ -m and its peptide fragments. A, recombinant intact  $\beta_2$ -m; B, K3-K7 peptide; C, K3 peptide. Amyloid fibrils of intact  $\beta_2$ -m were prepared by the extension reaction with seed fibrils whereas those of the peptide fragments were prepared by the spontaneous reactions. The scale bars indicate a length of 200 nm.

cantly shortened by increasing the concentration of K3 peptide to  $35 \mu\text{M}$  (Fig. 6A, curve 3).

As spontaneous fibril formation of the K3 peptide at  $35 \mu\text{M}$  was observed after incubation for several hours (Fig. 3B), it is likely that the fibril formation of  $\beta_2$ -m was triggered by the spontaneous formation of K3 fibrils. In accordance with this, when preformed K3 fibrils were added instead of monomeric K3 peptide, the lag phase was further shortened. However, the lag phase was seen even in the presence of K3 fibrils and was similar between the two peptide concentrations (Fig. 6A, curves 4 and 5). Here, the maximal ThT fluorescence intensity was still about 700 and slowly decreased to the value for the standard reaction (about 400). The fibrils of intact  $\beta_2$ -m formed with K3 fibrils as seeds were indistinguishable from those prepared by the standard extension reaction with  $\beta_2$ -m with respect to the subsequent extension reaction with intact monomeric  $\beta_2$ -m (Fig. 6B).

We then examined the extension reaction of K3 peptide with different seeds (Fig. 6C). K3 peptide at  $35 \mu\text{M}$  exhibited spontaneous fibril formation with a lag phase of several hours (see also Fig. 3B). The intensity of ThT fluorescence (about 50) was lower than that of intact  $\beta_2$ -m fibrils (about 400) at the same molar concentration, as expected from the small size of the K3 peptide. The addition of intact  $\beta_2$ -m fibrils at  $0.5 \mu\text{M}$  reduced the lag time, although it was still present. The maximal fluorescence intensity was slightly higher than that in the absence of seed fibrils. In contrast, upon addition of K3 fibrils at  $0.5 \mu\text{M}$ ,

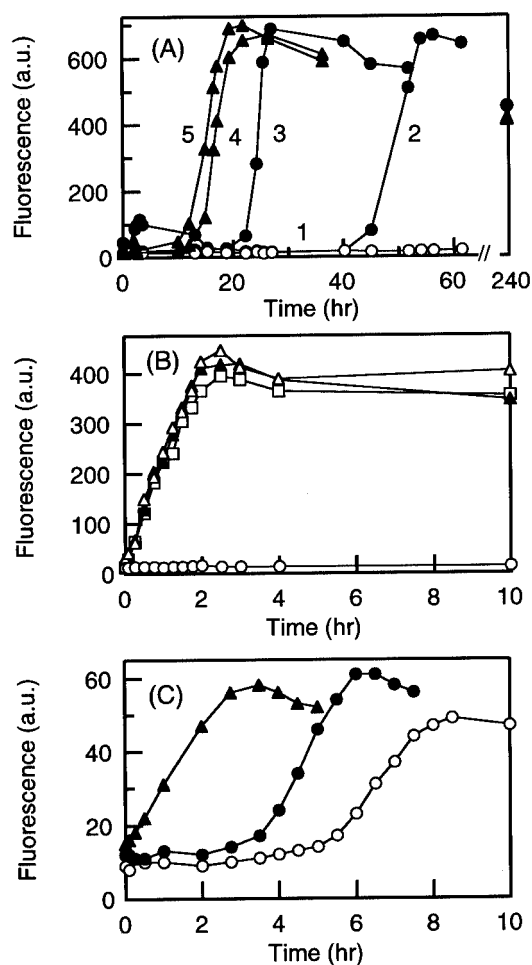


FIG. 6. Cross-reactions of amyloid fibril formation between intact  $\beta_2$ -m and its peptide fragments. A, effects of K3 peptide on the spontaneous fibril formation of  $35 \mu\text{M}$   $\beta_2$ -m. 1, in the absence of K3 peptide; 2,  $10 \mu\text{M}$  freshly prepared K3 peptide; 3,  $35 \mu\text{M}$  freshly prepared K3 peptide; 4,  $10 \mu\text{M}$  K3 fibrils; 5,  $35 \mu\text{M}$  K3 fibrils. The data points at 240 h for the reactions in the presence of K3 peptide (2–4) were clustered at the y-value of 400. B, effects of  $0.5 \mu\text{M}$   $\beta_2$ -m fibrils prepared in A on the fibril extension reaction with  $35 \mu\text{M}$  intact  $\beta_2$ -m.  $\circ$ , in the absence of seed fibrils;  $\blacktriangle$ , standard intact  $\beta_2$ -m fibrils;  $\triangle$ ,  $\beta_2$ -m fibrils prepared with  $10 \mu\text{M}$  freshly prepared K3 peptide;  $\square$ ,  $\beta_2$ -m fibrils prepared with  $10 \mu\text{M}$  K3 fibrils. C, effects of various seed fibrils at  $0.5 \mu\text{M}$  on the fibril formation of  $35 \mu\text{M}$  K3 peptide.  $\circ$ , in the absence of seed fibrils;  $\bullet$ , intact  $\beta_2$ -m fibrils;  $\blacktriangle$ , K3 fibrils. The concentration of seed fibrils was expressed in terms of monomer. The reaction was monitored by fluorometric analysis with ThT.

the ThT fluorescence increased smoothly without a lag phase, and the final intensity was slightly higher than that in the absence of seed fibrils. These results indicated that K3 fibrils, but not intact  $\beta_2$ -m fibrils, worked directly as seeds in the extension reaction of the K3 peptide.

**Stability of Amyloid Fibrils**—Amyloid fibrils of  $\beta_2$ -m prepared at pH 2.5 were unstable at neutral pH and depolymerized spontaneously with a few hours at pH 8.5 (Fig. 7; see also Ref. 33). Intriguingly, the depolymerization of K3 fibrils occurred much faster than that of intact  $\beta_2$ -m fibrils, completing in several minutes. These results indicated that K3 fibrils are less stable than those of intact  $\beta_2$ -m fibrils.

#### DISCUSSION

Human  $\beta_2$ -m consists of 99 amino acid residues (14, 15). It is likely that even short peptides including key residues can form amyloid fibrils. In accordance with this expectation, we found that K3 peptide made of 22 amino acid residues retained the potential to form amyloid fibrils. We could not distinguish the

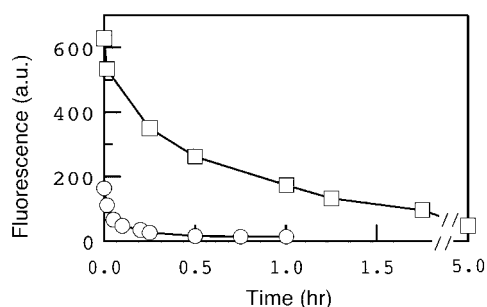


FIG. 7. **Time course of depolymerization of amyloid fibrils.** 50  $\mu\text{M}$   $\beta_2$ -m fibrils ( $\square$ ) and 200  $\mu\text{M}$  K3 fibrils ( $\circ$ ) were incubated in 50 mM glycine NaOH buffer, pH 8.5, at 37 °C. The reaction was monitored by fluorometric analysis with ThT.

fibrils of K3 and intact  $\beta_2$ -m on CD (Fig. 4) or electron microscopy (Fig. 5), although we believe that they probably differ in structural details. On the other hand, there were clear differences between K3 peptide and intact  $\beta_2$ -m in their kinetics of fibril formation (Figs. 3 and 6) and depolymerization (Fig. 7). These differences as well as the results of cross-reactions between K3 peptide and intact  $\beta_2$ -m (Fig. 6) can be explained satisfactorily on the basis of the schematic mechanisms as described below (Fig. 8).

The  $\beta_2$ -m molecule is assumed to consist of two regions: *i.e.* essential (or minimal) and non-essential regions. The essential region even after isolation can form fibrils by itself. We considered the K3 peptide to accommodate such a region. We started experiments with various synthetic peptides to narrow the minimal sequence. Preliminary results indicated that a shorter peptide within the K3 region still retains the amyloidogenic potential (data not shown), suggesting that even a limited sequence in K3 peptide can form the amyloid fibrils. Although the non-essential region cannot form the amyloid fibrils by itself, it can participate in fibril formation passively once it is associated with the essential region. In other words, the core  $\beta$ -sheet formed in the essential region can propagate by itself to the rest of the molecule. However, because of its larger size, the nucleation process of  $\beta_2$ -m may require more extensive and cooperative conformational changes than that of K3 peptide, *i.e.* there is a high free energy barrier. This high energy barrier of intact  $\beta_2$ -m may explain the difficulty of spontaneous fibril formation of  $\beta_2$ -m but not of the K3 peptide. On the other hand, because  $\beta_2$ -m has interaction sites more than the K3 peptide, fibrils of  $\beta_2$ -m, once formed, would be stabilized to a greater extent than those of K3 peptide, as demonstrated by depolymerization reactions at neutral pH (Fig. 7).

This two-region model (essential and non-essential regions) can explain most of the cross-extension reactions between  $\beta_2$ -m and K3 peptide (Fig. 6). In the extension reaction with seeds, we can focus only on the extension process by removing the nucleation process. Extension of  $\beta_2$ -m with the  $\beta_2$ -m fibril seeds is rapid without a lag phase. The same is likely to be true for the homogeneous extension of K3 with the K3 seeds (Fig. 8B). On the other hand, the heterogeneous reactions between intact  $\beta_2$ -m and K3 peptide exhibited a lag phase. The conformations of fibrils are probably different between them, and the heterogeneous association of monomers onto the end(s) of the seed fibrils would be thermodynamically unfavorable. After a certain number of the heterogeneous monomers have polymerized onto the ends of seeds, the ends would become similar to those of fibrils of the monomers so that the extension becomes favorable in terms of free energy and rate. In the heterogeneous extension reaction of  $\beta_2$ -m with K3 seeds, we observed a maximum in ThT fluorescence intensity, the value higher than that of typical  $\beta_2$ -m fibrils (Fig. 6A). The two-region model does not

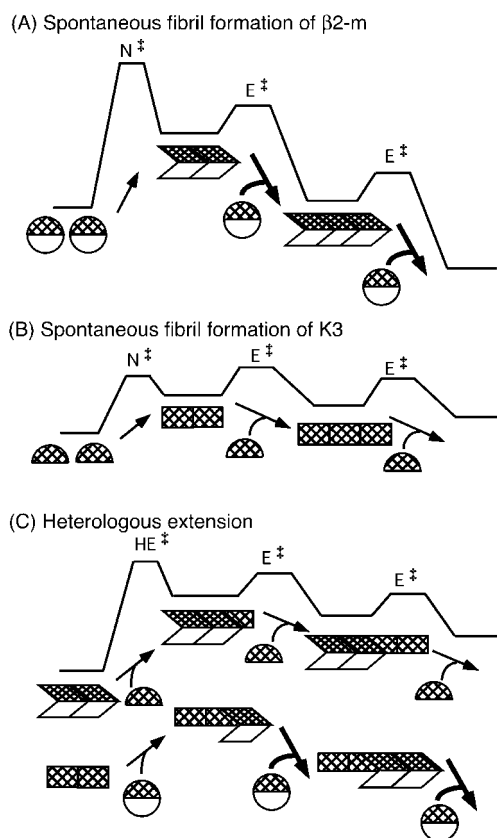


FIG. 8. **Schematic representation of the mechanisms of spontaneous amyloid fibril formation of intact  $\beta_2$ -m (A) and K3 peptide (B) and heterologous extension reactions between their fibrillar and monomeric forms (C).** The free energy profiles for the reactions except the heterologous reaction between the K3 seeds and intact  $\beta_2$ -m monomers are indicated.  $N^\ddagger$ ,  $E^\ddagger$ , and  $HE^\ddagger$  represent the transition states for the nucleation reaction, homologous extension reaction, and heterologous extension reactions, respectively. *Shaded* and *open* regions correspond to the essential and non-essential regions, respectively. The *different shapes* indicate the different conformations. Spontaneous fibril formation (A and B) consists of the nucleation and extension processes. The nucleation process of  $n$ -mers is represented by the association of two monomers. Only two extension processes are shown. In the heterogeneous extensions (C), after a certain number of the heterogeneous monomers have polymerized onto the ends of seeds, the ends would become similar to those of fibrils consisting of the monomers so that the free energy barrier decreases.

explain this complicated behavior, suggesting that the exact mechanism for the heterogeneous extension includes several fibril conformations different in their affinity to ThT. It is clear that, once the fibrils of intact  $\beta_2$ -m are formed with K3 seeds, they are indistinguishable from  $\beta_2$ -m fibrils made with intact  $\beta_2$ -m seeds. We do not need to assume different fibril conformations depending on the type of seeds as observed for yeast prions (8, 9).

The results observed here are very similar to the cross-reactions between  $A\beta$ -(1–42) and  $A\beta$ -(1–40) in Alzheimer's  $\beta$ -amyloid fibril formation *in vitro* reported by Hasegawa *et al.* (22). They examined homogeneous and heterogeneous extensions with  $A\beta$ -(1–42) and  $A\beta$ -(1–40). When the species used for seeds was the same as the species of monomers, no lag phase was observed. In contrast, when the two species were different, the lag phase was observed. They argued the importance of a conformational change in order to start the heterogeneous extension reaction between different  $A\beta$  peptides. The morphology of the fibrils formed was governed by the major component in the reaction mixture, not by the morphology of preexisting fibrils. This was also the case for  $\beta_2$ -m. Therefore, when the

different species cross-react, the requirement of the conformational change at the extending end(s) of the seeds will be common to various cases of amyloid fibril formation.

In conclusion, we showed that K3 peptide (Ser<sup>21</sup>–Lys<sup>41</sup>) constitutes the essential region of  $\beta_2$ -m-related amyloid fibril formation. Although K3 peptide of  $\beta_2$ -m formed amyloid fibrils more readily than intact  $\beta_2$ -m, the stability of K3 fibrils was less than that of intact  $\beta_2$ -m fibrils, implying that the high free energy barrier of the nucleation event is important for the high stability of amyloid fibrils. Amyloid fibril formation of globular proteins is considered to be an intriguing example of the complex landscape of protein folding. Because of its moderately small size as a globular protein, we may be able to clarify the topological and topographical relationships between the native, unfolded (monomeric), and fibrillar conformations of  $\beta_2$ -m more convincingly than in the cases of other amyloidogenic proteins.

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## REFERENCES

- Gillmore, J. D., Hawkins, P. N., and Pepys, M. B. (1997) *Br. J. Haematol.* **99**, 245–256
- Koo, E. H., Lansbury, P. T., Jr., and Kelly, J. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 9989–9990
- Kelly, J. W. (1998) *Curr. Opin. Struct. Biol.* **8**, 101–106
- Brange, J., Andersen, L., Laursen, E. D., Meyn, G., and Rasmussen, E. (1997) *J. Pharm. Sci.* **86**, 517–525
- Guijarro, J. I., Sunde, M., Jones, J. A., Campbell, I. D., and Dobson, C. M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95**, 4224–4228
- Ohnishi, S., Koide, A., and Koide, S. (2000) *J. Mol. Biol.* **301**, 477–489
- MacPhee, C. E., and Dobson, C. E. (2000) *J. Mol. Biol.* **297**, 1203–1215
- Santoso, A., Chien, P., Osheerovich, L. Z., and Weissman, J. S. (2000) *Cell* **100**, 277–288
- Chien, P., and Weissman, J. S. (2001) *Nature* **410**, 223–227
- Gejyo, F., Yamada, T., Odani, S., Nakagawa, Y., Arakawa, M., Kunitomo, T., Kataoka, H., Suzuki, M., Hirasawa, Y., Shirahama, T., Cohen, A. S., and Schmid, K. (1985) *Biochem. Biophys. Res. Commun.* **129**, 701–706
- Casey, T. T., Stone, W. J., Diraimondo, C. R., Barantley, B. D., Diraimondo, C. V., Gorevic, P. D., and Page, D. L. (1986) *Human Pathol.* **17**, 731–738
- Koch, K. M. (1992) *Kidney Int.* **41**, 1416–1429
- Gejyo, F., and Arakawa, M. (1990) *Contrib. Nephrol.* **78**, 47–60
- Bjorkman, P. J., Saper, M. A., Samraoui, B., Bennett, W. S., Strominger, J. L., and Wiley, D. C. (1987) *Nature* **329**, 506–512
- Okon, M., Bray, P., and Vucelic, D. (1992) *Biochemistry* **31**, 8906–8915
- Esposito, G., Michelutti, R., Verdona, G., Viglino, P., Hernández, H., Robinson, C. V., Amoresano, A., Dal Piaz, F., Monti, M., Pucci, P., Mangione, P., Stoppini, M., Merlini, G., Ferri, G., and Bellotti, V. (2000) *Protein Sci.* **9**, 831–845
- Chiti, F., Mangione, P., Andreola, A., Giorgetti, S., Stefani, M., Dobson, C. M., Bellotti, V., and Taddei, N. (2001) *J. Mol. Biol.* **307**, 379–391
- Naiki, H., Hashimoto, N., Suzuki, S., Kimura, H., Nakakuki, K., and Gejyo, F. (1997) *Amyloid: Int. J. Exp. Clin. Invest.* **4**, 223–232
- Naiki, H., Hasegawa, K., Yamaguchi, I., Nakamura, H., Gejyo, F., and Nakakuki, K. (1998) *Biochemistry* **37**, 17882–17889
- Naiki, H., and Gejyo, F. (1999) *Methods Enzymol.* **309**, 305–318
- Naiki, H., and Nakakuki, K. (1996) *Lab. Invest.* **74**, 374–383
- Hasegawa, K., Yamaguchi, I., Omata, S., Gejyo, F., and Naiki, H. (1999) *Biochemistry* **38**, 15514–15521
- Naiki, H., Higuchi, K., Hosokawa, M., and Takeda, T. (1989) *Anal. Biochem.* **177**, 244–249
- Naiki, H., Higuchi, K., Nakakuki, K., and Takeda, T. (1991) *Lab. Invest.* **65**, 104–110
- McParland, V. J., Kad, N. M., Kalverda, A. P., Brown, A., Kirwin-Jones, P., Hunter, M. G., Sunde, M., and Radford, S. E. (2000) *Biochemistry* **39**, 8735–8746
- Morgan, C. J., Gelfand, M., Atreya, C., and Miranker, A. D. (2001) *J. Mol. Biol.* **309**, 339–345
- Höggovist, B., Näslund, J., Sletten, K., Westermark, G. T., Mucchiano, G., Tjernberg, L. O., Nordstedt, C., Engström, U., and Westermark, P. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96**, 8669–8674
- Serpell, L. C. (2000) *Biochim. Biophys. Acta* **1502**, 16–30
- Balbach, J. J., Ishii, Y., Antzutkin, O. N., Leapman, R. D., Rizzo, N. W., Dyda, F., Reed, J., and Tycko, R. (2000) *Biochemistry* **39**, 13748–13759
- von Bergen, M., Friedhoff, P., Biernat, J., Heberle, J., Mandelkow, E.-M., and Mandelkow, E. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97**, 5129–5134
- Hoshino, M., Hagihara, Y., Nishii, I., Yamazaki, T., Kato, H., and Goto, Y. (2000) *J. Mol. Biol.* **304**, 927–939
- Akhrem, A. A., and Drozhdenyuk, A. P. (1989) *Anal. Biochem.* **179**, 86–89
- Yamaguchi, I., Hasegawa, K., Takahashi, N., Gejyo, F., and Naiki, H. (2001) *Biochemistry* **40**, 8499–8507
- Kraulis, P. J. (1991) *J. Appl. Crystallogr.* **24**, 946–950

**Investigation of a Peptide Responsible for Amyloid Fibril Formation of  $\beta_2$ -Microglobulin by *Achromobacter* Protease I**  
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