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Optimum Amyloid Fibril Formation of a Peptide Fragment Suggests the Amyloidogenic Preference of β_2-Microglobulin under Physiological Conditions*

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β_2-Microglobulin (β_2m) is a major component of amyloid fibrils deposited in patients with dialysis-related amyloidosis. Although full-length β_2m readily forms amyloid fibrils *in vitro* by seed-dependent extension with a maximum at pH 2.5, fibril formation under physiological conditions as detected in patients has been difficult to reproduce. A 22-residue K3 peptide of β_2m, Ser^{20}–Lys^{41}, obtained by digestion with *Acromobacter* protease I, forms amyloid fibrils without seeding. To obtain further insight into the mechanism of fibril formation, we studied the pH dependence of fibril formation of the K3 peptide and its morphology using a ThT fluorescence assay and electron microscopy, respectively. K3 peptide formed amyloid fibrils over a wide range of pH values with an optimum around pH 7 and contrasted with the pH profile of the seed-dependent extension reaction of full-length β_2m. This suggests that once the rigid native-fold of β_2m is unfolded and additional factors triggering the nucleation process are provided, full-length β_2m discloses an intrinsic potential to form amyloid fibrils at neutral pH. The fibril formation was strongly promoted by dimerization of K3 through Cys^{25}. The morphology of the fibrils varied depending on the fibril formation conditions and the presence or absence of a disulfide bond. Various fibrils had the potential to seed fibril formation of full-length β_2m accompanied with a characteristic lag phase, suggesting that the internal structures are similar.

Amyloid fibrils are recognized as being associated with the pathology of more than 20 serious human diseases and the responsible peptides or proteins specific to these diseases have been identified (1–5). These fibrils are characterized by a cross-β structure where β-strands are perpendicularly oriented to the axis of the polymeric fibril (6–8). Moreover, various proteins and peptides that are not related to diseases can also form amyloid-like fibers, implying that formation of amyloid fibrils is a general property of polypeptides (7, 9–14). Clarifying the mechanism of amyloid fibril formation is essential not only for understanding the pathogenesis of amyloidosis but also for improving our understanding of the mechanism of protein folding.

Dialysis-related amyloidosis is a common and serious complication among patients on long term hemodialysis (15, 16), in which β_2-microglobulin (β_2m)[1] forms amyloid fibrils. Native β_2m, made of 99 amino acid residues, corresponds to a typical immunoglobulin domain (Fig. 1) and is a component of the type I major histocompatibility antigen (17–19). Although the increase in β_2m concentration in blood over a long period is the most critical risk factor causing amyloidosis, the molecular details remain unknown. Recently β_2m, because of its relatively small size, which makes it suitable for physicochemical studies, has been used as a target for extensive studies addressing the mechanism of amyloid fibril formation in the context of protein conformation (20–28). We have studied the mechanism of fibril formation and their conformation with fibrils prepared by a seed-dependent extension reaction (29–36). Intriguingly, the seed-dependent reaction has an optimum at pH 2.5 (37, 38), which is far from the physiological pH at which amyloid fibrils are deposited in patients.

In many amyloidogenic proteins, short peptides called minimal or essential sequences can form amyloid fibrils by themselves (7, 9, 39–41). We found that a 22-residue K3 peptide, Ser^{20}–Lys^{41} (Fig. 1), obtained by digestion of β_2m with *Acromobacter* protease I, forms amyloid fibrils (30). Recently, we further identified an 11-residue peptide, Asn^{21}–His^{31}, in the K3 region that also forms amyloid fibrils (42). On the other hand, Jones *et al.* (43) reported that another region, Asp^{59}–Thr^{71}, forms amyloid fibrils.

The minimal sequence provides various pieces of information useful for addressing amyloid fibril formation. It is likely that the minimal sequence includes the initiation site for amyloid fibril formation of the whole molecule. Moreover, it may constitute the core of the consequent amyloid fibrils. We found under conditions without agitation that although efficient fibril formation of whole β_2m at pH 2.5 requires seeding, K3 peptide can form amyloid fibrils spontaneously, suggesting that the free energy barrier for fibril formation by the K3 peptide is lower than that of whole β_2m (30). In this paper, to obtain further insight into the mechanism of amyloid fibril formation of β_2m, we studied the pH dependence and role of the disulfide bond on amyloid fibril formation of the K3 peptide. The results show that an optimum for K3 fibril formation exists at neutral pH, suggesting that, although unfolding of the rigid native-fold and additional factors triggering the nucleation process are

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[1] The abbreviations used are: β_2m, β_2-microglobulin; Me_2SO, dimethyl sulfoxide; K3 peptide, a 22-residue peptide Ser^{20}-Lys^{41} of β_2m; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid.

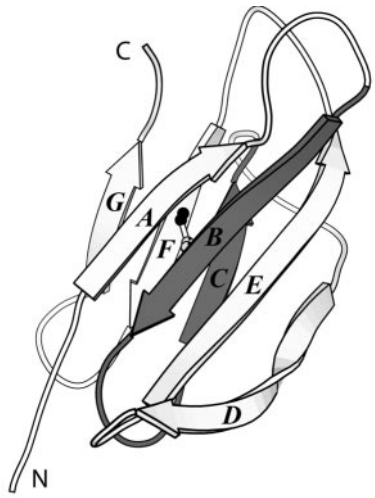

FIG. 1. **Structure of β_2m and amino acid sequence of K3 peptide.** The location of the K3 peptide is indicated by *shading*. The diagram was created using Molscript (55) with the structure reported by Bjorkman *et al.* (17).

necessary, the intrinsic amyloidogenic preference of β_2m has an optimum at neutral pH.

EXPERIMENTAL PROCEDURES

Recombinant β_2m and K3 Peptide—Recombinant human β_2m was expressed in the methylotrophic yeast, *Pichia pastoris,* and purified as described (29, 30). Three fractions with 6 (Glu-Ala-Glu-Ala-Tyr-Val-), 4 (Glu-Ala-Tyr-Val-), and 1 (Val-) additional amino acid residues added to the N-terminal (Leu) of intact β_2m were obtained. These additional residues were derived from the signal sequence in the expression vector. The second peak with 4 additional amino acid residues was the major peak, and this fraction was used as the intact β_2m.

K3 peptide was obtained by digestion of β_2m with lysyl endopeptidase from *Achromobacter lyticus* (*Achromobacter* protease I, Wako Pure Chemical, Osaka, Japan) as described previously (30). For preparation of the K3 peptide, we used a crude β_2m fraction made of a mixture of species with different N-terminals. The K3 peptide has a cysteine residue, Cys^{25} (Fig. 1). Alkylated K3, in which Cys^{25} was alkylated by iodoacetamide, was prepared by incubating 100 μM K3 with 10 mM iodoacetamide in 50 mM Tris-HCl buffer at pH 9.0 for 1 h. A K3 dimer with a disulfide bond between Cys^{25} was prepared by air oxidation for 3 days in the same buffer. Both K3 derivatives were separated by reverse phase high performance liquid chromatography (30).

Polymerization Assay—Fibril formation of K3 and its derivatives were performed at 37 °C using a final peptide concentration of 100 μM for the K3 monomers and 50 μM for the K3 dimer. The buffers used were glycine-HCl (pH 1.5–3.0), sodium acetate (pH 3.5–5.5), MES-NaOH (pH 6.0–7.0), and Tris-HCl (pH 7.5–8.5). The concentration of the buffers was 50 mM and all contained 100 mM NaCl. The lyophilized K3 and its derivatives were dissolved in 100% Me_2SO and then diluted into the buffer solutions. The final concentration of Me_2SO was less than 1% (v/v). We confirmed that 1% (v/v) Me_2SO does not affect the standard seed-dependent extension reaction with intact β_2m. For seeding experiments, the sonicated K3 fibrils at a concentration of 5 μg/ml were used as seeds.

Amyloid fibril formation of the full-length β_2m was carried out using a standard seed-dependent extension at 25 μM β_2m at 37 °C (37, 38). It must be noted that the protein solution was not agitated during the standard extension reaction. The seed fibrils of full-length β_2m used were the 6th generation from original fibrils purified from patients and were extended with monomeric recombinant β_2m.

ThT Fluorescence Assay—The polymerization reaction was monitored by fluorometric analysis with ThT at 25 °C as described previously (37). The excitation and emission wavelengths were 445 and 485 nm, respectively. From each reaction tube, 5 (for time course measurements) or 7.5 μl (for pH-dependence measurements) was taken and mixed with 1.5 ml of 5 μM ThT in 50 mM glycine-NaOH buffer (pH 8.5) and the fluorescence of ThT was measured using a Hitachi fluorescence spectrophotometer, F4500. We confirmed that the pH of the ThT assay solution is not affected significantly by the addition of the protein solution at acidic pH.

Transmission Electron Microscopy—Reaction mixtures (2.5 μl) were diluted with 25 μl of distilled water. These diluted samples were spread on carbon-coated grids and were allowed to stand for 1 to 2 min before excess solution was removed with filter paper. After drying the residual solution, the grids were negatively stained with 1% phosphotungstic acid (pH 7.0) and once more excess solution on the grids was removed by filter paper and dried. These samples were examined under a Hitachi H-7000 electron microscope with an acceleration voltage of 75 kV.

CD—Far UV CD measurements were carried out with a Jasco spectropolarimeter, J-720, at 20 °C using a cell with a light path of 1 mm. The protein concentration was 25 μM and the results are expressed as the mean residue ellipticity [θ] (deg cm^2 $dmol^{-1}$).

RESULTS

pH Dependence of K3 Fibril Formation—In the present study the fibril formations were examined at various pH conditions, and the amounts of fibril formed were determined from ThT binding using the standard method at pH 8.5 (see "Experimental Procedures"). Although fibrils prepared at different pH conditions might have different ThT binding capabilities, thus producing some uncertainty in estimating the amounts, we consider that the present method is the most reliable available. Purified and lyophilized K3 peptide was difficult to completely dissolve in water under acidic pH conditions, exhibiting turbidity and a high background as monitored by ThT fluorescence. Among the various solvent conditions examined, we found that a high concentration of Me_2SO, which was also useful for dissolving β_2m amyloid fibrils (31, 44), could completely dissolve the K3 peptide. Therefore, we used 100% Me_2SO to prepare a stock solution of the K3 peptide, which was mostly used within the same day.

Under acidic pH conditions, full-length recombinant β_2m forms amyloid fibrils by the seed-dependent extension reaction with an optimum at pH 2.5 (29, 37, 38). We observed no fibril formation without seeding even after incubating for several days. However, it has been shown that agitation can induce fibril formation of β_2m without seeding (45). In accordance with this, we confirmed that agitation of the solution with seeds further accelerated seed-dependent fibril formation.[2] In contrast, the K3 peptide formed amyloid fibrils spontaneously at pH 2.5 (30). The kinetics depended on peptide concentration whereby the higher the peptide concentration the faster the reaction. At 100 μM K3, a lag phase of 3 h was observed and the reaction ended after about 6 h incubation (Fig. 2*A*). The lag phase shortened with increasing K3 concentration, such that the overall reaction accelerated (data not shown; see Fig. 3*B* of Ref. 30). Although it is likely that agitation also accelerates fibril formation of the K3 peptide, all the experiments were carried out without agitation, where most of the reactions ended after 24 h (Fig. 2).

We examined the pH dependence of the spontaneous fibril formation of the K3 peptide at 100 μM as monitored by ThT fluorescence (Fig. 3*A*). Because most of the reactions ended at 24 h (Fig. 2), the profile was constructed by measuring the fibril formation at 24 h. Intriguingly, the ThT fluorescence intensity after 24 h incubation increased gradually with increasing pH to a maximum at pH 7.5. At pH values above 7.5, a sharp drop in ThT fluorescence was noted. A time course of fibril formation showed a lag phase for all the pH values examined (Fig. 2).

The Role of the Disulfide Bond—K3 peptide has a free thiol group at Cys^{25}, such that it can form a homodimer with an intermolecular disulfide bond. Disulfide bond formation by air oxidation is promoted by increasing pH. Thus, it is likely that

[2] Y. Ohhashi and Y. Goto, unpublished results.

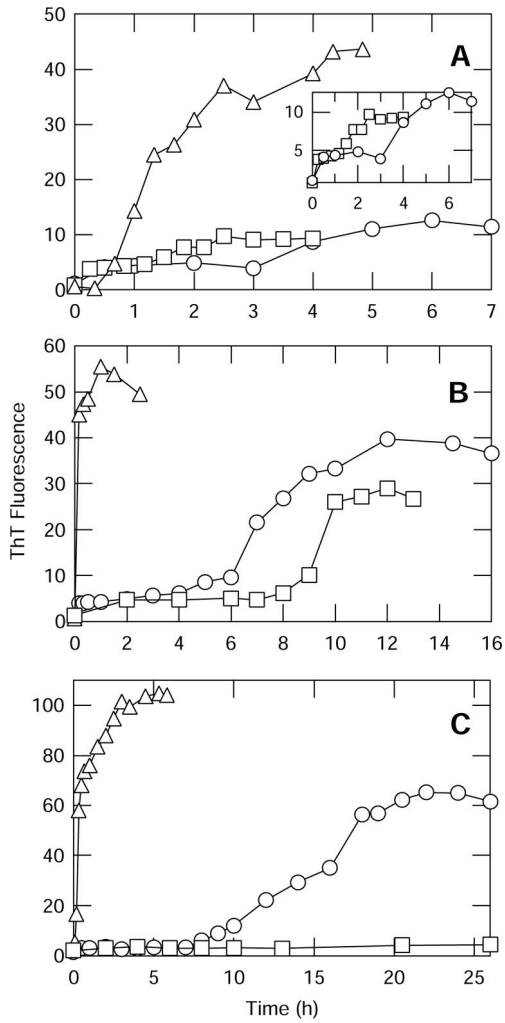

FIG. 2. **Fibril formation of K3 and its modified peptides at pH 2.5 (*A*), 6.0 (*B*), and 7.5 (*C*) at 37 °C.** Kinetics of spontaneous fibril formation of K3 (*circles*), K3 in the presence of 10 mM DTT (*squares*), and dimeric K3 (*triangles*) were monitored by fluorometric analysis with ThT. The K3 peptide concentrations were 100 μM for the monomer and 50 μM for the dimer. The inserted figure in *panel A* enlarges the *ordinate* to clarify the kinetics of K3 (*circles*) and K3 in the presence of 10 mM DTT (*squares*).

disulfide bond formation occurs during incubation of K3 under neutral and alkaline conditions, although in our study we started the reaction with monomeric K3 that was purified by high performance liquid chromatography. In fact, analysis of K3 fibrils prepared in alkaline pH regions by high performance liquid chromatography revealed a significant amount of dimer formation during incubation (*i.e.* 50% at pH 7.5). A small fraction of dimer was observed even for fibrils prepared in weakly acidic pH conditions (data not shown).

To analyze fibril formation of the K3 monomer separated from the dimer, we added 10 mM DTT to the reaction mixture. Between pH 2.0 and 7.0, the profile of the ThT fluorescence intensity after 24 h (Fig. 3*B*) was similar to that without DTT (Fig. 3*A*). Consistent with this, both at pH 2.5 and 6.0, the kinetics of fibril formation did not depend largely on the presence or absence of DTT, although a small change of lag time was observed (Fig. 2, *A* and *B*). On the other hand, the pH profile of the ThT fluorescence after 24 h showed a considerable difference between pH 7.0 and 8.0 (Fig. 3*B*). The kinetics of fibril formation at pH 7.5 showed that addition of DTT suppressed fibril formation significantly (Fig. 2*C*). These results indicated that at pH 7.5 the fibrils are formed predominantly by a disulfide bonded dimer. The pH profile of the fibril formation of monomeric K3 peptide in 10 mM DTT revealed a maximum at pH 5.5–6.5.

To confirm the role of the disulfide bond, the K3 dimer was prepared by air oxidation, purified by high performance liquid chromatography, and fibril formation was examined (Fig. 3*D*). As anticipated, ThT fluorescence after 5 h showed a dramatic increase. The pH region showing strong ThT fluorescence extended widely from pH 2 to 8, with an optimum at pH 6.0–7.5. The maximal ThT value at pH 7.5 was about 2-fold larger than that of the K3 monomer, although we used the same peptide concentrations with respect to the monomer: 100 μM for the K3 monomer and 50 μM for the K3 dimer. ThT fluorescence was minimum at pH 3, and increased slightly at pH values below 3. The kinetics of fibril formation showed that the lag phase disappeared at pH 6.0 and 7.5 and a shortened lag phase of 30 min was observed at pH 2.5 (Fig. 2). The rapid kinetics at pH 6.0 and 7.5 resemble the standard fibril extension reaction of the full-length β_2m at 25 μM and pH 2.5, which was completed in 2 h (29, 30). Thus, formation of the disulfide bond accelerated fibril formation as well as increasing the amount of fibrils monitored by the ThT fluorescence binding at pH 8.5.

As a control, we prepared an alkylated K3 peptide in which the thiol group of Cys^{25} was alkylated by iodoacetamide. Below pH 5.0, fibril formation of the alkylated K3, as monitored by ThT fluorescence (Fig. 3*C*), was similar to that of K3 in 10 mM DTT (*i.e.* K3 with a free thiol) (Fig. 3*B*). However, no increases in ThT fluorescence above pH 6.5 were observed, suggesting that the acetamide group introduced steric hindrance preventing the formation of tightly packed amyloid fibrils above pH 6.5. The results also suggested the presence of at least two types of fibrils made of monomeric K3 peptide. The first, with thiol groups exposed to the solvent, was mainly formed at pH values below 6.5 and was not affected by alkylation of the thiol groups. The other, with thiol groups buried and formed above pH 6.5, could not be formed once the thiol group was alkylated because of steric hindrance introduced by the alkyl groups. On the other hand, alkylation of Cys^{25} prevents its ionization. It is possible that the prevention of ionization also contributes to the reduced amyloidogenicity above pH 6.5, although the details are unknown.

Fibril Morphology—Figs. 4 and 5 show electron micrographs of fibrils of K3 and its derivatives prepared at pH 2.5 and 6.5, respectively. At pH 2.5, two types of monomer K3 fibrils were mainly observed. One of the monomer K3 fibrils (Fig. 4*F*) was similar to fibrils of intact β_2m prepared at pH 2.5 (Fig. 4*E*), *i.e.* twisted thick fibrils with maximal and minimal diameters of 16.5 and 10 nm, respectively, and relatively short in length. The other was clustered thin filaments with a variety of diameters (Fig. 4*G*). The overall morphology of K3 (Fig. 4*A*), K3 in the presence of 10 mM DTT (Fig. 4*B*), and alkylated K3 fibrils (Fig. 4*C*) appeared similar to each other. Under these conditions, clustered filaments predominated and the fraction of thick filament was low.

At pH 6.5, the monomer K3 fibrils observed (Fig. 5, *A–C*) were again categorized into two types: one with a diameter of ~10 (Fig. 5*E*) and the other with a diameter of about 5.5 nm (Fig. 5*F*). The former had a slightly twisted morphology with a longitudinal periodicity of about 60 nm, whereas the twist was not clear in the latter. The former are likely to consist of two latter filaments judging from their observed diameters. In general, the length of the fibrils prepared at pH 6.5 was longer than fibrils prepared at pH 2.5.

The K3 dimer produced thin fibrils both at pH 2.5 (Fig. 4*D*) and 6.5 (Fig. 5*D*), whereas the fibril length was shorter (100–200 nm) than those of the K3 monomer fibrils at both pH

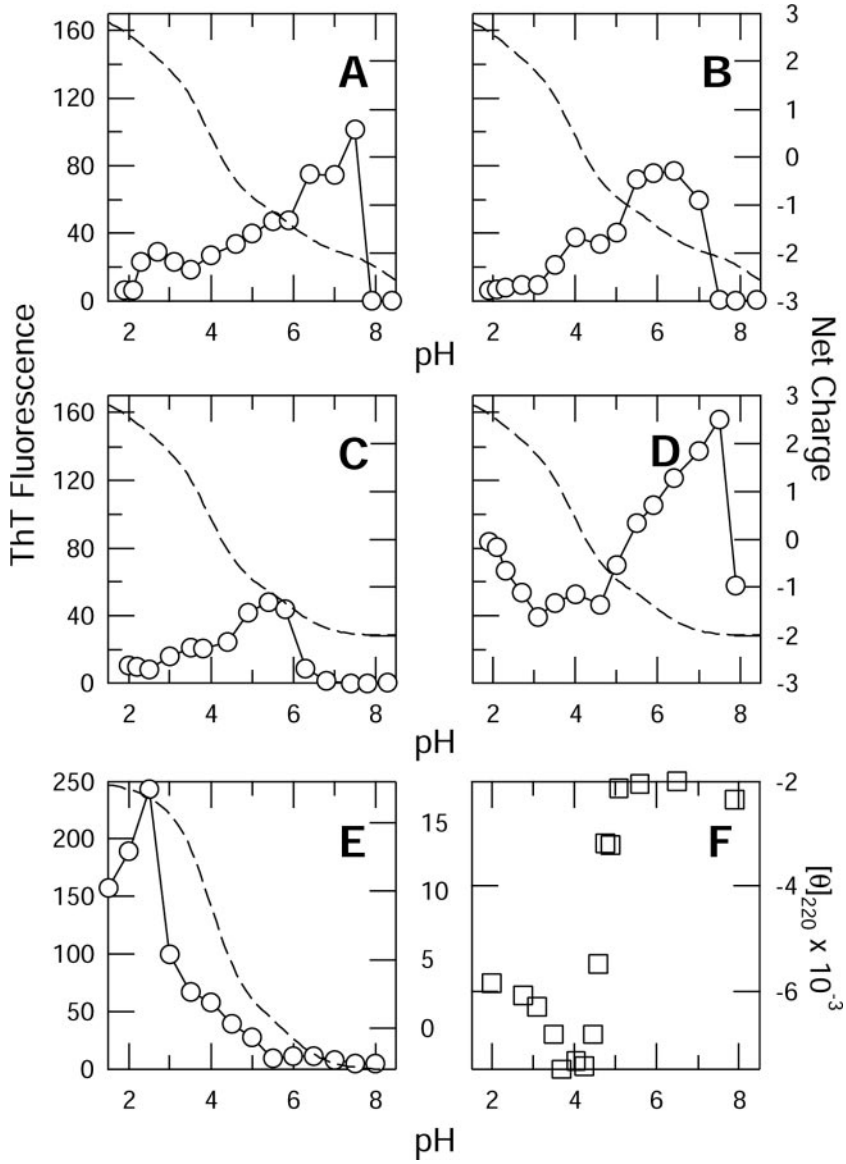

FIG. 3. **pH dependence of fibril formation of K3 and its modified peptides at 37 °C.** ThT fluorescence assay after 24 h incubation of 100 μM K3 in the absence (*A*) and presence (*B*) of 10 mM DTT, alkylated K3 (*C*) and after 5 h of 50 μM disulfide-bonded K3 dimer (*D*). *E*, for comparison, ThT fluorescence after 4 h of seed-dependent extension reaction of 25 μM intact β_2m is shown. In *A–E*, pH dependence of the net charge is indicated by *broken lines* (*right ordinate*). *F*, acid denaturation curve of intact β_2m monitored by the ellipticity at 220 nm.

values. The kinetics of fibril formation at pH 6.0 and 7.5 (Fig. 2, *B* and *C*) was rapid without a lag phase, similar to the standard fibril extension reaction of the full-length β_2m, suggesting that the K3 dimer had a strong potential for initiating fibril formation. It is likely that the shorter length of the fibrils produced by the K3 dimer is related to its strong potential of fibril formation.

Seeding Effects—In a previous paper (30), we showed that the K3 fibrils prepared at pH 2.5 work directly as seeds in the fibril formation of the K3 peptide at the same pH (*i.e.* the homogeneous extension of K3 with the K3 seeds). Upon addition of preformed K3 fibrils to the K3 peptide at pH 2.5, the ThT fluorescence increased smoothly without a lag phase, in contrast to the fibril formation of the K3 peptide alone with a lag phase of several hours (see Fig. 6 of Ref. 30). Here, we examined if the same is true of the fibrils prepared at neutral pH.

K3 seeds were prepared by sonicating the K3 fibrils extended at pH 6, and fibril formation was examined at pH 6.0 in the presence of seeds (Fig. 6*A*). The lag phase disappeared, indicating the seeding effects of preformed K3 fibrils. The absence of a lag phase suggested a smooth homogeneous extension reaction without a high energy barrier (30). Similar experiments were carried out at pH 7.5, where the reduced K3 cannot form fibrils spontaneously (Fig. 6*B*). In the presence of 10 mM DTT at pH 7.5, although no fibril formation was detected for 26 h, a gradual increase of ThT fluorescence was observed in the presence of K3 seeds, indicating that seeding enables fibril formation even for the reduced K3 at pH 7.5. As described before, in the absence of DTT and seeds, an increase in ThT fluorescence occurred with a lag phase (*open circles* in Fig. 6*B*, see also Fig. 2*C*) because of the slow oxidation of thiol groups and consequent fibril formation. Under these conditions, we observed acceleration of fibril formation by seeding (*solid circles* in Fig. 6*B*). The difference of the reaction curves in the absence and presence of DTT (*i.e. line with solid circles*, *line with solid squares*) arises from the contribution to fibril formation of disulfide-bonded K3, which occurs with time at pH 7.5.

We also examined cross-reaction between K3 fibrils and full-length β_2m by the standard fibrils extension reaction at pH 2.5 (Fig. 6*C*). Without agitation, full-length β_2m requires seed fibrils for smooth amyloid fibril formation at pH 2.5, where β_2m is substantially unfolded (29, 33). Although no reaction occurred in the absence of seeds, fibril extension proceeded rapidly in the presence of β_2m seeds and was completed in 3 h. We previously reported that K3 fibrils prepared at pH 2.5 work as seeds in the extension reaction of β_2m at 35 μM and pH 2.5, although the lag phase of about 10 h still remained (30). The

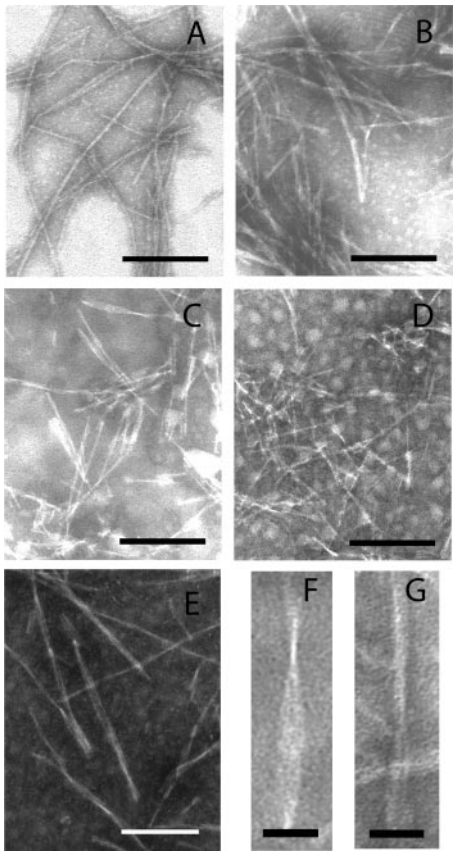

FIG. 4. **Electron micrographs of the amyloid fibrils of K3 and its modified peptides prepared at pH 2.5.** *A*, K3; *B*, K3 in 10 mM DTT; *C*, K3 alkylated with iodoacetamide; and *D*, K3 dimer. *E*, for comparison, electron micrograph of amyloid fibrils of intact β_2m prepared at pH 2.5 is shown. The *bar* in *A–E* indicates a length of 200 nm. *F* and *G*, enlarged images of K3 fibrils taken from *C* (*F*) and *A* (*G*). The *bars* in *F* and *G* indicate a length of 40 nm.

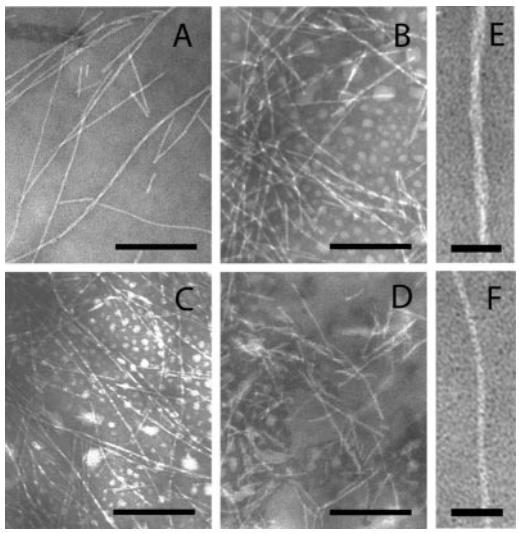

FIG. 5. **Electron micrographs of the amyloid fibrils of K3 and its modified peptides prepared at pH 6.5.** *A*, K3; *B*, K3 in 10 mM DTT; *C*, K3 alkylated with iodoacetamide; and *D*, K3 dimer. The *bar* indicates a length of 200 nm. *E* and *F*, enlarged images of K3 fibrils taken from *A*. The *bars* in *E* and *F* indicate a length of 40 nm.

remaining lag phase suggested the difficulty of heterogeneous reaction between K3 seed and monomeric β_2m, in contrast to the homogeneous extension of the K3 seed with monomeric K3. Here, we examined whether or not the K3 fibrils prepared at

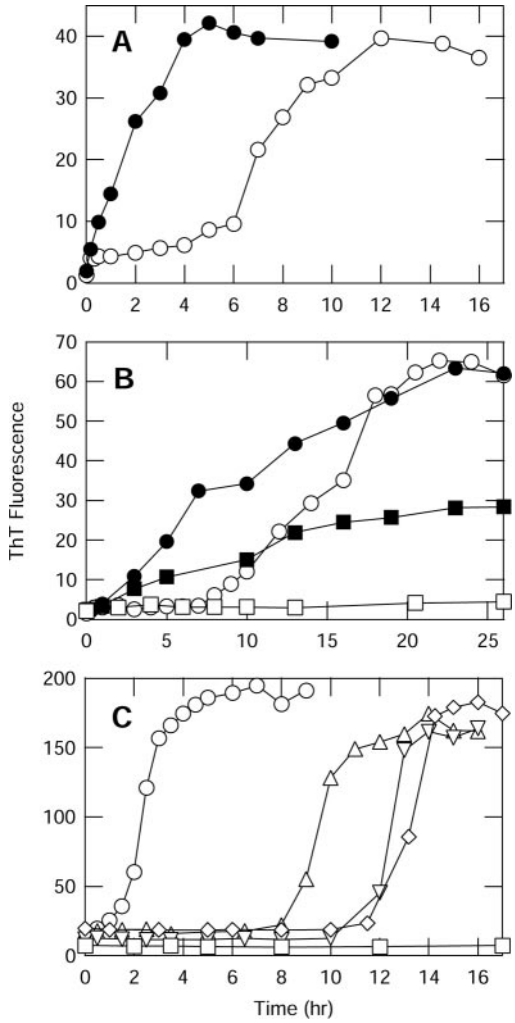

FIG. 6. **Cross-reactions of amyloid fibril formation between intact β_2m and K3 peptide by seeding experiments.** *A*, kinetics of fibril formation of the K3 monomers (100 μM) at pH 6.0 with K3 seeds (5 μg/ml) prepared at pH 6.0 (*filled circles*). Kinetics without seeds (*open circles*), taken from Fig. 2*B*, is also shown. *B*, kinetics of fibril formation of the K3 monomers (100 μM) at pH 7.5 with K3 seeds (5 μg/ml) prepared at pH 7.5 (*filled circles*, *filled squares*). Kinetics without seeds (*open circles*, *open squares*), taken from Fig. 2*C*, are also shown. The reactions were carried out in the absence (*filled circles*, *open circles*) or presence (*filled squares*, *open squares*) of 10 mM DTT. *C*, fibril formation of intact β_2m (25 μM) with intact β_2m seeds (*circles*), with K3 seeds prepared at pH 2.5 (*triangles*), with K3 seeds extended at pH 6.5 (*inverted triangles*), with dimeric K3 seeds extended at pH 7.5 (*diamonds*), and without seeds (*squares*). The concentration of seeds was 5 μg/ml.

neutral pH were able to work as seeds for formation of the β_2m fibrils at pH 2.5.

First, K3 seeds prepared at pH 2.5 were added to a solution of monomeric β_2m at pH 2.5. Fibril formation of β_2m with a lag phase of 8 h was observed in the presence of K3 seeds (Fig. 6*C*), which is consistent with previous results (30). The K3 seeds prepared at pH 6.5 also showed the seeding effect, although it was accompanied by a slightly longer lag phase of 10 h (Fig. 6*C*). K3 dimer seeds prepared at pH 7.5 seeded β_2m fibril formation to an extent similar to K3 monomer seeds prepared at pH 6.5. These results indicate that fibrils of K3 although they were formed at different pH conditions have similar internal structures, even though they are not the same, which makes seeding of β_2m fibrils possible.

DISCUSSION

pH Dependence of Fibril Formation—For many amyloidogenic globular proteins, destabilization of the native globular

state by either mutations or introduction of unstable conditions is highly correlated with formation of amyloid fibrils, suggesting that denaturation or unfolding of the native state is a critical event in triggering amyloid formation (46–48). In other words, denaturation of the native state is assumed to be a necessary condition of amyloid fibril formation although it is not a sufficient condition in itself. Moreover, because the nonnative state is an intermediate or precursor of amyloid fibril formation, the conformational propensity of the denatured state is likely to be a determinant for amyloidogenicity. Consistent with this, studies with various mutants of acylphosphatase have demonstrated that hydrophobicity and β-sheet propensity of key regions, which are distinct from those parts important for protein folding, as well as the net charge of the protein, are critical factors for aggregation (49). These intrinsic properties are proposed as being key factors in determining aggregation rates of various proteins and peptides (50).

Naiki *et al.* (37) established with β_2m that amyloid fibrils similar to those purified from patients can be formed *in vitro* by the seed-dependent extension reaction at pH 2.5. Because β_2m is substantially unfolded at pH 2.5 (Fig. 3*F*), the optimal fibril formation at pH 2.5 (Fig. 3*E*) is consistent with the idea that unfolding or destabilization of the native state is required to initiate fibril formation. However, in patients with dialysis-related amyloidosis, amyloid fibrils are formed under physiological conditions at neutral pH. Although an increased concentration of β_2m is the most critical risk factor, other factors that destabilize the structure of β_2m and, moreover, trigger the fibril formation are not elucidated, and the mechanism of how amyloid fibrils are formed under physiological conditions remains unknown. Importantly, several reports have proposed the *in vitro* formation of β_2m amyloid fibrils under physiological conditions (20, 23, 27).

In the present paper, we used the K3 peptide, a 22-residue proteolytic peptide, to explore the mechanism of amyloid fibril formation under physiological conditions. K3 peptide can form amyloid fibrils without seeding, indicating that the free energy barrier for the nucleation process of K3 fibril formation is lower than that of the whole β_2m molecule. The pH dependence of K3 fibril formation revealed that pH 2.5, which is the optimal pH for the seed-dependent extension reaction of full-length β_2m (Fig. 3*E*), is not optimal for K3. Instead, a maximum was observed at pH 7.5 for the K3 peptide without DTT (Fig. 3*A*). Consideration of the role of the disulfide bond revealed that reduced and oxidized K3 peptides have optimal fibril formation at pH 6 and 7.5, respectively, and that the disulfide bond accelerates fibril formation significantly. Thus, it is likely that the optimal pH for fibril formation of unfolded β_2m is also close to physiological conditions. One of the most important obstacles for fibril formation of whole β_2m at neutral pH is the folding to the native state. Moreover, it is likely that additional factors are required to trigger fibril formation considering the high free energy of the nucleation process. The present results imply that, once unfolded and the additional factors are provided, whole β_2m has an optimal potential to form amyloid fibrils at neutral pH.

Recently, Radford and co-workers (27, 28) studied the relationship between structural stability and amyloid formation with various mutants of β_2m. They indicated that, whereas destabilization of the native state is important for the generation of amyloid fibrils, the population of specific denatured states is a pre-requisite for amyloid formation. They proposed that perturbation of the N- and C-terminal edge strands (*i.e.* βA and βG) is an important feature in the generation of assembly-competent states of β_2m (27). The unpairing of βA strand has also been suggested by other groups (18, 20) to be a critical event leading to the amyloidogenic partially unfolded intermediates. These results are basically consistent with ours in that β_2m has an intrinsic potential to form amyloid fibrils under physiological conditions once the rigid native structure is destabilized. We also prepared various mutants of β_2m, in which proline was introduced to each of the β-strands (36). These mutations affected the seed-dependent amyloidogenic potential at pH 2.5 to various degrees. The amyloidogenicity of mutants showed a significant correlation with stability of the amyloid fibrils, and little correlation was observed with that of the native state, indicating that stability of the amyloid fibrils is an additional key factor determining the amyloidogenic potential of the proteins.

To obtain further insight, we considered the net charge of the β_2m and K3 peptide (Fig. 3). K3 peptide has three positive (*i.e.* α-amino group, His^{31}, and Lys^{41}) and five negative (*i.e.* Cys^{25}, Asp^{34}, Glu^{36}, Asp^{38}, and α-carboxyl group) titratable groups, and its isoelectric point, which is calculated on the basis of their pK_a values, is around pH 4.5 (Fig. 3). The net charge of the Cys-reduced K3 peptide at pH 6 is −1 and −2 at pH 7. Although slightly different, the net charge profiles of other K3 derivatives are similar to that of the reduced K3 peptide. Under acidic and basic pH regions, repulsion between the charged residues becomes a barrier for forming fibrils. On the other hand, under optimal pH conditions for fibril formation of K3, the net charge is −1 to −2. López de la Paz *et al.* (7) showed with a series of amyloidogenic peptides that a net charge less than ±2 is one of the requisites for determining amyloidogenicity. Optimal fibril formation of the K3 peptide at pH 7 is consistent with their observations. However, the pH profile for fibril formation does not agree exactly with that of the net charge, suggesting that although the net charge is an important factor, additional factors determine the pH profile.

We also plotted the pH dependence of the net charge of the whole β_2m molecule (Fig. 3*E*). The protein is highly positively charged (*i.e.* +17) at pH 2.5. This is one of the reasons why β_2m is acid-unfolded at pH 2.5. Moreover, because of this high net charge, β_2m remains unfolded in the absence of seeds. The addition of seed affects the energetics, dramatically transforming β_2m to the fibrillar state even in the presence of high net charge repulsion. This suggests that, under favorable conditions, even peptides and proteins with a high net charge can form amyloid fibrils. Because the net charge of whole β_2m at neutral pH is close to zero, fibril formation at neutral pH is not surprising in the context of electrostatics. The intriguing observation in this context is the depolymerization of fibrils at pH 7 (38). Refolding of the monomeric β_2m to the native state is an important driving force for depolymerizing the fibrils at pH 7. Therefore, additional factors for stabilizing the fibrils, *e.g.* apolipoprotein E, are required to prevent depolymerization, thus promoting deposition of fibrils under physiological conditions (38). The depolymerization of K3 fibrils at pH 7, which were prepared at pH 2.5, was also noted (30). On the other hand, K3 fibrils prepared at pH 7 were stable at pH 2.5.[3] These results suggest that detailed structures of amyloid fibrils depend on the pH at which they were prepared. Whereas the basic folds of protofilaments are the same, the modes of association to form the mature fibrils might be different depending on pH. Further study is necessary to clarify the pH-dependent stability of amyloid fibrils.

Role of the Disulfide Bond—For several amyloidogenic proteins including prion, intermolecular disulfide bond formation has been suggested to be linked with amyloid fibril formation (51–54). Intriguingly, we observed a dramatic increase in amy-

[3] Y. Ohhashi, K. Hasegawa, H. Naiki, and Y. Goto, unpublished results.

loidogenicity by disulfide bond formation. The lag phase of the spontaneous fibril formation was shortened at pH 2.5 and disappeared at pH 6 and 7.5 (Fig. 2). The intensity of ThT fluorescence increased markedly for all pH values examined. The increase in amyloidogenicity because of disulfide bond formation has also been observed for an 11-residue peptide, Asn^{21}–His^{31}, in the K3 region (42). As measured by EM, amyloid fibrils of disulfide bonded K3 are short at both pH 2.5 and 6.5 compared with those of the reduced or alkylated K3 monomers (Figs. 4 and 5). This suggests that, although the disulfide bond promotes fibril formation, rapid and extensive reactions fail to cooperatively form longer fibrils.

Previously, we reported the role of the disulfide bond in conformation and amyloid fibril formation of intact β_2m (29). In amyloid fibrils of β_2m, more than 50% of amide protons mostly located at the central regions of the protein are highly protected from H/D exchange, which is distinct from the protected amide protons of the native state protein (31). However, the reduced β_2m, in which the disulfide bond between Cys^{25} and Cys^{80} (Fig. 1) is reduced, cannot form straight needle-like amyloid fibrils, although this molecule has both of the minimal sequence regions. Instead, the reduced β_2m forms flexible, thin filaments, suggesting that the mature fibril is made of several filaments. Recovery of rigid fibril formation of the K3 peptide (Ser^{20}–Lys^{41}) upon removal of non-essential regions indicates that regions other than the minimal region prevent fibril formation of the reduced β_2m. We have suggested that flexibility of non-core regions, which increases when the disulfide bond in reduced, inhibits formation of mature rigid fibrils (33). The increased amyloigodenicity of the dimeric K3 peptides connected by a disulfide bond indicate that linkage of the minimal region by the disulfide bond without introducing a non-essential flexible region contributes positively to fibril formation. The amyloid fibrils are formed by the intermolecular association of amyloidogenic peptides or proteins. Thus, dimer formation by the disulfide bond is anticipated to increase effectively the chance of intermolecular encounters by increasing the effective concentration of monomers, unless the disulfide bond introduces inhibitory effects by steric constraint. It is emphasized that in our case increased fibril formation resulted in formation of a large amount of short filaments.

Conclusion—Intact full-length β_2m forms amyloid fibrils *in vitro* by seed-dependent extension with a maximum at pH 2.5. However, pH 2.5 is far from the physiological conditions, and the validity of the results from the pH 2.5 experiments with regard to addressing the mechanism of fibril formation under physiological conditions has to be questioned. In the present paper, we showed that a 22-residue K3 peptide, which probably constitutes both the initiation and core region of the β_2m amyloid fibrils, forms amyloid fibrils with an optimum at around pH 7. This strongly suggests that, although the rigid native-fold of β_2m at neutral pH values prevents amyloid fibril formation, β_2m has an intrinsic amyloidogenic preference at neutral pH. Thus, once the native structure is destabilized under conditions where the seeds or yet unknown additional factors triggering the fibril formation are available, it is likely that β_2m starts to form amyloid fibrils in a manner similar to that observed at pH 2.5. Moreover, the present results exhibit a variety of fibril morphologies of K3 depending on pH and disulfide bond. Although different, the K3 fibrils can all promote fibril formation of the whole β_2m by seeding effects. The seeding effects varied and the K3 fibrils prepared under the same pH conditions were more effective, suggesting that, whereas the basic internal structures are common, slight differences in the higher order structures determines the efficiency of heterogeneous seeding.

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Optimum Amyloid Fibril Formation of a Peptide Fragment Suggests the Amyloidogenic Preference of β_2-Microglobulin under Physiological Conditions

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