

Reversible Heat-Induced Dissociation of β_2 -Microglobulin Amyloid Fibrils

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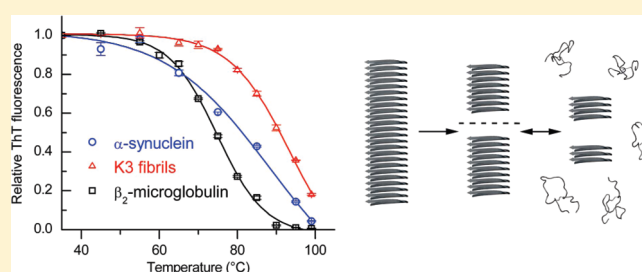
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S Supporting Information

ABSTRACT: Recent progress in the field of amyloid research indicates that the classical view of amyloid fibrils, being irreversibly formed highly stable structures resistant to perturbing conditions and proteolytic digestion, is getting more complex. We studied the thermal stability and heat-induced depolymerization of amyloid fibrils of β_2 -microglobulin (β_2 m), a protein responsible for dialysis-related amyloidosis. We found that freshly polymerized β_2 m fibrils at 0.1–0.3 mg/mL concentration completely dissociated to monomers upon 10 min incubation at 99 °C. Fibril depolymerization was followed by thioflavin-T fluorescence and circular dichroism spectroscopy at various temperatures. Dissociation of β_2 m fibrils was found to be a reversible and dynamic process reaching equilibrium between fibrils and monomers within minutes. Repolymerization experiments revealed that the number of extendable fibril ends increased significantly upon incubation at elevated temperatures suggesting that the mechanism of fibril unfolding involves two distinct processes: (1) dissociation of monomers from the fibril ends and (2) the breakage of fibrils. The breakage of fibrils may be an important *in vivo* factor multiplying the number of fibril nuclei and thus affecting the onset and progress of disease. We investigated the effects of some additives and different factors on the stability of amyloid fibrils. Sample aging increased the thermal stability of β_2 m fibril solution. 0.5 mM SDS completely prevented β_2 m fibrils from dissociation up to the applied highest temperature of 99 °C. The generality of our findings was proved on fibrils of K3 peptide and α -synuclein. Our simple method may also be beneficial for screening and developing amyloid-active compounds for therapeutic purposes.



An increasing number of serious diseases including Alzheimer's, Parkinson's, and prion disease have been linked to amyloid deposition in the human body.^{1–3} A characteristic feature of amyloid formation is that protein molecules aggregate to non-native intermolecular cross- β -sheet structures by the formation of extensive hydrogen bond networks.^{4–6} *In vitro*, under carefully chosen conditions, many proteins assemble into amyloid fibrils, suggesting that amyloid formation is a general property of the polypeptide chain.^{7–9} In the last years, information on the structure of the amyloid fibrils has been accumulating swiftly including the first atomic resolution data of amyloid conformations of short peptides.^{10–12} Amyloid formation is a complex and hierarchical process which requires nucleation and proceeds eventually to mature amyloid fibrils through different stages of assembly. Non-native intermediates of the precursor protein is often involved in fibrillogenesis.^{3,13–22} Recent studies have revealed the importance of general physicochemical properties

of the polypeptide chain in amyloid formation, such as hydrophobicity, overall charge, and secondary structure propensity.^{23,24}

To develop effective therapies to prevent or reverse amyloid deposition, it is crucial to understand the factors underlying the kinetic and thermodynamic stability of amyloid fibrils. However, our knowledge on the basis of the stability of the amyloid form of proteins is still limited. Unfolding studies of globular proteins have been well established by various physicochemical methods and have provided detailed characterization of the important intramolecular interactions maintaining the native conformation.²⁵ The application of these techniques to probe the stability of the amyloid structure, however, is complicated in different aspects. The disruption of the amyloid structure is not a simple

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monomolecular reaction making difficult the theoretical description and interpretation of the experimental parameters. The amyloid solution might be heterogeneous and the experimental results are not reproducible. The study of the physicochemical properties of amyloid fibrils also challenge the limitations of the experimental techniques in sensitivity, measurement range, or resolution because of the unique properties of amyloid structures, such as insolubility, large size, often increased stability against pressure, temperature, or chemical denaturants. Chemical stability of amyloid fibrils were probed in a number of studies.^{26–31} The use of high pressure in fibril dissociation experiments is progressing dynamically.^{32–35} Only a small number of studies investigated the thermal stability of amyloid fibrils.^{36–42} These studies showed that, compared to globular proteins, amyloid fibrils are highly resistant to thermal-induced dissociation. In many cases, to achieve complete dissociation, a temperature far beyond 100 °C is necessary to use.^{36,37}

β_2 -Microglobulin (β_2m), the light chain of the major histocompatibility complex class I, is a 99-residue β -sandwich protein consisting of seven β -strands organized into two antiparallel β -sheets linked by a disulfide bond.⁴³ As a serious complication of long-term hemodialysis, β_2m forms amyloid deposits in the joints.⁴⁴ In vitro, β_2m readily forms straight, long, and well-ordered amyloid fibrils at pH 2.5 in a seed-dependent manner.^{21,45–47} Structural and thermodynamic studies revealed the unique structural features of the amyloid form compared to the native globular structure: a rigid amyloid core, maintained by backbone hydrogen bonds, coupled with a lower level of overall internal packing and the possible presence of unfavorable side-chain contributions.^{48–53}

In this work, we investigate the thermal stability and mechanism of dissociation of amyloid fibrils. Recently, our group reported the kinetically controlled thermal response of β_2m amyloid fibrils upon gradual heating in the cell of the differential scanning calorimeter (DSC).⁴² This unusual heat-effect, suggested to arise from the association of fibrils, is observable even at low fibril concentration, and characteristic for the amyloid form of β_2m . The effect increases until a certain temperature, depending on the protein concentration and heating rate.^{42,54} This temperature proved to be around 68 °C by heating up fibrils at a concentration of 0.3 mg/mL with a heating rate of 1 °C/min. Above this temperature, dissociation of fibrils commences.⁴² In the present work, we focus on this second process, the dissociation of β_2m fibrils at elevated temperatures. β_2m fibrils were formed by seeded polymerization reactions at pH 2.5 to decrease the inhomogeneity of the sample and achieve good reproducibility. Circular dichroism (CD) spectroscopy and the fluorescence assay using thioflavin T (ThT), an amyloid specific dye, were used to quantify the fibril content in the solution.

The main goals of the present study are to unravel the important factors of the fibril stability and explore the mechanism of fibril depolymerization. First, we found that upon incubation at high temperature β_2m fibrils can be completely dissociated to monomers. Carefully designed measurements revealed that the thermal-induced dissociation of β_2m fibrils is a reversible process reaching rapid equilibrium with monomers. The heat stability of the fibrils depends on the concentration, growth time, growth temperature, and the presence of additives. The mechanism of depolymerization possibly involves two simultaneous processes, the dissociation of monomers from the fibril ends and the breakage of fibrils into pieces, producing increased number of fibrils.

EXPERIMENTAL PROCEDURES

Fibril Polymerization Reaction. Recombinant β_2m was expressed and purified as described previously.^{55,56} The protein concentration was determined using an extinction coefficient of $1.91 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ at 280 nm. Amyloid fibril formation of β_2m was carried out using the fibril extension method established by Naiki et al.^{57,58} in which the seed fibrils were extended by the monomeric β_2m at pH 2.5 at 37 °C. Generally 0.1–0.3 mg/mL monomeric β_2m was prepared in 50 mM sodium citrate buffer containing 100 mM NaCl, pH 2.5 at 4 °C. The reaction was started by placing the sample to a water bath at 37 °C and by adding seed fibrils at a final concentration of 5 $\mu\text{g/mL}$. Seeds (i.e., fragmented fibrils) were prepared by sonication of 100 μL aliquots from a 0.1 mg/mL fibril stock solution using a Microson sonicator (Misonix, Farmingdale, NY) at intensity level 2 with 50 1-s pulses on ice. The reaction was monitored by fluorometric analysis with ThT. In some experiments, monomer and seed concentrations were altered as indicated in the text. Amyloid fibrils of K3 peptide, the 21–41 fragment of β_2m , were grown at a concentration of 0.06 mg/mL in 6 mM HCl and 7 mM NaCl in a seed-dependent manner.⁵⁹ α -Synuclein fibrils were promoted by ultrasonication at a concentration of 0.1 mg/mL in 20 mM Na-phosphate buffer containing 100 mM NaCl at pH 7.4 by adapting the protocol of Chatani et al.⁶⁰

Thioflavin T Fluorescence Measurements. Depending on the protein concentration and experiment, an aliquot of 5–20 μL was taken from the sample and mixed with 1.0 mL of 5 μM ThT in 50 mM glycine NaOH buffer at pH 8.5.⁵⁷ The fluorescence of ThT was monitored at 485 nm with excitation at 445 nm using Hitachi F4500 fluorescence spectrophotometer or Perkin-Elmer Fluoromax-3 instrument in a cell thermostatted to 25 °C. Each point was measured three times and averaged. All experiments were repeated several times.

Thermal Unfolding of Fibrils. Incubation of the samples at various temperatures in the 25–99 °C range were carried out in a Hybaid PCR Sprint Thermal Cycler (Mandel Scientific, Canada). PCR-02 200 μL -type thin walled PCR tubes (Axygen Scientific, CA, USA) were used to ensure a prompt temperature jump in the experiments applying 10–30 μL sample volumes. After 30 s to 60 min incubation, the samples were put on ice or diluted into the ThT assay solution with no delay. To obtain the steepest temperature jump, essential for short time measurements, the sample tubes were put to the tempered PCR machine and taken back to ice manually.

Circular Dichroism Spectroscopy. CD measurements were carried out on a J-720 spectropolarimeter (Jasco) equipped with a Neslab RTE-110 thermostat. A cylindrical water-jacketed quartz cell of 0.1 cm path length was used for recording the spectra in the far UV region. Considering the weakness of the CD spectroscopy in the estimation of β -structure, a specific method was used for the investigation of the heat-treated β_2m sample. The spectra of the monomeric acid-denatured β_2m and mature fibrils at pH 2.5 were taken as basis spectra for acid denatured and fibrillar states of β_2m , respectively. The linear combination of these spectra resulted in a good fit for the heat-treated samples.

Ultracentrifuge Analysis. A Beckman Optima XL-I analytical ultracentrifuge (Beckman Instruments Inc., Palo Alto, USA) was used. Sedimentation experiments were carried out in the sedimentation velocity mode at 40 000 and 55 000 rpm. Data obtained were analyzed using Origin 4.0 (MicroCal Inc., USA). Protein concentration of 0.35 mg/mL was used. β_2m monomers

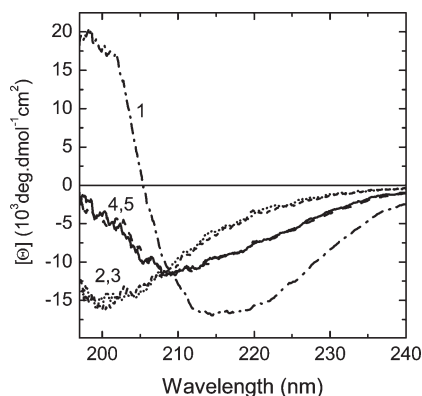


Figure 1. CD spectroscopy of heat-treated β 2m fibril samples. (1) Spectrum of freshly polymerized β 2m fibrils at 0.1 mg/mL concentration in 50 mM Na-citrate buffer containing 100 mM NaCl at pH 2.5 by the addition of 5 μ g/mL seeds, (2) acid-denatured monomer β 2m solution, (3) fibril solution after 10 min incubation at 99 °C, (4) fibrils after 5 min incubation at 80 °C, and (5) the best fit to (4) by linear combination of the original fibril solution and the acid denatured monomer β 2m (curves 1, 2) with factors of 0.32 and 0.68, respectively.

in 50 mM citrate buffer at pH 2.5 and β 2m monomer and fibril solutions in the absence and presence of heat treatment (10 min at 99 °C) in 50 mM citrate buffer containing 100 mM NaCl at pH 2.5 were measured. A low temperature, 4 °C, was used to keep the aggregation slow in the presence of salt, and to prevent the reformation of fibrils in the heat-treated samples. For reference, a native, monomer β 2m sample was measured in 50 mM Na-phosphate, 100 mM NaCl at pH 7.0.

RESULTS AND DISCUSSION

In this work, we studied the thermal stability and the heat-induced dissociation of β 2m amyloid fibrils. To achieve a homogeneous starting fibril solution, fibrils were grown in a seed-controlled reaction in 50 mM Na-citrate buffer containing 100 mM NaCl at pH 2.5, where β 2m forms straight and rigid amyloid fibrils without other types of aggregates, that is, protofibrils or amorphous aggregates. To quantify the fibril content in the solution, a fluorometric assay of ThT was used. ThT, a fluorescent dye is specific for the amyloid structure, especially in the case of β 2m fibrils, producing high fluorescence intensity in the bound form.⁵⁷ Although it binds to other type of aggregates as well, the resulting intensity is significantly lower.⁵¹ ThT shows no binding to monomers. To verify the validity of the assay, reference measurements were carried out as a function of protein concentration, seed concentration, and polymerization time (data not shown). We found that the final ThT fluorescence intensity in the polymerization reaction was remarkably linear with the protein concentration of the fibril solution in the 0.1–0.3 mg/mL β 2m concentration range used.

β 2m Amyloid Fibrils Dissociate at Elevated Temperatures. β 2m fibrils were freshly polymerized for 3 h at a concentration of 0.1 mg/mL using 5 μ g/mL seed. We tested the resistance of the fibrils against high temperature incubation. After 10 min incubation at 99 °C, the ThT fluorescence intensity was assayed with no delay. The sample showed no remaining ThT intensity indicating the loss of the amyloid structure. CD spectrum of the heat-treated sample showed a mostly random coil structure very similar to the spectrum of an acid denatured monomeric β 2m

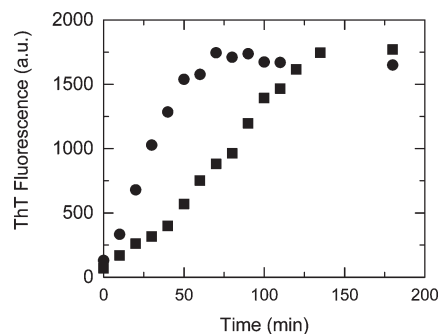


Figure 2. Re-extension of the thermal-dissociated β 2m fibril solution. Fibrils were regrown at 37 °C after 5 min incubation at 95 °C. (■) ThT fluorescence intensity change of the heat-treated sample at 37 °C, (●) fibril regrowth accelerated by the addition of fibril seeds at a concentration of 5 μ g/mL.

sample prepared for fibrillization (Figure 1, curves 2, 3). These results indicate that the cross- β -sheet structure of β 2m fibrils (Figure 1 curve 1) can be completely destroyed by heat.

To investigate if the heat treated sample consists of amorphous aggregated material or monomeric protein, which might be difficult to distinguish by CD, sedimentation velocity experiments were carried out at 4 °C with a fibril sample of 0.35 mg/mL concentration. We found that the sedimentation velocity profile of the heat treated sample was identical to an acid denatured monomeric β 2m sample exhibiting a sedimentation coefficient of 1.9 S. The sample was proved to be homogeneous with no sign of oligomers and 1–2% content of large aggregates spun down during the acceleration of the centrifuge to 55 000 rpm (data not shown). β 2m fibrils in a sample without heat-treatment were spun down during the acceleration period with a remaining low absorption in the supernatant that might be related to a low monomer concentration difficult to estimate precisely. These results suggest that β 2m fibrils can be completely dissociated to monomers by heat treatment. We have to note that in our measurements an abrupt temperature jump was applied by placing the low volume samples in thin-walled 200 μ L PCR tubes into a tempered PCR machine to minimize the extent of processes other than depolymerization, such as the association of fibrils observed in samples heated up gradually to 68 °C in the calorimeter cell.^{42,54}

Reversible Thermal Unfolding of β 2m Amyloid Fibrils. To investigate whether the heat-treated β 2m fibril solution contains monomers capable of re-extension of amyloid fibrils, we further incubated the heat-treated samples at 37 °C and measured the change of ThT fluorescence intensity. Figure 2 shows ThT intensity change in a 0.1 mg/mL β 2m fibril sample pretreated at 95 °C for 5 min. The sample exhibited a relatively slow increase in ThT intensity (Figure 2, ■), which might either be the result of a low concentration of functional monomers or a low number of seeds, that is, extendable fibril-ends. It is also possible that the extendable fibril-end face structure was changed. However, after several hours, ThT intensity reached the same maximum measured before the heat treatment. Addition of 5 μ g/mL seeds to the heat-treated solution resulted in fast re-extension with a similar rate observed in the standard polymerization reaction (Figure 2, ●). Under the conditions used, spontaneous nucleation and fibrillization were not observed during the time of the experiments; that is, monomeric β 2m sample did not form fibrils without seeds. Thus, we can conclude that upon heat treatment,

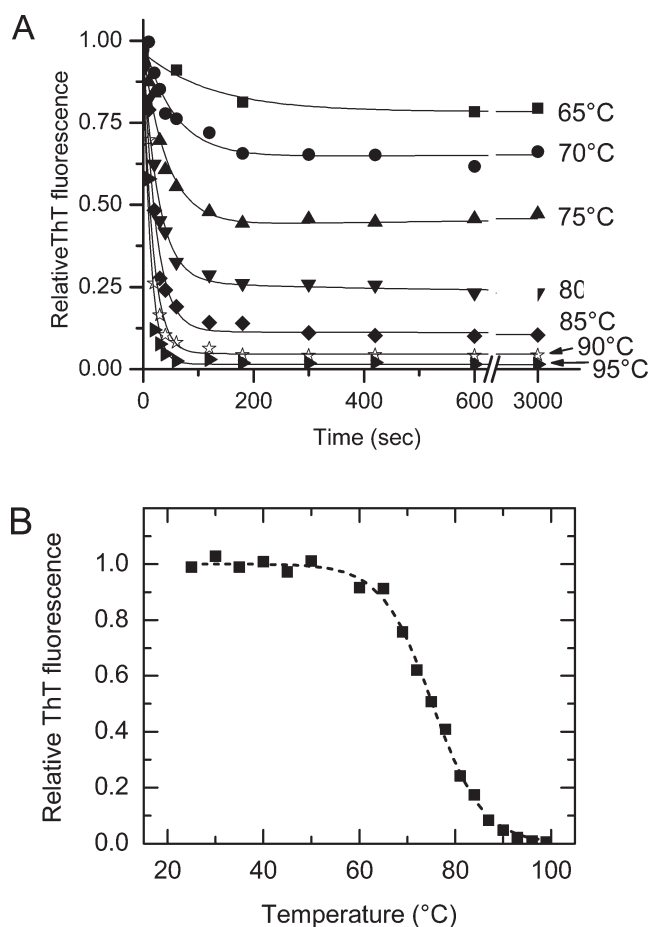


Figure 3. Thermal stability of $\beta 2m$ amyloid fibrils. (A) Kinetics of the thermal dissociation of $\beta 2m$ fibrils at various temperatures. Remaining ThT intensities of 0.1 mg/mL $\beta 2m$ fibril samples are plotted as a function of incubation time. The temperatures are indicated along with the dissociation profiles. (B) The stability was probed by 5 min incubation at various temperatures and detected by the remaining ThT fluorescence intensity. The dashed line is a sigmoidal fit to the data.

$\beta 2m$ fibrils dissociated to monomers capable of extending amyloid fibrils. The remaining 2–3% ThT fluorescence intensity makes it probable that the observed slower re-extension after high temperature incubation is caused by the loss of the extendable fibril-ends.

Kinetics of the Thermal Unfolding and the Stability Profile of $\beta 2m$ Fibrils. To investigate the kinetics of the thermal unfolding of the fibrils, the remaining ThT intensity was measured as a function of the incubation time at various temperatures. Figure 3A shows a rapid decrease in ThT intensity after incubation at elevated temperatures, reaching a certain equilibrium value depending on the temperature within 1–3 min of incubation. This value is apparently stable as presented by the data of 50 min incubation. These results prove that 5 min incubation is sufficient for the dissociation process to take place. Therefore, the thermal stability profile of 0.1 mg/mL $\beta 2m$ fibril solution was recorded by 5 min incubation at various temperatures and by the subsequent measurement of the remaining ThT fluorescence intensities, indicative of the remaining fibril content. Figure 3B shows that a ThT fluorescence intensity decrease occurs above 60 °C and the fibril content decreases with temperatures leading to a complete loss at 99 °C. The midpoint,

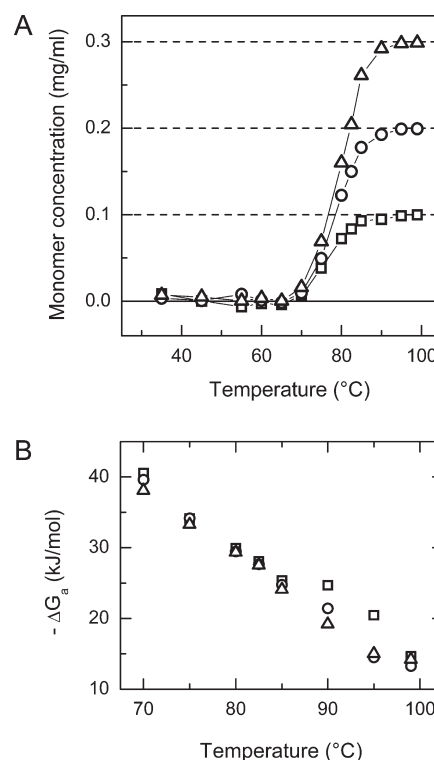


Figure 4. Heat-induced dissociation of fibril solutions at different $\beta 2m$ concentrations. (A) Monomer concentrations at various temperatures were calculated from the ThT fluorescence loss upon thermal denaturation of $\beta 2m$ fibrils. Starting fibril concentrations were 0.1 mg/mL (\square), 0.2 mg/mL (\circ), 0.3 mg/mL (Δ), prepared by dilution from the same 0.3 mg/mL fibril solution grown at 37 °C using 5 μ g/mL seeds. (B) Calculated apparent standard Gibbs free energy changes of association according to the linear polymerization model as a function of temperature at different $\beta 2m$ concentrations ($\Delta G_a = -RT \ln K_a$). Symbols are the same as in panel A.

corresponding to 50% of dissociation is observed around 75 °C. The dissociation profile is similar to the well-known denaturation curve of the globular proteins and can be fitted with a sigmoid profile (Figure 3B). The two reactions, that is, unfolding of globular proteins and dissociation of $\beta 2m$ amyloid fibrils at pH 2.5 are also similar from the point of view of the unfolding or denaturation of well-ordered protein structures to disordered polypeptide chains. However, the unfolding of fibrils may not be a single step reaction due to the polymeric nature of the amyloid structure.

Thermal destabilization of amyloid fibrils were reported for amyloid A, fibrils of fibronectin type III module, and an α -spectrin SH3 domain mutant, all disaggregated under 100 °C and for insulin fibrils formed between 100 and 140 °C.^{36,39–41} High temperature incubation decreased prion infectivity.⁶¹

Aspects of Fibril–Monomer Equilibrium. We investigated if the apparent equilibrium observed upon incubation at elevated temperatures represents equilibrium between the fibrils and the monomer $\beta 2m$ molecules as suggested in equilibrium models of fibril growth and dissociation.^{30,52,62–64} The CD spectra of the $\beta 2m$ fibril samples were recorded after 5 min incubation at different temperatures. The cell was thermostatted to 3 °C to quench the fibril regrowth. In Figure 1 curve 4, the CD spectrum of a fibril sample after 5 min incubation at 80 °C is presented. The

spectrum could be fitted with good agreement by the linear combination of the acid denatured monomer spectrum and the original fibril spectrum with factors of 0.68 and 0.32 (Figure 1 curve 5) suggesting that indeed, the sample is a mixture of fibrils and monomers. The control measurement of the ThT fluorescence intensity indicated similar extent of depolymerization.

To explore the nature of the monomer–fibril equilibrium and to investigate its dependence of the monomer and the overall protein concentration, we repeated the thermal dissociation study of 5 min incubations at different temperatures, at protein concentrations of 0.1, 0.2, and 0.3 mg/mL. First, to avoid any difference in the properties of the fibrils (size distribution and morphology) at different concentrations, one single fibril stock of 0.3 mg/mL was prepared. The samples with different concentrations were prepared from this solution by dilution. We calculated the free monomer concentrations of the heat-treated samples from the decrease in ThT fluorescence intensity assuming linear dependence, considering being negligible at the highest starting ThT values, and reaching the maximum overall protein concentration as the ThT intensity disappears. Figure 4A presents the calculated monomer concentrations as a function of temperature. Although the equilibrium monomer concentration is not zero at lower temperatures, its value, being a few $\mu\text{g/mL}$ at most,⁵² may be negligible compared to that in the dissociation temperature region above 60 °C.

Different models have been applied for the formation and equilibrium of amyloid fibril solutions. The simplest model, which is suitable in the case of the seed-induced polymerization, assumes that the number of seeds, that is, the number of extendable fibril-ends is constant in the reaction.^{52,62,64} The monomer molecules maintain equilibrium with the fibril-ends and the association constant (K_a) depends only on the monomer concentration, and independent of the length of fibrils or the overall protein concentration: $K_a = 1/[M]$. However, this model might not be acceptable to handle a process accompanied by significant dissociation and possible change in the number of fibrils. In case equilibrium only depends on the monomer concentration, one can expect that the curves of the different overall concentrations should be overlapping until the monomer concentrations reach their possible maximum values. The monomer concentration profiles of the three fibril solutions in Figure 4A show clear deviation from the overlapping shape, suggesting that this model is insufficient for the characterization of fibril depolymerization.

Generally, amyloid formation is a nucleation-dependent polymerization process. The first polymerization steps resulting in the formation of the nucleus such as the smallest stable fibril is highly unfavorable. Once the critical nucleus is reached, the subsequent assembly of the monomers to the fibril-ends is a favorable process. A model established originally for helix polymerization by Oosawa and Kasai^{65,66} is accommodating the two processes, the first with an unfavorable association constant and the second with a favorable one and thus might be suitable to interpret amyloid fibril polymerization and dissociation. According to the model, one expects to observe equilibrium between long polymers and free monomers. Our results, showing equilibrium between $\beta 2\text{m}$ fibrils and monomers without the presence of oligomers, suggest that the helix-model might be appropriate for the treatment of thermal-induced fibril dissociation. However, the experimental determination of the association constant of the unfavorable nucleation process is difficult. For simplicity, here we apply the linear polymerization

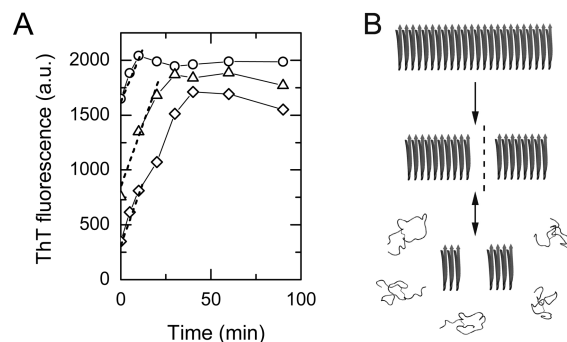
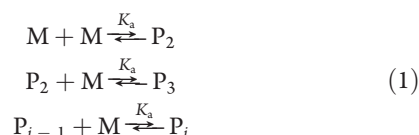


Figure 5. Measurement of the rate of repolymerization. (A) $\beta 2\text{m}$ fibril samples were repolymerized at 37 °C after 5 min incubation at 65 °C (O), 75 °C (Δ), and 85 °C (\diamond). Dashed lines show the linear fittings for the determination of the initial rate of ThT fluorescence increase. A typical experiment of three determinations is shown. The experimental error was less than 10%. (B) Schematic representation of the possible thermal-induced depolymerization of $\beta 2\text{m}$ amyloid fibrils. Breakage of the fibrils occurs simultaneously with depolymerization.

model supposing that the equilibrium constant for adding a monomer to the end of a chain, K_a , is independent of length:



where M is the monomer, P_i is polymer with size of i . The association constant, K_a is defined by

$$K_a = \frac{[P_i]}{[P_{i-1}][M]} \quad (2)$$

Thus, the total concentration of $\beta 2\text{m}$, $[M]_0$, is expressed by

$$[M]_0 = \sum_{i=1}^{\infty} i[P_i] = \sum_{i=1}^{\infty} iK_a^{i-1}[M]^i = \frac{[M]}{(1 - K_a[M])^2} \quad (3)$$

The advantage of this simplified model is that we can determine K_a value, which provides us an apparent standard Gibbs free energy change of association (ΔG_a).

$$K_a = \frac{1 - \sqrt{[M]/[M]_0}}{[M]} \quad (4)$$

Using the calculated monomer and the overall protein concentrations, presented in Figure 4A, the apparent ΔG_a values were determined for the 70–99 °C dissociation transition range by this linear polymerization model (Figure 4B). Intriguingly, the samples with the three different overall concentrations provided similar ΔG_a values suggesting that the linear polymerization model offers a reasonable description of the thermal-induced dissociation process of $\beta 2\text{m}$ fibrils. Moreover, these results are close to $\beta 2\text{m}$ fibril stability data from GdnHCl denaturation studies reporting a -44 kJ/mol ΔG_a value at 37 °C.³⁰

Mechanism of Heat-Induced Unfolding. The absolute value of the ThT fluorescence intensity provides information only about the fibril content but is not capable of determining the number or size distribution of fibrils. The rate of ThT intensity increase in a polymerization reaction, however, is expected to be proportional to the rate of extension and thus proportional to the number of extendable fibril ends. In the seeded extension

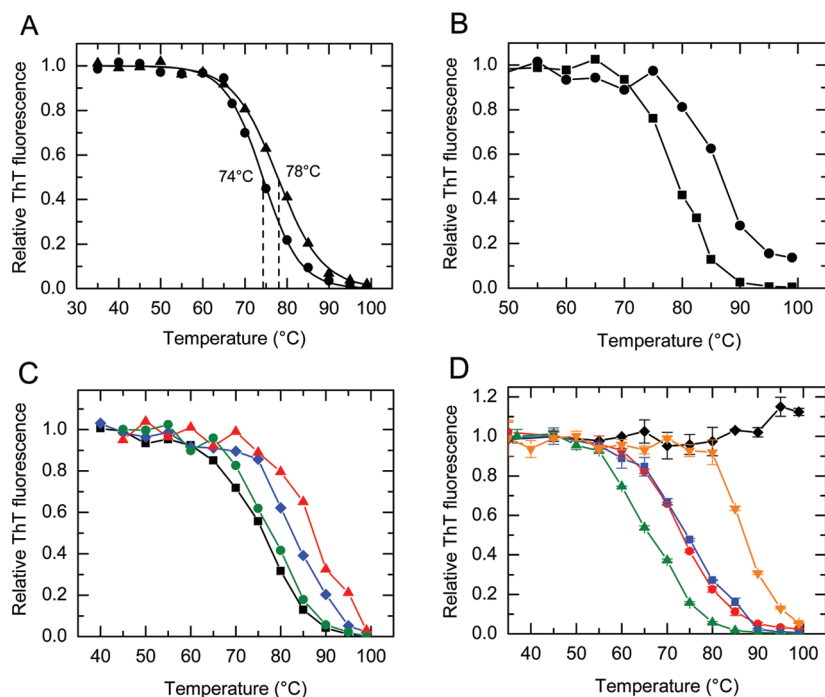


Figure 6. Effect of different conditions and various additives on the thermal stability of $\beta 2m$ fibrils. Samples were incubated for 5 min at various temperatures and the remaining ThT intensities were recorded with no delay. (A) Stability of fibrils grown in the presence of seeds at 1 $\mu g/mL$ (●) and 10 $\mu g/mL$ (▲) concentrations in order to produce fibril solutions with different average length. (B) Effect of aging on the stability of $\beta 2m$ fibrils polymerized at 37 °C for 3 h (■), and 48 h (●). (C) Stability of fibril solutions grown at 37 °C (■), 45 °C (green circles), 55 °C (blue diamonds), and 65 °C (red triangles). (D) Effect of some additives on the stability of $\beta 2m$ fibrils. 0.5 mM SDS stabilizes $\beta 2m$ fibrils, completely preventing them from dissociation (◆). 10% glycerol has a negligible effect on the thermal stability (red circles) compared to the reference fibril solution (blue squares). The absence of 100 mM NaCl in the buffer decreases stability significantly (green triangles), while the addition of 2 mM Na-sulfate greatly increases stability indicating specific interactions between sulfate and $\beta 2m$ fibrils (downfacing orange triangles). Protein concentrations were set to 0.1 mg/mL in (A), (C), and (D) and 0.3 mg/mL in (B).

reaction, the monomer molecules extend the fibril-ends taking up the ordered cross- β -sheet structure of the fibrils. In the reverse process induced by heat, however, it is questionable if the monomers dissociate from the ends of the destabilized fibrils or the breakage of the fibrils into shorter pieces might also happen. We measured the re-extension rates of the fibrils after 5 min incubation at 65, 75, and 85 °C. A fibril solution grown with a seed concentration as low as 2 $\mu g/mL$ in order to produce long fibrils was used for the experiment. After incubation at the temperatures specified, the samples were placed back to 37 °C and the ThT fluorescence intensity was followed until the complete regrowth of the fibrils (Figure 5A). Upon significant depolymerization, we may expect the decrease of the number of fibrils and thereby a decreasing repolymerization rate. However, as shown in Figure 5A, the repolymerization rates were significantly higher than the original growth rate, suggesting the existence of an increased number of extendable fibril ends in the heat-treated solutions. A reasonable explanation is that the breakage of the fibrils might also occur together with monomer dissociation upon incubation at high temperatures. In Figure 5B, we show the schematic representation of the breakage-and-dissociation mechanism for fibril depolymerization of $\beta 2m$. The possible role of breakage in prion propagation was discussed in theoretical studies and was experimentally evidenced.^{67–69} Recently, Xue and co-workers reported the importance of the fibril breakage in the amyloid formation of $\beta 2m$.⁷⁰ The observed fast disaggregation and regrowth enlightens the dynamic nature

of amyloid fibrils suggested previously by Carulla et al., studying H/D exchange of amyloid fibrils of an SH3 domain.⁷¹

In the case of different conditions and other proteins, the presented simple mechanism for thermal-induced fibril dissociation may be complicated by other processes, such as aggregation of preformed fibrils, a possible structural reorganization within the fibrils resulting higher stability, repolymerization in a different fibril form, or nonspecific aggregation of the monomer molecules and thus might have limited validity. Although we cannot provide a universal description of disaggregation, the characterization of the basic principles of fibril dissociation may lead to a more comprehensive understanding of amyloid formation and stability.

The Role of Different Factors in the Thermal Stability of $\beta 2m$ Fibrils. To further characterize the thermal stability of $\beta 2m$ fibrils, the effects of seed concentration, aging, and growth temperature were investigated (Figure 6). The use of higher seed concentration for preparation of the fibril solution resulted in a slightly increased stability against heat treatment. The midpoint of the dissociation profile shifted from 74 to 78 °C when the seed concentration was increased from 1 to 10 $\mu g/mL$ (Figure 6A). The stabilization effect is more expressed at the high temperature region. This experiment also revealed the importance of the standardization of seed preparation. Fibril seeds are prepared by sonication of preformed fibrils. The efficiency of sonication influences the number of fibril fragments. Because we were not able to determine the exact number of seeds, in every comparative experiment the parallel samples were prepared using the same fresh seed solution and treated identically. A reference

sample with 5 $\mu\text{g/mL}$ seed concentration was always included. Comparison between the properties of fibril solutions of different series of experiments was less reliable.

To study the effect of aging on the thermal stability of $\beta 2\text{m}$ fibrils, the stability of a freshly polymerized 0.3 mg/mL fibril sample was compared to the same fibril solution after 48 h incubation at 37 $^{\circ}\text{C}$. The aged sample showed significantly increased resistance against high temperature incubation (Figure 6B). The dissociation was not complete at the highest applied temperature of 99 $^{\circ}\text{C}$, with a remaining 14% of ThT intensity, suggesting that after the polymerization reaction a secondary, slower process takes place resulting in higher resistance against elevated temperatures. Studies using high pressure as perturbation reported increased stability with aging of transthyretin and prion protein aggregates and suggested a possible reorganization of the fibril structure into more densely packed and hydrogen-bond stabilized forms.^{72–74} Because in our study mature fibrils were prepared by seeding with short fibrils and the experiments were carried out after the ThT intensity reached its maximum value, we do not expect a significant reorganization within the structure of the mature fibrils with time. In the background of the stabilization upon long-time storage at 37 $^{\circ}\text{C}$ there might rather be the slow association or aggregation of fibrils. The same effect was observed upon long-term storage of the samples at 4 $^{\circ}\text{C}$ (data not shown). The association of fibrils was suggested to occur in experiments heating the samples in the calorimeter cell with a relatively slow heating rate of 0.25–1.5 $^{\circ}\text{C/min}$ ⁴² that might be considered as an accelerated aging reaction. Although Sasahara et al. also suggested the existence of a reversible and temperature-dependent compaction of the fibrils at moderate temperatures, that process was supposed to be slower than the association.⁴² Analytical ultracentrifuge experiments showed the susceptibility of $\beta 2\text{m}$ fibrils for association (Chatani and Goto, unpublished results).

The effect of growth temperature on the thermal stability was investigated using fibril solutions grown at 37, 45, 55, and 65 $^{\circ}\text{C}$. The normalized ThT fluorescence intensities as a function of incubation temperature are presented in Figure 6C and reveal an increased stability of the fibrils polymerized at higher temperatures. We have to note that the fibril solution grown at 65 $^{\circ}\text{C}$ exhibited approximately 50% lower initial ThT fluorescence intensities than the samples grown at lower temperatures. This is surprising, taking into consideration that fibrils grown at 37 $^{\circ}\text{C}$ are essentially stable at 65 $^{\circ}\text{C}$ and keep their ThT intensity. Explanations might be that an altered fibril structure is formed at 65 $^{\circ}\text{C}$ with lower ThT binding ability, or because of an extensive association of already formed fibrils, the surface of the fibrils is partly buried from ThT binding. To investigate this question, we compared the morphology of $\beta 2\text{m}$ fibrils formed at 37 and 65 $^{\circ}\text{C}$ by transmission electron microscopy (TEM) (Supplementary Figure 1, Supporting Information). The same and well reported $\beta 2\text{m}$ fibril types^{17,46,51,80} were observed in both samples. However, in the sample prepared at 65 $^{\circ}\text{C}$ the thicker types of fibrils with an average diameter of 18 ± 1 nm and a clear helical twist of 130 ± 10 nm crossover repeat were dominant (Supplementary Figure 1, Supporting Information). At 37 $^{\circ}\text{C}$, the so-called type I and type II fibrils were observed in higher proportion with average diameters of 5 ± 1 and 8 ± 1 nm, respectively. The thicker fibrils might expose a decreased relative surface for ThT binding that explains the lower fluorescence intensity of fibrils formed at 65 $^{\circ}\text{C}$. At the same time, we can observe a higher amount of aggregated fibrils that might further decrease the surface accessible for ThT. It is intriguing that the fibrils formed

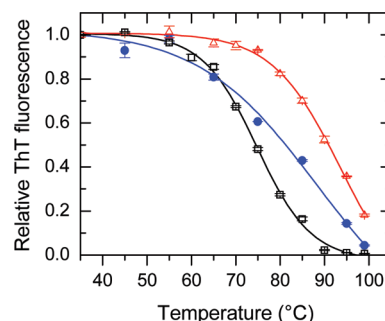


Figure 7. Comparison of the stabilities of amyloid fibrils of different proteins. α -synuclein fibrils (blue circles) and K3 fibrils (open red triangles) were proved to be more resistant to thermal dissociation than $\beta 2\text{m}$ fibrils (\square). However, all three amyloids could be significantly dissociated at elevated temperatures indicating that this phenomenon is not attributed solely to $\beta 2\text{m}$. α -Synuclein amyloid sample at 0.1 mg/mL concentration was prepared in 20 mM Na-phosphate buffer containing 100 mM NaCl at pH 7.4 and 0.06 mg/mL K3 fibril sample was prepared in 6 mM HCl, 7 mM NaCl as described in Experimental Procedures. $\beta 2\text{m}$ concentration was 0.1 mg/mL in a buffer of 50 mM Na-citrate, 100 mM NaCl at pH 2.5. Data points are mean \pm standard error of 3 independent measurements. As guiding for the eye, the data was fitted with sigmoidal curves.

at 65 $^{\circ}\text{C}$ within half an hour in our experiments are similar to those formed after 2–8 weeks incubation at 37 $^{\circ}\text{C}$ in the work of White et al.⁸⁰ in 25 mM sodium phosphate and 25 mM sodium acetate buffer.

Effect of Additives on the Thermal Stability of Fibrils. In the field of the amyloid research, there is an increasing interest for molecules that influence the fibril formation process. Several papers were published on the effect of different molecules on amyloid formation of $\beta 2\text{m}$.^{75–79} Our method might be suitable for the study of the effect of different additives on the fibril formation through probing the change in thermal stability. Recently, the effect of sodium dodecyl sulfate (SDS), an anionic detergent, on the fibril formation of $\beta 2\text{m}$ was reported.⁷⁷ SDS destabilizes the native monomer structure of $\beta 2\text{m}$ while stabilizes amyloid fibrils. This effect is clearly manifested in the thermal stability experiment where 500 μM SDS completely protected $\beta 2\text{m}$ fibrils from dissociation in the full temperature range studied (Figure 6D). For comparison, 10% of glycerol exhibited negligible effect on the fibril stability. In the absence of 100 mM NaCl, $\beta 2\text{m}$ fibrils showed decreased heat-resistance suggesting the importance of ionic strength in the fibril stability. Addition of ammonium-sulfate at a concentration as low as 2 mM increased fibril stability significantly (Supplementary Figure 2, Supporting Information). Additional measurements showed that ammonium chloride had no effect on the stability, while Na-sulfate exhibited an effect identical to that of ammonium-sulfate (Figure 6D) revealing the presence of specific stabilizing interactions between sulfate and $\beta 2\text{m}$ fibrils. This observation might be helpful in understanding the stabilization effect of sulfated glycosaminoglycans (GAGs) and heparan sulfate on the structure of amyloid fibrils.

The Generality of Our Findings. To investigate the usability of our simple method for the study of amyloid fibrils of other protein molecules, we carried out experiments on fibrils of the K3 fragment of $\beta 2\text{m}$ and on fibrils of α -synuclein. As shown in Figure 7, K3 fibrils exhibit an increased stability compared to $\beta 2\text{m}$ fibrils with a transition-midpoint 15 degrees higher. K3

peptide is one of the fragments of $\beta 2m$ (21–41 fragment) that is capable of fibril formation in itself and supposed to be the part of the amyloid core region of $\beta 2m$ fibrils.⁵⁰ This increased stability of K3 fibrils might be explained by the fact that in $\beta 2m$ fibrils, only approximately 50% of the polypeptide chain participates in the cross- β -sheet amyloid core;^{50,51} the rest of the protein molecule might decrease fibril stability. α -Synuclein fibrils showed a thermal dissociation profile less steep than that of $\beta 2m$ or K3 fibrils, however the dissociation is apparently complete at 99 °C (Figure 7). These results suggest that $\beta 2m$ is not a unique exception among amyloidogenic proteins and our findings would be also applicable to other amyloid fibrils.

CONCLUSION

One of the major challenges in the current medicine and pharmaceutical industry is to develop the effective treatments against amyloid-related degenerative diseases such as Alzheimer's disease. To prevent the onset or reverse the process of amyloid formation, it is of vital importance to understand the kinetic and thermodynamic basis of the fibril stability. Probing the fibril stability against different destabilizing conditions, high pressure, denaturing agents, or heat, may reveal the possible mechanisms and important aspects of fibril dissociation. In the present work, we studied the thermal stability and mechanism of heat-induced dissociation of $\beta 2$ -microglobulin amyloid fibrils. We found that $\beta 2m$ fibrils, freshly prepared in a seeded polymerization reaction, can be dissociated by a heat-treatment. Kinetic experiments carried out at various temperatures suggested that the depolymerization process leads to a rapid and dynamic equilibrium between the fibrils and the monomers. Repolymerization experiments showed an accelerated regrowth compared to the original rate of fibril formation, indicating that the number of extendable fibril-ends increased significantly upon incubation at elevated temperatures. This observation suggests that the mechanism of fibril unfolding might involve two distinct processes, the dissociation of monomers from the fibril ends and the breakage of fibrils into pieces and draws our attention to the possible difficulties of in vivo disaggregation when the incomplete dissociation of fibrils may lead to an unwanted rapid regrowth and metastases. The thermal stability of $\beta 2m$ fibrils depends on the protein concentration, the seed concentration used for preparation of the fibril solution, and the growth temperature. Sample aging increases the fibril stability significantly. The possible rationale behind the change in stability may lie in the association of the fibrils.

Although, in vivo, the mechanism of amyloid depolymerization may be more complicated than in our reproducible and simple model system and may be different for other proteins, our results further strengthened the view that amyloid fibril formation is not necessarily irreversible and the disaggregation of fibrils might be a possible aspect in future therapies. Our fast and simple method can be useful for the study of the effect of different additives on the stability of amyloid fibrils offering a technique for the screening and developing amyloid-active compounds.

ASSOCIATED CONTENT

S Supporting Information. We compared the morphology of fibrils formed at 37 and 65 °C by transmission electron microscopy. We found that in the 65 °C sample, fibril types with higher complexity and thickness were dominant (Supplementary

Figure 1). We observed that ammonium sulfate exhibits a strong effect on fibril stability. To clarify the background of this effect, we also probed the thermal stability of $\beta 2m$ amyloid fibrils in the presence of ammonium chloride and Na-sulfate. These experiments revealed that the stabilization effect can be exclusively attributed to the sulfate ions. The results are presented in Supplementary Figure 2. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ABBREVIATIONS USED

$\beta 2m$, $\beta 2$ -microglobulin; DRA, dialysis-related amyloidosis; DSC, differential scanning calorimetry; SDS, sodium dodecyl sulfate; ThT, thioflavin T.

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