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Seeding-dependent Maturation of β_2-Microglobulin Amyloid Fibrils at Neutral pH*

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Miho Kihara‡, Eri Chatani‡§, Miyo Sakai‡, Kazuhiro Hasegawa¶, Hironobu Naiki¶, and Yuji Goto‡‖

From the ‡Institute for Protein Research, Osaka University and CREST, Japan Science and Technology Agency, Suita, Osaka 565-0871, Japan and the ¶Faculty of Medical Sciences, University of Fukui and CREST, Japan Science and Technology Agency, Matsuoka, Fukui 910-1193, Japan

β_2-Microglobulin (β2-m) is a major component of amyloid fibrils deposited in patients with dialysis-related amyloidosis. Recent studies have focused on the mechanism by which amyloid fibrils are formed under physiological conditions, which had been difficult to reproduce quantitatively. Yamamoto *et al.* (Yamamoto, S., Hasegawa, K., Yamaguchi, I., Tsutsumi, S., Kardos, J., Goto, Y., Gejyo, F. & Naiki, H. (2004) *Biochemistry* 43, 11075–11082) showed that a combination of seed fibrils prepared under acidic conditions and a low concentration of sodium dodecyl sulfate below its critical micelle concentration enabled extensive fibril formation at pH 7.0. Here, we found that repeated self-seeding at pH 7.0 with fibrils formed at the same pH causes a marked acceleration of growth, indicating the maturation of fibrils. The observed maturation can be simulated by assuming the existence of two types of fibrils with different growth rates. Importantly, some mutations of β2-m or the addition of a low concentration of urea, both destabilizing the native conformation, were not enough to extend the fibrils at pH 7.0, and a low concentration of sodium dodecyl sulfate (*i.e.* 0.5 mM) was essential. Thus, even though the first stage fibrils in patients are unstable and require stabilizing factors to remain at neutral pH, they can adapt to a neutral pH with repeated self-seeding, implying a mechanism of development of amyloid deposition after a long latent period in patients.

Amyloidosis results from the deposition of normally soluble proteins into insoluble amyloid fibrils that are long, unbranched, and often twisted fibrillar structures a few nanometers in diameter and predominantly composed of cross-β-sheets (1–3). Currently, >20 proteins are known to be associated with human amyloid diseases (1–3). Moreover, various proteins and peptides that are not related to diseases can also form amyloid-like fibrils, implying that the formation of amyloid fibrils is a general property of polypeptides (2, 3). Most of the amyloidogenic proteins studied to date have been shown to unfold or refold (if they are initially unfolded), such that one or more partially unfolded intermediate(s) are produced prior to the formation of amyloid fibrils (2, 3). These structural alterations are often brought about by changes in the solution, such as a switch to low pH (4–6), high temperature (7, 8), or by the addition of organic solvents (9, 10). However, the mechanism by which native proteins form amyloid fibrils under physiological conditions is still unclear.

Among the various amyloidogenic proteins, β_2-microglobulin (β2-m),[1] which is responsible for dialysis-related amyloidosis, is a target of extensive study because of its clinical importance and suitable size for examining the relationship between protein folding and amyloid fibril formation (10–18). Dialysis-related amyloidosis is a common and serious complication in patients receiving hemodialysis for >10 years (11, 12). β2-m, a typical immunoglobulin domain made of 99 residues and seven β-strands, is present as the non-polymorphic light chain of the class I major histocompatibility complex (MHC-I) (19). As part of its normal catabolic cycle, β2-m dissociates from the MHC-I complex and is transported in serum to the kidneys, where the majority (95%) of it is degraded (12). Renal failure disrupts the clearance of β2-m from the serum and, moreover, the β2-m does not pass through the dialysis membrane, resulting in an increase in the β2-m concentration by up to 50-fold in the blood circulation (12). By a mechanism that is currently not well understood, β2-m then self-associates to form amyloid fibrils.

The incubation of β2-m *in vitro* under acidic conditions in the presence or absence of seed fibrils results in the formation of high yields of amyloid fibrils with a range of different morphologies (5, 13–17). In contrast, the generation of amyloid fibrils under physiological conditions at neutral pH has been difficult. Although there are several reports of fibril formation at neutral pH (20–24), most of the experiments were qualitative, and the amount of fibrils formed was much less than that prepared under acidic conditions.

Several groups have suggested that lipid molecules are involved in the conformational change of various amyloid precursor proteins, as well as in the formation of amyloid fibrils (25–27). SDS is an anionic detergent that mimics some characteristics of biological membranes and is considered to be a good model for anionic phospholipids. It has been reported that SDS induces some proteins or peptides to form aggregates (28) or amyloid-like fibrils *in vitro* (29). Recently, Yamamoto *et al.* (30) found that low concentrations of SDS around the critical micelle concentration (CMC) (0.7 mM) not only stabilize the fibrils but also induce the extensive growth of β2-m amyloid fibrils at neutral pH, probably through the SDS-induced con-

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‖ To whom correspondence should be addressed: Inst. for Protein Research, Osaka University, Yamadaoka 3-2, Suita, Osaka 565-0871, Japan. Tel.: 81-6-6879-8614; Fax: 81-6-6879-8616; E-mail: ygoto@protein.osaka-u.ac.jp.

[1] The abbreviations used are: β2-m, β_2-microglobulin; CD, circular dichroism; CMC, critical micelle concentration; ThT, thioflavin T.

formational change of β2-m monomers. In the present study, we further examined the formation of fibrils at neutral pH in the presence of SDS, showing that the fibril formation at neutral pH is markedly accelerated by self-seeding. The observed acceleration can be reproduced theoretically by a simple model that assumes the existence of two types of fibrils, implying that the maturation of fibrils is a common mechanism leading to the development of amyloidosis. We also address the role of SDS and corresponding substances in promoting the formation of fibrils under physiological conditions.

MATERIALS AND METHODS

Proteins—The expression and purification of human recombinant β2-m and the V9P mutant were achieved as described previously (31). The molecular mass of the purified proteins was measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Applied Biosystems, Foster City, CA). Monomer concentrations of wild-type and V9P mutant proteins were determined from the extinction coefficient (ϵ = 19,300 M^{-1} cm^{-1}) at 280 nm based on the amino acid composition as described previously (31).

β2-m Amyloid Fibrils and Seeds—Throughout the experiments, we incubated the solutions for fibril formation or depolymerization without agitation. The standard buffers used were 50 mM sodium citrate buffer (pH 2.5) containing 100 mM NaCl and 50 mM sodium phosphate buffer (pH 7.0) containing 100 mM NaCl. β2-m fibrils were originally purified from patients suffering from dialysis-related amyloidosis (13). Fibrils were prepared by a repeated seed-dependent extension at pH 2.5 with human recombinant β2-m expressed in *Escherichia coli* (31) or yeast *Pichia pastoris* (32). Seeds (*i.e.* fragmented fibrils) were prepared by the sonication of 200-μl aliquots of a fibril stock solution using a Microson sonicator (Misonix, Farmingdale, NY) at intensity level 2 and 20 1-s pulses on ice. The fibril concentrations were measured with a Micro BCA™ protein assay reagent kit (Pierce).

Depolymerization Assay of Seeds at Neutral pH—The stability of seeds prepared at pH 2.5 was examined at pH 7.0 and 37 °C in the presence of various concentrations of SDS by the fluorometric assay with thioflavin T (ThT) in which an aliquot of 5 μl was taken from each reaction tube and mixed with 1 ml of 5 μM ThT in 50 mM sodium glycine buffer (pH 8.5). The fluorescence of ThT was measured using a Hitachi F-4500 spectrofluorometer at 25 °C with excitation at 445 nm and emission at 485 nm (13, 32).

Extension Assay at Neutral pH—Seeds incubated in the presence of 0.5 mM SDS at pH 7.0 and 37 °C were recovered by centrifugation and resuspended in the pH 7.0 buffer to prepare the SDS-stabilized seeds (S0 seeds). The extension reaction mixture designed to obtain F1 fibrils contained 25 μM monomeric β2-m, 30 μg/ml S0 seeds, and 0.5 mM SDS at pH 7.0. The reaction was monitored by ThT assay. By repeating the algorithmic protocol at pH 7.0, F2, F3, and F4 fibrils were obtained from the extension reaction with S1, S2, and S3 seeds, respectively. Electron microscopy images of the fibrils were recorded with a Hitachi H-7000 electron microscope as described (31).

The extension reaction of β2-m with reduced stability was examined in the absence of SDS at pH 7.0. First, the reaction in the presence of 4.2 M urea was measured with 25 μM wild-type β2-m and 30 μg/ml S3 seeds at pH 7.0 and monitored using ThT fluorescence. Second, the extension reactions of the V9P mutant were carried out with wild-type S3 seeds (30 μg/ml) and 25 μM V9P monomers at pH 7.0 and 37 °C.

Equilibrium Denaturation of Monomers and Fibrils—Urea-dependent unfolding of wild-type β2-m in the monomeric state at pH 7.0 was measured using the change in the intrinsic tryptophan fluorescence at 0.05 mg/ml β2-m in the presence and absence of 0.5 mM SDS. Before the measurements, the samples were incubated for 24 h at 25 °C. Fluorescence spectra were measured at 25 °C with excitation at 295 nm. Urea-dependent unfolding of amyloid fibrils was measured by the same method as monomeric β2-m.

It was difficult to obtain the smooth transition curves, in particular for the amyloid fibrils, by plotting the fluorescence intensity at a particular wavelength. In such a case, a mathematically exact transition curve can also be obtained by performing deconvolution of the spectra (31). Emission spectra from 310 nm to 450 nm were represented by a combination of the spectra of the native (or amyloid) and unfolded states, so that fractions of the two states were obtained at respective concentrations of urea. Because the protein-SDS interaction seems to affect the fluorescence spectrum of monomeric β2-m in the absence of urea but not in 1 M urea, we used the spectrum acquired in 1 M urea as the reference native state. The spectrum in the presence of 10 M urea was used as a reference spectrum for the unfolded state. The fraction of unfolded species (f_U) was plotted against urea concentration. Although the gradual changes of f_U occurred before the cooperative unfolding was observed, we did not correct them because the unfolding transitions were used only for comparing the global stability of the different β2-m conformational states.

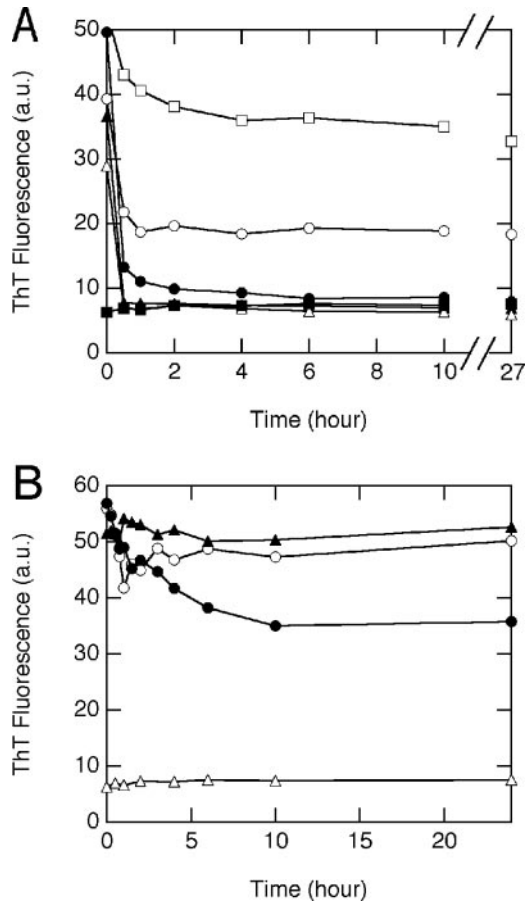

FIG. 1. **Stability of β2-m seed fibrils in the presence and absence of SDS monitored by the ThT fluorescence at 37 °C.** *A*, depolymerization of sonicated S0 (pH 2.5) seeds (30 μg/ml) at pH 7.0. Concentration of SDS is 0 (●), 0.5 (○), 1 (▲), or 5 (△) mM. For controls, the ThT fluorescence of monomeric β2-m (■) at pH 7.0 and that of pH 2.5-seeds at pH2.5 (□) are shown. *B*, depolymerization of sonicated pH 7.0 seeds (30 μg/ml S4) at pH 2.5. Concentrations of SDS are 0 (●) and 0.5 (○) mM. For controls, the ThT fluorescence of sonicated fibrils at pH 7.0 (▲) and that of monomeric β2-m (△) are shown. *a.u.*, arbitrary units.

CD Measurements—Far-UV CD spectra of β2-m in the monomeric and fibrillar states were measured with an AVIV model 215s spectropolarimeter (Lakewood, NJ) at 37 °C at a protein concentration of 0.05 mg/ml β2-m.

Ultracentrifuge Measurements—Sedimentation velocity and sedimentation equilibrium measurements were performed using a Beckman-Coulter Optima XL-1 analytical ultracentrifuge (Fullerton, CA) with an An-60 rotor and two-channel charcoal-filled Epon cells at 25 μM β2-m, pH 7.0, and 37 °C in the presence and absence of 0.5 mM SDS. The centrifugation experiments were performed after incubation of samples for 24 h, and the data were analyzed using the software Ultrascan 6.01 (www.ultrascan.uthscsa.edu/).

RESULTS

Extension of β2-m Amyloid Fibrils at Neutral pH—First, we examined the pH-dependent stability of seeds prepared by ultrasonication of the pH 2.5 fibrils. When the pH 2.5 S0 seeds were incubated at pH 7.0 in the absence of SDS, the ThT fluorescence decreased immediately to the control value without fibrils, showing the complete depolymerization (*i.e.* unfolding) of the seed fibrils (Fig. 1). In the presence of 0.5 mM SDS at pH 7.0, however, the ThT fluorescence indicated that ~30–40% of the seeds remain. In the presence of 1 and 5 mM SDS,

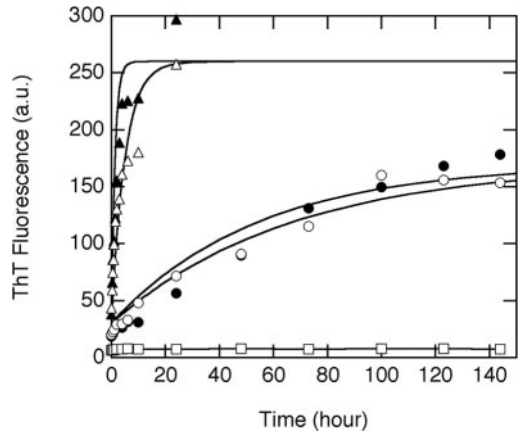

FIG. 2. **Self-seeding dependent maturation of amyloid fibril growth monitored on the basis of ThT fluorescence.** The reaction mixture contained a 25 μM β2-m monomer and 30 μg/ml seed fibrils in 50 mM phosphate buffer (pH 7.0), 100 mM NaCl, and 0.5 mM SDS. The types of seeds are S0 (●), S1 (○), S2 (△), and S3 (▲). Whereas S0 seeds were prepared at pH 2.5, others were prepared at pH 7.0 with the fibrils formed by the preceding extension reaction. For a control, the time course without seeds (□) is shown. The expanded time course with S2 seeds is shown in Fig. 6. The *solid lines* indicate the simulated kinetics, assuming the fraction of neutral pH from the S0 seeds to be 1×10^{-4}. The growth rates of the acidic and neutral pH forms were assumed to be 0.015 and 0.75 h^{-1}, respectively, and the ThT intensities of the acidic and neutral pH forms were 170 and 260, respectively. *a.u.*, arbitrary units.

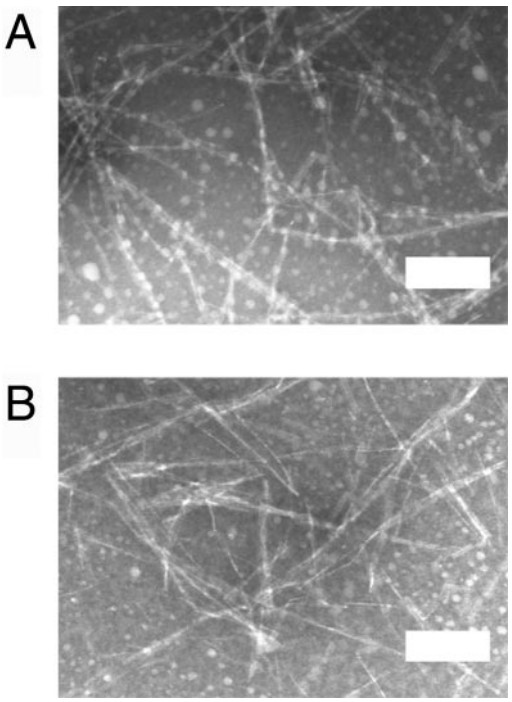

FIG. 3. **Electron micrographs of β2-m amyloid fibrils.** *A*, F0 fibrils prepared at pH 2.5. *B*, F3 fibrils prepared at pH 7.0. *Bars* indicate a length of 200 nm.

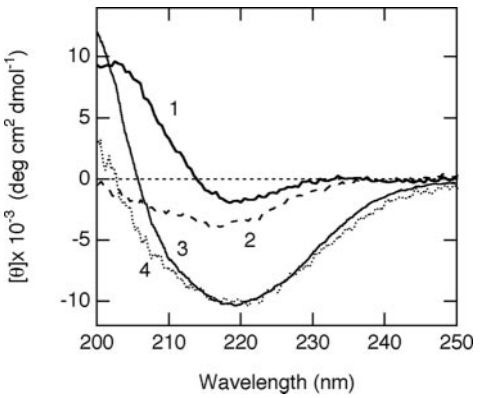

FIG. 4. **Far-UV CD spectra of various conformational states of β2-m measured at 37 °C.** *Line 1*, monomer at pH 7.0; *line 2*, monomer in the presence of 0.5 mM SDS; *line 3*, F0 fibrils at pH 2.5; and *line 4*, F4 fibrils in the presence of 0.5 mM SDS at pH 7.0.

the fluorescence disappeared completely, similarly as in the case of 0 mM SDS. These results show that 0.5 mM SDS, slightly lower than the CMC value of SDS (0.67 mM), is useful to protect seeds from depolymerization, which is consistent with a previous report in which almost full protection of "unsonicated" fibrils from depolymerization was observed with 0.5 mM SDS at pH 7.0 (30). The decreased stability of the fragmented S0 seeds in comparison with that of intact pH 2.5 fibrils is probably due to the increased amount of fibril ends after fragmentation.

As was found by Yamamoto *et al.* (30), we observed a substantial fibril (F1) extension at pH 7.0 with the SDS-stabilized S0 seeds (Fig. 2). The seed-dependent extension in the presence of 0.5 mM SDS at pH 7.0 showed that the ThT fluorescence increases slowly but without a lag phase, reaching equilibrium after 120 h. We observed no extension without SDS, indicating that the role of SDS is essential. In the absence of the seeds, no increase in fluorescence was observed throughout the reaction, showing that seeds are also necessary even in the presence of SDS. It is noted that we incubated the solutions without agitation. Similar fibril (F2) growth was observed for the second cycle of the extension reaction at pH 7.0 with S1 seeds prepared with the product (F1) of the first cycle at pH 7.0.

Surprisingly, the third cycle of the extension reaction at pH 7.0 with S2 seeds showed that the rate of fibril (F3) growth increased significantly. The ThT value increased markedly, reaching, at 6 h, a much higher value than the final value for F2 fibrils (Fig. 2; see also Fig. 6 below). Further repeats of the seeding reaction with the S3 seeds did not accelerate fibril growth notably, although some increase in ThT fluorescence was observed for the fourth cycle of the extension reaction to make F4 fibrils (Fig. 2). These observations were reproducible; we repeated a series of experiments four times, obtaining essentially the same results. Electron microscopic images of the fibrils formed at different cycles of seeding showed a typical morphology of β2-m amyloid fibrils with a helical filament structure (Fig. 3). These fibrils were indistinguishable from each other and also similar to the fibrils prepared at pH 2.5. In addition, the far-UV CD spectrum of fibrils prepared at pH 7.0 had a large minimum at ~220 nm (Fig. 4), which is typical for β2-m amyloid fibrils, confirming the formation of amyloid fibrils at pH 7.0. These results indicate that, with the repeated seeding at pH 7.0, the acidic pH fibrils adapted to the neutral pH.

In contrast to the increased stability at pH 7.0, the β2-m fibrils adapted to pH 7.0 were partially unfolded at pH 2.5 (Fig. 1*B*), suggesting that the stability of amyloid fibrils is optimized at the pH of fibril formation. Thus, although we cannot distinguish the acidic pH and neutral pH forms of fibrils from structures measured by electron microscopy and CD, they are distinct in terms of growth rate and stability, and both are optimized for the pH of fibril formation.

Effects of SDS on the Conformational Stability of β2-m—A low concentration of SDS was essential for the fibril extension at pH 7.0 even when the pH 7.0 seeds made from the adapted fibrils were used. It has been suggested that a role of SDS is to destabilize the native structure of β2-m in addition to stabilizing the seed and fibrils (30). To investigate this possibility, the stability of the β2-m monomer in the presence of 0.5 mM SDS was examined by monitoring the urea-induced unfolding.

First, the far-UV CD spectra of the β2-m monomer and fibrils were measured at pH 7.0 in 0.5 mM SDS (Fig. 4). Native β2-m in the absence of SDS has a spectrum with a markedly small intensity, suggesting some negative contribution to the apparent CD intensity of the β-sheet structure. After incubation overnight in the presence of 0.5 mM SDS, the spectrum exhibited a negative peak at 218 nm with an intensity larger than that in the absence of SDS. These results were consistent with the report by Yamamoto *et al.* (30), suggesting a slight conformational change upon interaction with SDS.

The urea-induced unfolding transitions of β2-m monomers in the presence and absence of 0.5 mM SDS at pH 7.0 were measured using tryptophan fluorescence. β2-m has two Trp residues at positions 60 and 95. Whereas in the native state Trp^{95}, located at the end of β-strand G, is partially exposed to the solvent, Trp^{60}, on the β-turn connecting β-strands D and E, is greatly exposed. The Trp fluorescence spectrum of the monomeric β2-m showed a maximum at 335 nm, consistent with the partial exposure of Trp residues (Fig. 5*A*). The addition of urea resulted in a red shift of the maximal wavelength to 348 nm. Although the fluorescence intensity increased below 4 M urea, it decreased above 4 M urea where a significant change in maximal wavelength was observed. The plot of the fraction of unfolding showed that the unfolding transition accompanied by the decrease in fluorescence intensity is cooperative with a transition midpoint at 6 M urea (Fig. 5*C*).

The Trp fluorescence spectrum of the β2-m monomer in 0.5 mM SDS showed a maximum at 330 nm, blue-shifted ~5 nm compared with that without SDS, suggesting that Trp residues become less solvent-accessible in the presence of 0.5 mM SDS (data not shown). Upon the addition of urea, a cooperative transition similar to that in the absence of SDS was observed. The maximal fluorescence wavelength was 335 nm in the presence of 1 M urea, suggesting that the dissociation of bound SDS occurs before the unfolding transition. The spectrum of the unfolded form in the presence of 0.5 mM SDS was the same as that in the absence of urea. The normalized transition curve showed that the apparent stability of β2-m in the presence of 0.5 mM SDS is essentially the same as that in the absence of SDS, indicating that 0.5 mM SDS does not affect the apparent stability of β2-m even though a slight change in conformation is introduced.

The β2-m fibrils at pH 7.0 in the presence of 0.5 mM SDS also showed a cooperative unfolding transition in which the maximum at 340 nm in the absence of urea, slightly longer than that of the native state, shifted to 350 nm accompanied by the increase in intensity (Fig. 5*B*). Intriguingly, the normalized transition curve was very similar to that of the native state (Fig. 5*C*).

Extension Assay with Destabilized β2-m—That there was no effect of SDS on the apparent stability of β2-m was surprising, because we anticipated the SDS-assisted formation of fibrils to be coupled with the SDS-induced destabilization of the rigid native structure. It is likely that the apparent lack of effect was caused by the dissociation of bound SDS molecules before the major unfolding transition of β2-m. To examine the role of the destabilization of protein structures in the fibril formation more directly, we measured the extension reaction of β2-m in the presence of 4.2 M urea, where monomer β2-m is slightly destabilized (Fig. 5*C*). Under conditions of 4.2 M urea and 0 mM SDS at pH 7.0, no fibril formation was observed despite the addition of maturated S3 seeds (Fig. 6*A*).

Furthermore, we examined the formation of fibrils with the V9P β2-m mutant (31). The stability of the V9P native structure was decreased substantially compared with that of the wild-type protein as monitored using urea-induced denaturation, although V9P still retains its native state in the absence of a denaturant (Fig. 5*C*). The fibril extension reaction of V9P β2-m at pH 7.0 in the absence of SDS was very slow, even if a notable increase of ThT fluorescence compared with the control was observed (Fig. 6*B*). The addition of 0.5 mM SDS recovered the rapid fibril growth of V9P β2-m with the ThT fluorescence intensity higher than that of the wild type β2-m, demonstrating that V9P β2-m retains a strong potential to form fibrils under optimal conditions. These results indicated that desta-

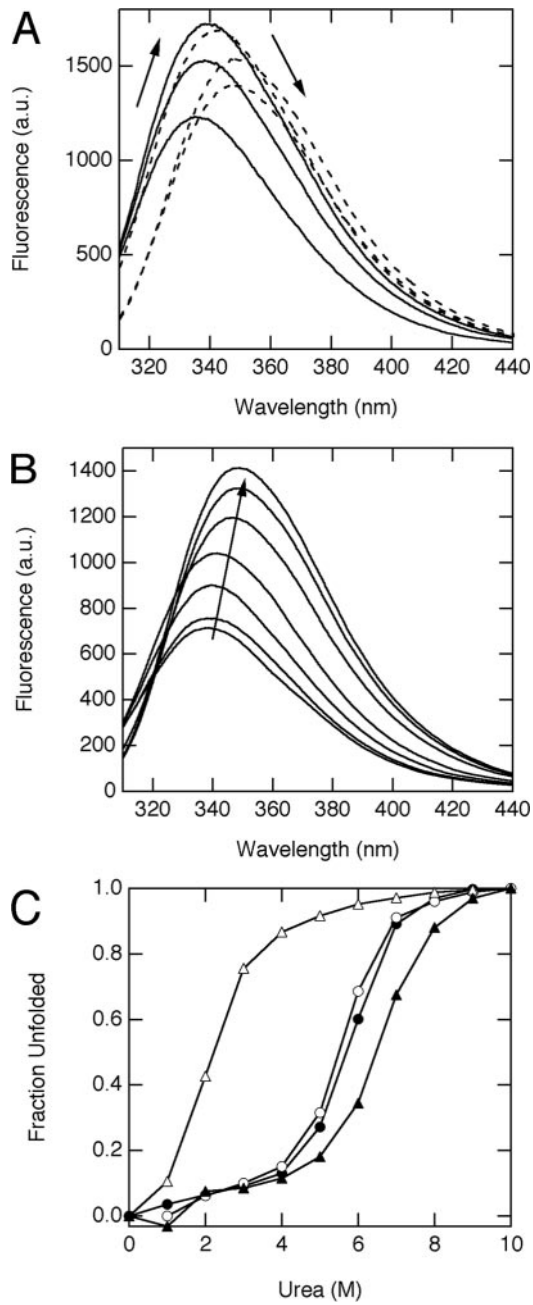

FIG. 5. **Urea-induced unfolding transitions of monomers and fibrils of β2-m measured from the tryptophan fluorescence spectrum at pH 7.0 and 25 °C.** *A*, urea-induced unfolding of the β2-m monomer. Concentrations of urea are 0, 1, 3, 5, 7, and 10 M with an increase in the maximal wavelength from 335 to 350 nm, as guided by *arrows*. *B*, urea-induced unfolding of β2-m fibrils (F4). Concentrations of urea are 0, 1, 3, 5, 7, 9, and 10 M with an increase in the maximal wavelength from 340 to 350 nm. *C*, normalized unfolding transition curves of β2-m monomers in the presence of 0.5 mM SDS (●) or the absence of SDS (○), β2-m fibrils (F4) (▲) in the presence of 0.5 mM SDS, and V9P β2-m monomers (△) in the absence of SDS. *a.u.*, arbitrary units.

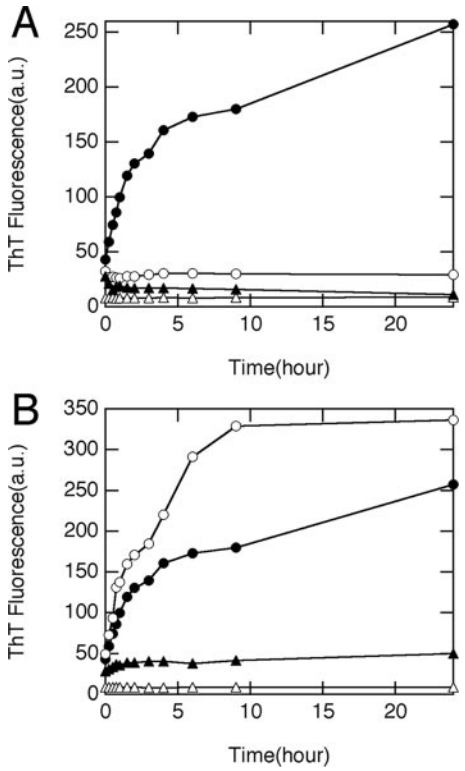

FIG. 6. **Essential role of SDS in the extension of β2-m amyloid fibrils at pH 7.0 and 37 °C.** *A*, the fibril extension of wild-type β2-m (25 μM) in 4.2 M urea with (●) and without (○) S3 seeds (30 μg/ml). The ThT control without monomers (▲) or seeds (△) is shown. *B*, the fibril extension of V9P β2-m (25 μM) in the presence (○) and absence (▲) of 0.5 mM SDS. The control experiment without seeds (△) is shown. For both panels, the efficient fibril extension of wild-type β2-m (●) in the presence of 0.5 mM SDS with S3 seeds taken from Fig. 2 is shown for comparison. *a.u.*, arbitrary units.

bilization of the monomeric form is not enough to induce efficient seed-dependent fibril growth at neutral pH.

Oligomer Formation Revealed by Analytical Centrifugation—A low concentration of SDS, below the CMC, has been suggested to induce the aggregation of proteins, including β2-m (28, 30). We examined this possibility by measuring sedimentation velocity and sedimentation equilibrium (Fig. 7). In the absence of SDS, the distribution of the sedimentation coefficient, $S_{20,w}$, of β2-m is ~2, which is consistent with a monomeric globular protein with a molecular weight of 12,000 (Fig. 7*A*). In the presence of 0.5 mM SDS, the distribution showed two main peaks, one at 2 and the other at 5.5 (Fig. 7*B*). Whereas the former corresponds to a monomer, the latter corresponds to oligomers consisting of about five monomers. The results of sedimentation equilibrium measurements were consistent with those of sedimentation velocity measurements (Fig. 7*C*). Whereas the apparent molecular weight was 12,000 in the absence of SDS, it depended on the location in the cell from ~10,000 at the top to 60,000 at the bottom, where the protein concentration was 1 mg/ml (0.1 mM). These results indicate that, upon incubation with 25 μM β2-m and 0.5 mM SDS at pH 7.0 and 37 °C, the standard conditions for fibril growth in the present study, about half of the molecules are converted to oligomers with an average size of five monomers.

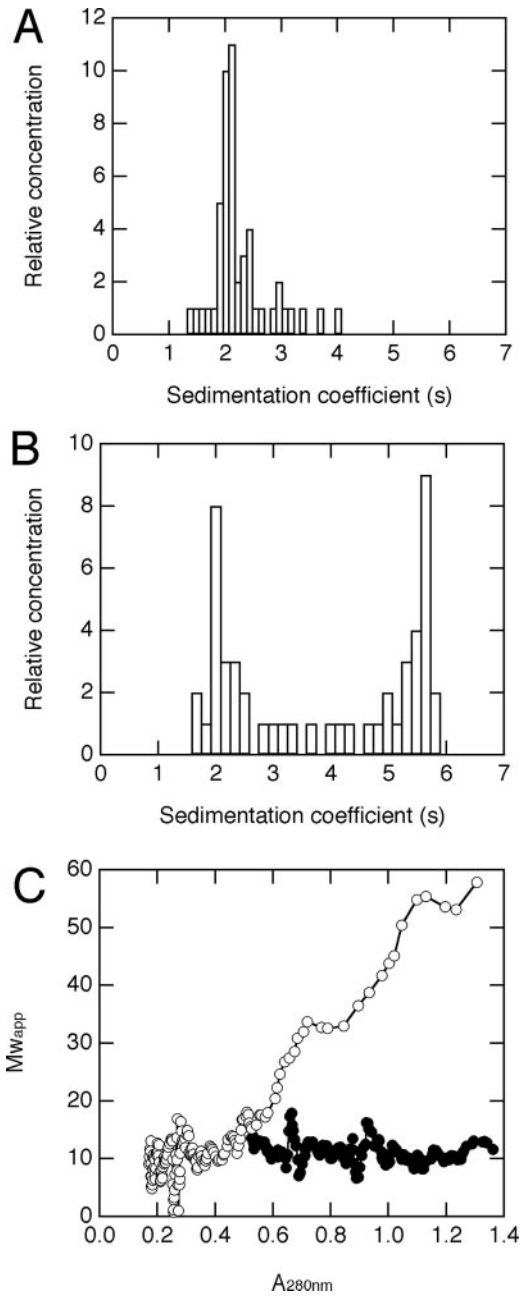

FIG. 7. **Sedimentation velocity and sedimentation equilibrium measurements on the effects of SDS.** Samples of 25 μM β2-m monomers in the presence and absence of 0.5 mM SDS at pH 7.0 were incubated at 37 °C for 24 h. The centrifugation experiments were performed at the same temperature. *A* and *B*, the distribution of sedimentation coefficients obtained from the sedimentation velocity measurements in the absence (*A*) and presence (*B*) of 0.5 mM SDS. *C*, the distribution of apparent molecular weight (MW_{app}) against the location in the cell obtained from the sedimentation equilibrium measurement in the absence (●) and presence (○) of 0.5 mM SDS. The apparent molecular weight was calculated at respective points in the cell, *i.e.* the higher the absorbance at 280 nm ($A_{280\ nm}$), the closer to the bottom of the cell.

DISCUSSION

Seeding-dependent Maturation of Amyloid Fibrils—One of the most urgent issues in the study of β2-m amyloid fibrils is reproducing the formation of fibrils under physiological conditions, which is the phenomenon in patients suffering from dialysis-related amyloidosis. To date, several groups have reported the formation of β2-m amyloid fibrils under physiological conditions at neutral pH (20–24) through the use of mutants with a deletion or mutation of N-terminal residues (20, 23), the addition of copper ions (21, 24), or the addition of pre-formed seeds during the refolding of β2-m monomers (22). However, in contrast to the rapid and extensive reaction under acidic conditions (5, 12–17), the generation of amyloid fibrils under the physiological conditions so reported is qualitative, and, moreover, reproducibility is ambiguous.

Yamamoto *et al.* (10) indicated that the addition of trifluoroethanol stabilizes the acidic fibrils and also promotes the fibril extension at neutral pH. Furthermore, Yamamoto *et al.* (30) showed that a low concentration of SDS combined with SDS-stabilized seeds results in substantial fibril formation, approaching the serious situation that occurs in patients. However, the fibril growth took several days, which is slower than the rapid growth under acidic conditions although much faster than the actual time course of development of amyloidosis. Here, we showed that repeated seeding with fibrils formed at pH 7.0 results in a marked acceleration of the fibril growth under physiological conditions.

Simulation of the Acceleration of the Growth Rate—The observed acceleration of the fibril growth by the repeated seeding can be simulated by assuming the existence of two types of fibrils, namely the acidic pH form with the slow growth rate and the neutral pH form with the fast growth rate. Although the population of the neutral pH fibrils might be very small at the initial stage, it can be increased by repeated seeding because of the faster growth rate. Fitting the first cycle of extension with the S0 seeds to exponential kinetics gave the rate constant of 0.015 h^{-1} and the final ThT fluorescence of 170, corresponding to the parameters of the acidic pH fibrils. Then, to reproduce the subsequent extension reactions with S1, S2, and S3 seeds, the fraction of the neutral pH fibrils in the S0 seeds, the growth rate, and the ThT value were estimated to be 1×10^{-4}, 0.75 h^{-1}, and 260, respectively (Fig. 2). The reasonable agreement of the observed and simulated curves indicates that the observed increase of the growth rate upon the repeated seeding can be explained by this simple mechanism.

Implication in Dialysis-related Amyloidosis—These results provide important insight into the formation of amyloid fibrils. First, the suggested mechanism of maturation might explain the development of amyloid deposits in patients. It is accepted that one of the most critical factors of dialysis-related amyloidosis is the 50-fold increased level of β2-m in blood (*i.e.* 50 mg/l). However, it takes on average >10 years to form substantial amyloid deposits. Once the deposition is started, the accumulative formation of amyloid develops in an accelerated manner. The general idea is that extensive amyloid deposits start only after >10 years because of unknown factors. The present study suggests an alternative possibility, *i.e.* that the maturation of amyloid fibrils is involved in the development of disease in patients. The fibrils of the first stage that are formed soon after the start of dialysis might be unstable, requiring additional stabilizing components such as glycosaminoglycans, proteoglycans, or collagens (10, 33). Repeated self-seeding with fragmented fibrils in patients causes the fibrils to mature with time and the amount of fibrils to increase. Consequently, the fibrils become stable by themselves and, moreover, the accelerated fibril growth results in a massive accumulation of amyloid deposits. It should be noted that we do not argue for a direct correspondence of the acidic fibrils to the first stage fibrils in patients but for the similarity of the two types of fibrils with respect to the low stability under the neutral pH conditions. Although the mechanism of the initial nucleation step in patients remains unknown, the maturation of fibrils via repeated self-seeding is likely to be involved in the development of dialysis-related amyloidosis.

Distinct Amyloid Fibrils with One Protein—As a second insight into the formation of amyloid fibrils, the present results show that β2-m can form distinct amyloid fibrils depending on the conditions. Previously, the depolymerization of acidic amyloid fibrils under physiological conditions has been puzzling, given the deposition of β2-m amyloid fibrils at neutral pH in patients (33). Although the participation of additional factors stabilizing the amyloid fibrils is evident, the present findings suggest that, by repeating the self-seeding, the β2-m amyloid fibrils can adapt themselves to the neutral pH conditions and transform into matured fibrils that are stable even in the absence of additional factors. On the other hand, the fibrils adapted to pH 7.0 are unstable at pH 2.5, which is the opposite situation to that of the pH 2.5-adapted fibrils.

Recently, it has been argued that one amyloidogenic protein or peptide can lead to distinct amyloid fibrils and that a particular type of fibril can be propagated by seeding (34–36). This has been used to explain the distinct phenotypes of prion diseases (37). Although electron microscopy images cannot distinguish the acidic and neutral pH forms of fibrils, the rate of fibril extension and the pH-dependent stability can clearly reveal the two types of amyloid fibrils. The conformational difference between the two types of fibrils remains unknown, although the difference may not be extensive.

Role of SDS in Fibril Formation—In the present experiments, a low concentration of SDS was essential. SDS has been used to stabilize the pH 2.5 seeds as well as to stabilize the fibrils formed at pH 7.0 (30). Additionally, SDS has been assumed to be important to destabilize the compact and β-sheet-rich native conformation at a neutral pH, which may resist the conversion to the fibrillar conformation (30). The importance of the destabilization of the native form has been argued for various proteins, including β2-m (3–10, 38). Contrary to our expectation, overall stability as measured by urea-induced unfolding was not affected by the presence of 0.5 mM SDS. Even a slight increase in stability in 0.5 mM SDS was observed by the thermal unfolding transition measured by differential scanning calorimetry.[2] More importantly, the inability of the V9P mutant to form amyloid fibrils in the absence of SDS clearly shows that destabilization of the native conformation is not enough to form the fibrils. A similar observation suggesting the participation of additional factors was reported for the N17D mutant of β2-m with decreased stability (14).

Our results argue that the oligomer of structurally perturbed β2-m is important to promote seed-dependent fibril growth under physiological conditions. SDS has been well known to bind to various kinds of proteins (39), producing, above the CMC, a stable complex of SDS micelles and proteins in which the proteins tend to denature and assume an α-helical conformation. There have been several reports that SDS at a concentration lower than the CMC value induces the aggregation of proteins and peptides (28). Furthermore, SDS has been reported to induce amyloid-like fibrils of a peptide from human complement receptor I (29). Below the CMC, the complexes between the proteins and SDS are unstable with an exposed hydrophobic surface, resulting in the additional intermolecular association of proteins via hydrophobic interactions. Intriguingly, the peptides tend to assume a β-sheet conformation upon interaction with SDS at concentrations below the CMC (40).

There have been reports that the formation of oligomers or spherical aggregates precedes spontaneous fibril formation without seeding (14, 24, 41, 42). The accumulation of spherical aggregates was observed preceding the fibril formation of β2-m under acidic conditions with agitation (14). On the other hand, Cu^{2+} has been indicated to mediate the oligomer formation of native-like precursors of β2-m, which precedes the amyloid formation at pH 7.4 (24). Although the exact mechanism of oligomer-assisted fibril elongation as observed here remains unknown, it is likely that oligomers can facilitate the interaction of monomers, with the growing ends of fibrils taking advantage of clustered binding sites.

[2] J. Kardos, H. Naiki, and Y. Goto, unpublished data.

Conclusions—We showed that several cycles of self-seeding at pH 7.0 resulted in rapid and extensive β2-m fibril growth, suggesting that the maturation of fibrils is an important part of the molecular mechanism of amyloidosis. Although the initial phase of the deposition proceeds slowly because of the difficulty in forming fibrils and a possible low stability of amyloid fibrils, the adaptation of fibrils to pH 7.0 conditions through repeated self-seeding can optimize the conformation with time, producing stable fibrils that can exist without additional factors. This may result in the accelerated development of amyloid deposits at the later stage of amyloidosis. We consider that this seeding-dependent maturation of amyloid fibrils is another manifestation of the intrinsic conformational properties of amyloid fibrils, *i.e.* the same amyloidogenic protein can assume distinct amyloid fibrils. In the case where the free energy barriers between distinct amyloid fibrils are high, the respective amyloid conformations propagate persistently even between distinct species as proposed for prion diseases (34–37). On the other hand, when the free energy barriers are not so high, the fibrils can adapt themselves to the environmental conditions resulting in maturation. These contrasting aspects of amyloid fibrils will help our understanding of the molecular pathology of amyloidosis.

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Seeding-dependent Maturation of β_2-Microglobulin Amyloid Fibrils at Neutral pH

Miho Kihara, Eri Chatani, Miyo Sakai, Kazuhiro Hasegawa, Hironobu Naiki and Yuji Goto

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