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Amyloidogenic synthetic peptides of β2-microglobulin—a role of the disulfide bond

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Abstract

To search for the essential regions responsible for the β2-microglobulin (β2-m) amyloid fibril formation, we synthesized six peptides corresponding to six of the seven β-sheets in the native structure of β2-m, and examined their amyloidogenicity. Among the peptides examined, peptide (21–31) (strand B) and the mixture of peptide (21–31) and (78–86) (strand F) showed fibril formation at both pH 2.5 and 7.5. Peptide (21–31) is the N-terminal half of the previously reported proteolytic fragment of β2-m, Ser21–Lys41 (K3), suggesting that this region may be the essential core. Interestingly, the dimer formation of peptide (21–31) by the disulfide bond substantially facilitated the fibril formation, indicating that the disulfide bond is important for the structural stability of the fibrils.

Keywords: β2-Microglobulin; Amyloid fibril; Synthetic peptide; Disulfide bond

β2-Microglobulin-related (Aβ2M) amyloidosis is a common and serious complication in patients on long-term hemodialysis [1]. Intact β2-microglobulin (β2-m) is a major structural component of Aβ2M amyloid fibrils [2–5]. The mechanism of the deposition of these Aβ2M amyloid fibrils is still unknown. Although the retention of β2-m in the plasma appears to be prerequisite, other factors, such as the age of the patient, the duration of dialysis, and the type of dialysis membrane used, may also be involved [6–8].

We and other groups have proposed that a nucleation-dependent polymerization model could explain the general mechanisms of amyloid fibril formation in vitro, in various types of human as well as murine amyloidosis [9–14]. This model consists of two phases, i.e., nucleation and extension phases. The extension of Aβ2M amyloid fibrils, as well as the formation of the fibrils from β2-m are greatly dependent on the pH of the reaction mixture, with an optimum pH of around 2.0–3.0 [13–15]. On the other hand, we have demonstrated that Aβ2M amyloid fibrils readily depolymerize into monomeric β2-m at a neutral to basic pH [16]. Using circular dichroism and nuclear magnetic resonance spectroscopy, we and Radford's group have revealed a reversible change in the conformation of β2-m in the pH-dependent cycle of the polymerization and depolymerization of Aβ2M amyloid fibrils [14,16,17]. β2-m at pH 2.5 loses much of the secondary and tertiary structures observed at pH 7.5 [14,16]. Once incorporated into Aβ2M amyloid fibrils at pH 2.5, β2-m becomes highly rich in β-sheet structure and obtains the secondary and tertiary structures strikingly different from monomeric β2-m at both pH 7.5 and 2.5 [16,17]. A recent H/D exchange study of amide protons combined with nuclear magnetic resonance spectroscopy indicated that most of the residues in the middle region, including the loop regions in the native structure, form a rigid β-sheet core [17]. These results suggest that the β2-m molecule may

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consist of two regions, i.e., essential (or minimal) and non-essential regions for the fibril formation, and the non-essential regions may participate in the fibril formation passively following the initiative conformational change of the essential regions. Therefore, the search for the essential regions is important for the study of the folding kinetics of the fibril formation. We previously reported that the proteolytic fragments of β2-m, both peptides K3 (Ser21–Cys25–Lys41) and K3–K7 (Ser21–Cys25–Lys41 and Asp76–Cys80–Lys91 linked by the disulfide bond between Cys25 and Cys80) formed amyloid fibrils at pH 2.5 [18]. These regions may be candidates for the essential regions. To narrow down the essential region in the K3 peptide as well as to search for other regions responsible for Aβ2M amyloid fibril formation, we have synthesized six peptides, corresponding to six β-sheets (A, B, C, D, F, and G) out of seven β-sheets in the native structure of β2-m (Fig. 1), and examined their amyloidogenicity.

Materials and methods

Peptides. The peptides were synthesized using Fmoc chemistry (Kurabo Industries, Osaka, Japan). The cleaved peptides were purified (>95%) by reversed phase HPLC. Molecular weights of the peptides were determined by ESI-mass spectrometry.

Polymerization assay. The lyophilized peptides were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10–30 mg/mL. Reaction mixtures were prepared on ice at 4 °C. Distilled water was first put into an Eppendorf tube (0.5 or 1.4 mL). Next, 500 mM phosphate buffer (pH 7.5) or citrate buffer (pH 2.5) was added to yield a final buffer concentration of 50 mM, and 5 M NaCl was added to a final concentration of 100 mM. Peptide solutions were then added to yield a final concentration of 100–300 μg/mL (approx. 70–230 μM for peptide (21–31)). The final DMSO concentration in the mixture was 0.7–1.0%. After briefly vortexed, the reaction tubes were incubated in an air incubator adjusted at 37 °C. Incubation times ranged between 0 and 7 days (as indicated in each figure). The reaction tubes were not agitated during the reaction.

Fluorescence spectroscopy. All studies were performed essentially as described elsewhere [13] using a Hitachi F-4500 fluorescence spectrophotometer. Optimum fluorescence measurements of Aβ2M amyloid fibrils were obtained at the excitation and emission wavelengths of 455 and 485 nm, respectively, with the reaction mixture containing 5 μM Thioflavin T (ThT) (Wako Pure Chemical Industries, Osaka, Japan) and 50 mM glycine–NaOH buffer (pH 8.5). From each reaction tube, 5 μL aliquots in triplicate were subjected to fluorescence spectroscopy. Fluorescence was measured immediately after the mixture was made, averaged for the initial 5 s, and the mean and the standard deviation of each triplicate were determined.

HPLC analysis of β2-m peptides. Peptide (21–31) was incubated in 50 mM phosphate (pH 7.5), 100 mM NaCl (PBS) at 37 °C for 0–120 h, and the reaction was stopped by placing the tubes on ice. After the tubes were centrifuged at 18,000*g* for 2 h at 4 °C, the supernatant was removed and 5 μL of 1 N HCl was added to lower the pH. The supernatant exhibited no ThT fluorescence, indicating that all of the amyloid fibrils formed were precipitated. The precipitate was washed with PBS and dissolved completely by adding 87.5 μL of 91% DMSO–85 mM HCl. The disulfide bond formation of the peptides was monitored by reversed phase HPLC performed on a GILSON series liquid chromatograph equipped with a COSMOSIL $5C_{18}$-AR-II column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan) [19]. Monomeric (reduced) and dimeric (oxidized) peptides were eluted separately with a gradient beginning with solvent A (0.05% trifluoroacetic acid) and an increasing percentage of solvent B (0.05% trifluoroacetic acid/acetonitrile). The flow rate was maintained at 0.8 mL/min. To identify the retention time of monomeric and dimeric peptides, the eluted peaks were collected and subjected to mass spectrometry.

Electron microscopy of amyloid fibrils. 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

Amyloid fibril formation of partial peptides of β2-m

As shown in Fig. 1, six peptides indicated in closed symbols were synthesized. Peptide (6–12), peptide (21–31), peptide (36–43), peptide (50–56), peptide (78–86), and peptide (91–99) corresponded to β-strand A, B, C, D, F, and G in the native structure, respectively. The peptide corresponding to strand E (VI) was not available because it was difficult to purify.

All peptides were incubated in PBS (pH 7.5) and in 50 mM citrate (pH 2.5), 100 mM NaCl at a concentration of 200 μg/mL, except the mixture of peptide (21–31) and (78–86), in which 200 μg/mL each of them was added (Fig. 2). Among the peptides examined, peptide (21–31) and the mixture of peptide (21–31) and (78–86) showed increased ThT fluorescence at both pH 7.5 and 2.5. At pH 7.5, ThT fluorescence of both reaction mixtures proceeded to equilibrium at 2 days post-initiation of the reaction (Fig. 2A). In contrast, the increase in the ThT fluorescence at pH 2.5 was relatively slow and small as compared with that at pH 7.5 (Fig. 2B). At pH 7.5, the fluorescence of the mixture of peptide (21–31) and (78–86) increased more

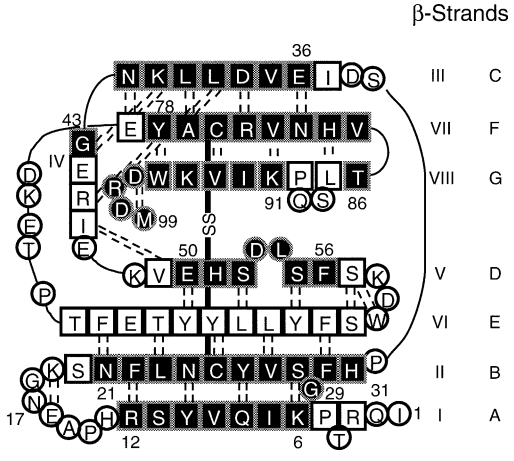

Fig. 1. Amino acid sequence of β2-m. Sequences of six peptides synthesized here are indicated by closed symbols. Secondary structures are indicated by hydrogen bonds and the numbering of β-strands [18].

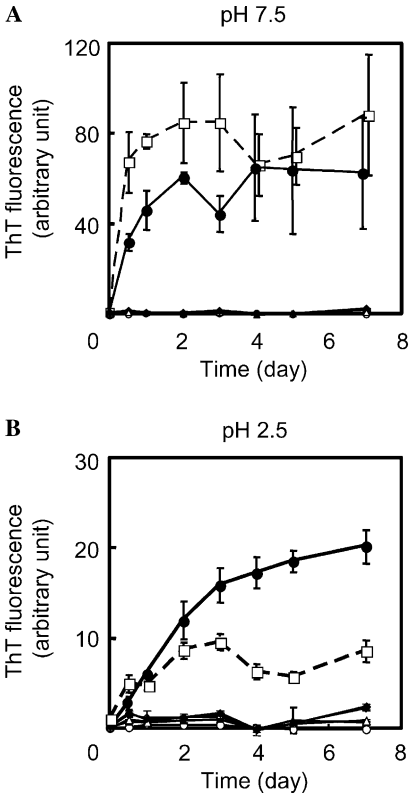

Fig. 2. Fibril formation of six peptides with time. The peptides were incubated in PBS (pH 7.5) (A) or 50 mM citrate (pH 2.5), 100 mM NaCl (B) at 37 °C. The reaction mixtures contained 200 μg/mL of peptide (6–12) (○), peptide (21–31) (●), peptide (36–43) (▲), peptide (50–56) (△), peptide (78–86) (◇), peptide (91–99) (◆) or 200 μg/mL each of peptides (21–31) and (78–86) (□). The fluorescence increase by the four peptides other than peptide (21–31) and the mixture of peptide (21–31) and (78–86) was negligible.

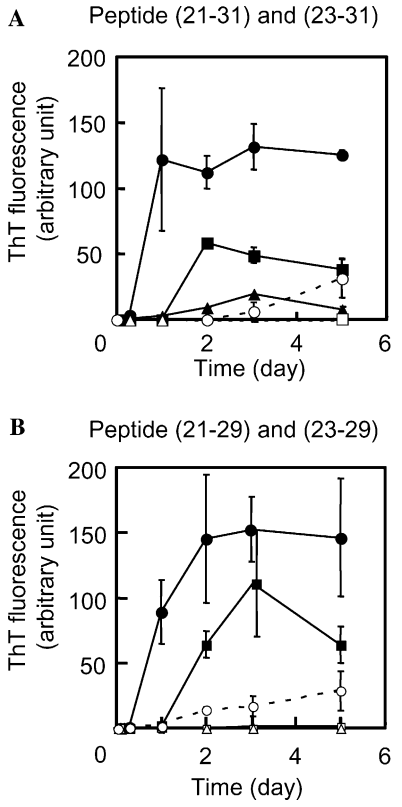

Fig. 3. Effect of truncation and DTT on the fibril formation of peptide (21–31) at 37 °C. (A) The reaction mixture contained 200 μg/mL of peptide (21–31) (closed symbols) or peptide (23–31) (open symbols), PBS (pH 7.5), and 0 (circle), 3 (square) or 10 mM (triangle) DTT. (B) The reaction mixture contained peptide (21–29) (closed symbols) or peptide (23–29) (open symbols) as in (A).

rapidly than that of peptide (21–31) alone (Fig. 2A). This indicates that addition of 200 μg/mL peptide (78–86) to 200 μg/mL peptide (21–31) may contribute significantly to fibril formation, even though peptide (78–86) alone cannot form amyloid fibrils. In contrast, at pH 2.5, the increase in the fluorescence of the mixture of peptide (21–31) and (78–86) was significantly smaller than that of peptide (21–31) alone (Fig. 2B). One possible explanation for these findings may be that the heterodimer formation of peptide (21–31) and (78–86) by the disulfide bond could readily occur at pH 7.5, which might facilitate the fibril formation (see Discussion).

Electron microscopic study confirmed the amyloid fibril formation of peptide (21–31) and the mixture of peptide (21–31) and (78–86) at pH 7.5 (Figs. 4A and C, respectively) and at pH 2.5 (data not shown). Since no aggregates other than the fibrillar structure were observed by electron microscopy, it may be reasonable to consider that the increase in ThT fluorescence corresponds to the amyloid fibrils formed during the reaction.

Effect of truncation and dithiothreitol (DTT) on the amyloid fibril formation of peptide (21–31)

Three truncated peptides of peptide (21–31), i.e. (23–31), (21–29), (23–29), were synthesized and incubated in PBS (pH 7.5) at a concentration of 200 μg/mL. As shown in Fig. 3, truncation of two residues at the N-terminal, i.e., peptides (23–31) and (23–29), reduced the ability of fibrillogenesis. However, these peptides formed amyloid fibrils after the incubation for 7 days (Fig. 4B). On the other hand, truncation of two residues at the C-terminal, i.e., peptide (21–29), did not affect the amyloidogenicity. Addition of DTT dose-dependently reduced both the rate of the fluorescence increase and the amount of amyloid fibrils formed. This effect is common to these Cys-containing peptides, suggesting that the prevention of oxidative dimerization by the disulfide bond may retard fibril formation.

HPLC analysis of the disulfide bond formation and the correlation with fibril formation

The correlation between the fibril formation and the oxidative dimerization of peptide (21–31) was

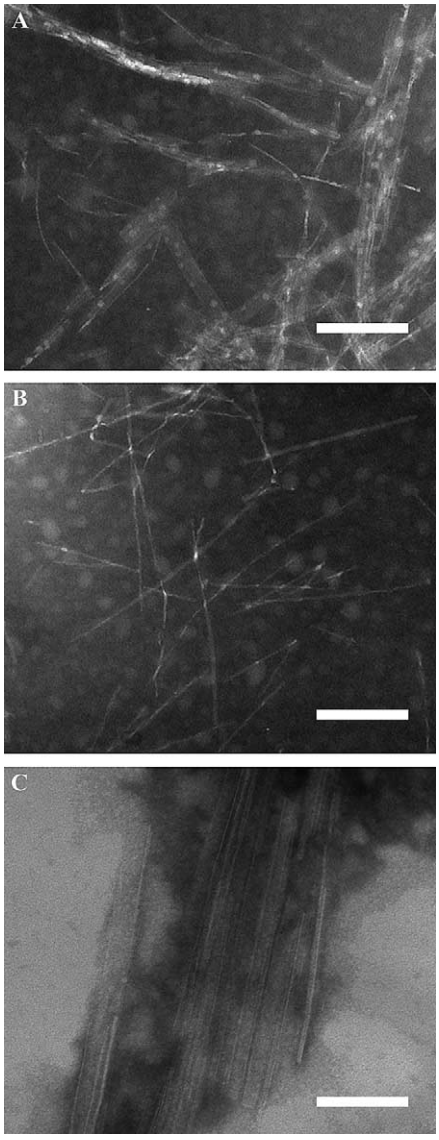

Fig. 4. Electron micrographs of the amyloid fibrils. Amyloid fibrils formed from peptide (21–31) (A), peptide (23–29) (B), and the mixture of peptide (21–31) and (78–86) (C). The peptides were incubated in PBS (pH 7.5) at 37 °C for 7 days (A, B) or 26 h (C). The bar indicates a length of 200 nm.

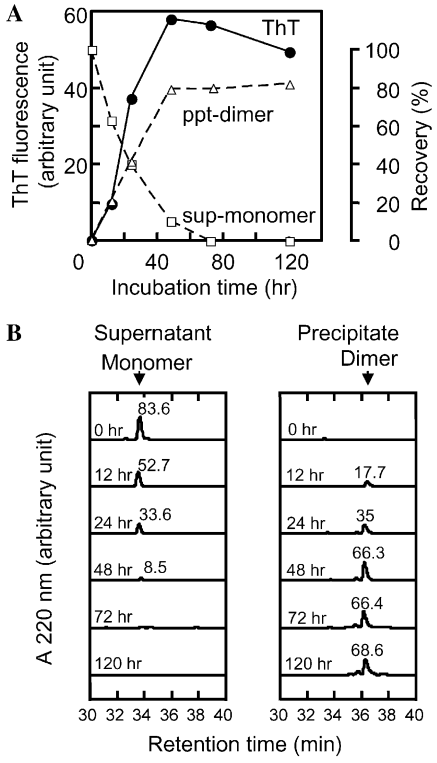

Fig. 5. HPLC analysis of the formation of the disulfide bond and its correlation with fibril formation. The fibril formation of peptide (21–31) in PBS (pH 7.5) at 37 °C was monitored by the ThT assay (A, (●)). The contents of monomeric and dimeric peptides both in the supernatant and in the precipitate fractions were analyzed by reversed phase HPLC as described in Materials and methods (B). The arrows indicate the retention times of monomer and dimer, and area of peak is indicated above each peak. The recoveries of the dimer in the precipitate (A, (△)) and the monomer in the supernatant (A, (□)) are plotted by regarding the area of monomer in the supernatant at 0 h as 100%.

investigated in PBS (pH 7.5) at 37 °C (Fig. 5). As shown in Fig. 5B, most of the peptide in the precipitate was dimeric (retention time 36.3 min), and the amount of dimeric peptide in the precipitate increased during the incubation (Fig. 5A). On the other hand, almost all of the peptide in the supernatant was monomeric (retention time 33.6 min) and the amount of monomeric peptide decreased during the incubation (Fig. 5A). The sum of the recovery of the monomer in the supernatant and the dimer in the precipitate exceeded 80% throughout the experiment. No aggregates other than the fibrillar structure were observed in the reaction mixture by electron microscopy (Fig. 4A) and all of the ThT fluorescence was precipitated by centrifugation (see Materials and methods). Moreover, the increase in the amount of dimeric peptide in the precipitate was well correlated with the increase in the ThT fluorescence (Fig. 5A). Thus, we consider that the precipitate is composed solely of the amyloid fibrils formed during the reaction. These results imply that most of the peptide (21–31) constituting the amyloid fibrils are homodimers formed by the disulfide bond.

Depolymerization of the fibrils by reducing the disulfide bond

When DTT was added to the preformed fibrils composed of peptide (21–31) and (21–29), the ThT fluorescence decreased in a dose-dependent manner, suggesting that the fibrils depolymerized by the reduction of the disulfide bond (Fig. 6). Fibrils of the shorter peptide (21–29) responded more sensitively to DTT than those of peptide (21–31).

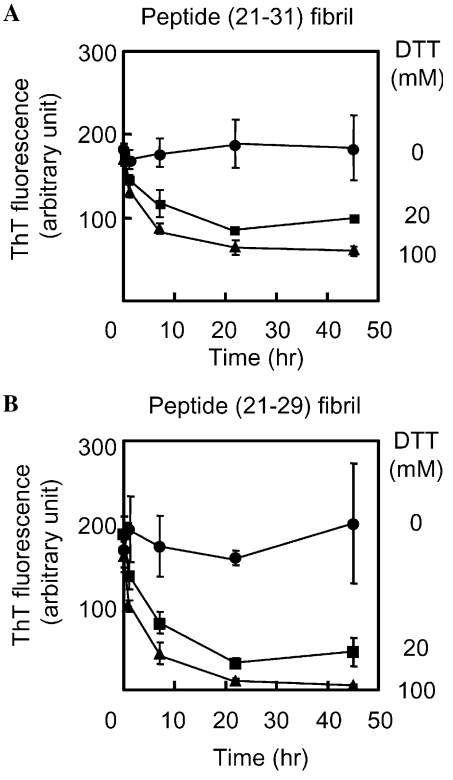

Fig. 6. Depolymerization of the fibrils by reducing the disulfide bond. Peptides (21–31) and (21–29) were polymerized in PBS (pH 7.5) at a concentration of 300 μg/mL for 48 h at 37 °C. Then the 0.1 volume of DTT solution was added to obtain the final concentrations of 0 (●), 20 (■), and 100 mM (▲). The reaction mixtures were then incubated at 37 °C and subjected to ThT assay as described in Materials and methods.

Discussion

Amyloid forming peptides

Among six peptides corresponding to six β-sheets of β2-m, we found that peptide (21–31) formed amyloid fibrils by itself or together with peptide (78–86). Peptide (21–31) is the N-terminal half of K3 peptide (Ser21–Lys41) [18]. Since peptide (36–43), corresponding to strand C (III), did not form fibrils by itself, peptide (21–31) may be the essential region in the K3 peptide. Although amyloid fibrils formed from peptide (21–31) were stable at pH 7.5 (Fig. 6A), amyloid fibrils formed from the K3 peptide readily depolymerize at pH 8.5 [18]. This suggests that the C-terminal half of K3 peptide may reduce the stability of the fibrils at a neutral to basic pH.

The inability of peptide (6–12) and (91–99) to form amyloid fibrils is consistent with our recent finding that the N- and C-terminals of β2-m are excluded from the hydrogen-bonded β-sheet core in Aβ2M amyloid fibrils [17].

Jones et al. [20] recently synthesized peptides equivalent to each of the seven β-strands of the native β2-m, together with an eighth peptide corresponding to the most stable region in the amyloid precursor conformation formed at pH 3.6, which includes residues in the native strand E plus the eight succeeding residues (named peptide E′), and investigated their ability to form fibrils in vitro. They reported that: (a) only two sequences, i.e., residues 59–71 (peptide E) and 59–79 (peptide E′) of intact β2-m are capable of forming amyloid-like fibrils under the acidic conditions shown previously to promote amyloid formation from the intact protein (pH < 5 at low and high ionic strength), and also at a neutral pH; (b) peptide (17–29), corresponding to strand B and C-terminal part of the loop between strands A and B, exhibited no amyloidogenicity at either pH 2.5 or 3.6. In contrast, we showed that peptide (21–29) and (21–31) can form amyloid fibrils at pH 7.5 (Fig. 3B) and at both pH 7.5 and 2.5 (Fig. 2), respectively. Although we and Jones et al. examined the amyloidogenicity of peptide (21–29 (or 31)) and (17–29) under the different experimental conditions, the N-terminal four residues (Asn17–Ser20) may affect greatly the amyloidogenicity of peptide (21–29). In this study, we could not evaluate the amyloidogenicity of the peptide corresponding to strand E (VI) because it was not available due to the difficulty of purification. Further studies are essential to elucidate the relative importance of each of these regions in the process of amyloid fibril formation in vitro.

Jones et al. [20] found no correlation between secondary structure propensity, peptide length, p*I* or hydrophobicity, and the ability of the peptides to associate into amyloid-like fibrils. On the other hand, they reported that the presence of a relatively high content of aromatic side-chains correlates with the ability of the peptides to form amyloid fibrils. We found that the grand average of hydropathicity [21] of peptide (21–31) is highest (0.309) among the six peptides studied, and peptide (21–31) contained no charged residues. However, the correlation of the properties of each peptide with the amyloidogenicity is poorly understood, and further investigation is essential to clarify the correlation.

The role of the disulfide bond formation on the amyloidogenicity of peptide (21–31)

The oxidative dimerization of peptide (21–31) at pH 7.5 enhanced the rate of amyloid fibril formation and the amount of fibrils formed (Fig. 3), as well as the stability of the preformed fibrils (Fig. 6). At present, we cannot determine whether this dimerization by the disulfide bond occurs before or after the polymerization of peptide (21–31) into amyloid fibrils. When intact β2-m was reduced in advance and incubated at an acidic pH, thinner and flexible fibrils were formed instead of straight typical amyloid fibrils [22,23]. As indicated by Katou et al. [24], reduction of the intramolecular disulfide bond, Cys25–Cys80 of intact β2-m increased the nano- to picosecond dynamics of the residues between these Cys residues. In the tertiary structure of intact

β2-m (light chain of MHC class I, 1A1O, in Protein Data Bank), the disulfide bond links strands B (II) and F (VII), and clumps these two β-sheet planes parallel [25]. By this arrangement, the backbone amide proton and carbonyl oxygen would be on each side of the β-sheet, and thus could participate in the formation of hydrogen bonds with adjacent β-sheets. These results suggest that the restriction of these backbone dynamics by the disulfide bond may facilitate the hydrogen bond formation between inter- or intra-molecular β-strands and ordered assembly of the molecules into amyloid fibrils.

Intermolecular disulfide bond formation between β2-m molecules does not occur during Aβ2M amyloid fibril formation in vitro [16,22]. Thus, the homodimer formation of peptide (21–31) described here may not have direct biological relevance. However, the disulfide bond may play an important role for the formation of amyloidogenic structure of β2-m. Moreover, the heterodimer of peptide (21–31) and (78–86) linked by a disulfide bond may represent the essential region for Aβ2M amyloid fibril formation. As indicated by the H/D exchange experiments of Aβ2M amyloid fibrils, most of the central region of β2-m except N- and C-terminals form a rigid amyloid structure [17]. Thus, a conformational change of the essential regions, such as peptide (21–31) and residues 59–71 (peptide E) [20], may result in the conformational change of the rest of the β2-m molecule in the process of amyloid fibril formation.

Acknowledgments

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