Heating during agitation of $\beta_2$-microglobulin reveals that supersaturation breakdown is required for amyloid fibril formation at neutral pH

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Amyloidosis-associated amyloid fibrils are formed by denatured proteins when supersaturation of denatured proteins is broken. $\beta_2$-Microglobulin ($\beta_2$m) forms amyloid fibrils and causes dialysis-related amyloidosis in patients receiving long-term hemodialysis. Although amyloid fibrils of $\beta_2$m in patients are observed at neutral pH, formation of $\beta_2$m amyloids in vitro has been difficult to discern at neutral pH because of the amyloid-resistant native structure. Here, to further understand the mechanism underlying in vivo amyloid formation, we investigated the relationship between protein folding/unfolding and misfolding leading to amyloid formation. Using thioflavin T assays, CD spectroscopy, and transmission EM analyses, we found that $\beta_2$m efficiently forms amyloid fibrils even at neutral pH by heating with agitation at high-salt conditions. We constructed temperature- and NaCl concentration-dependent conformational phase diagrams in the presence or absence of agitation, revealing how amyloid formation under neutral pH conditions is related to thermal unfolding and breakdown of supersaturation. Of note, after supersaturation breakdown and following the law of mass action, the $\beta_2$m monomer equilibrium shifted to the unfolded state, destabilizing the native state and thereby enabling amyloid formation even under physiological conditions with a low amount of unfolded precursor. The amyloid fibrils deposited at both lower and higher temperatures, resembling cold- or heat-induced denaturation of globular proteins. Our results suggest an important role for heating in the onset of dialysis-related amyloidosis and related amyloidoses.

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Amyloid fibrils, fibrillar aggregates of denatured proteins or peptides stabilized by intermolecular cross-$\beta$ structures, are associated with many amyloidoses, including neurodegenerative disorders, such as Alzheimer’s and Parkinson’s diseases, and dialysis-related amyloidosis (DRA). The kinetics of amyloid formation are common to amyloidogenic proteins and are typically separated into nucleation, elongation, and equilibrium phases. The nucleation phase, the duration of which is considered a lag time, is often long because of its high energy barrier. Once amyloid nuclei form, the elongation of fibrils occurs rapidly. The equilibrium phase is a thermodynamic state where the monomer concentration has reached its solubility. Seed-dependent elongation is a common property of amyloid formation, in which the nucleation phase is shortened or escaped. Another type of protein misfolding leads to amorphous aggregates. Amorphous aggregates with no ordered structure are seen in cataracts, in inclusion bodies, or during protein preparation. Rapid amorphous aggregation without a lag time competes with the slow nucleation-dependent amyloid formation, producing kinetic complexity of amyloid formation.

$\beta_2$-Microglobulin ($\beta_2$m), a typical amyloidogenic protein with a folded native structure, is a component of the major histocompatibility complex 1. It is filtered by the glomerulus and catabolized by proximal tubular cells in the kidneys in healthy persons. However, $\beta_2$m remains in the blood and sometimes forms amyloid fibrils in patients receiving long-term hemodialysis. Accumulation and deposition of these amyloids in tissues cause DRA. It has been established that amyloid formation of acid-unfolded $\beta_2$m readily occurs. The atomic structure of $\beta_2$m fibrils under acidic conditions has been reported. In contrast, amyloid formation under neutral pH conditions, under which patients develop diseases, does not occur easily because of the amyloid-resistant native structure. Based on previous studies performed with N-terminal truncated or mutant $\beta_2$m or those in the presence of additives, such as collagen, heparin, or SDS, or monomeric unfolded state, N native state.
glycosaminoglycans (14), the denaturation or local enrichment of β2m on biological membranes or extracellular matrix molecules has been suggested to induce amyloid formation in vivo. Recently, Cremers et al. (6) reported that polyphosphates with linearly linked phosphate units are markedly effective in inducing amyloid fibrils of various proteins. We found that polyphosphates effectively induced amyloid formation of β2m at neutral pH (22). However, the exact mechanism of DRA, in particular the factors inducing amyloid nucleation in patients, remains unclear.

Studies on protein aggregation have been also performed from the thermodynamic perspective. Otzen and colleagues (23, 24) reported the relationship between thermodynamic parameters and polymorphism of glucagon amyloid fibrils. Dzwolak and colleagues (25) reported the thermal stability and (23, 24) reported the relationship between thermodynamic from the thermodynamic perspective. Otzen and colleagues (23) reported the thermal stability and degradation of insulin fibrils. We also investigated the effects of heat on the amyloid formation of β2m and other proteins using calorimetry, and compared thermodynamic parameters with those of protein folding (26–29). Recently, we examined the effects of heating on β2m at low pH (12). Acid-unfolded β2m formed amyloid fibrils and amorphous aggregates competitively in a temperature- and NaCl concentration–dependent manner. A unified phase diagram of conformational states suggested that amyloid fibrils depolymerize at both low and high temperatures, reminiscent of cold and heat denaturation of globular proteins (30, 31). The phase diagram also accommodated a region of amorphous aggregates, which were formed under the strong forces of precipitation. Taken together, amyloid fibrils and amorphous aggregates may correspond to crystals and glasses of solutes, respectively, and the breakdown of supersaturation may lead to the formation of crystal-like amyloid fibrils (12, 32). However, such a diagram has not been constructed at neutral pH where DRA develops, and the relationship between protein folding/unfolding and amyloid formation is not clear. Recently, Wang et al. (33) reported the thermodynamic phase diagram of β-amyloid peptide based on a coarse-grained molecular dynamic simulation, in which amyloid fibrils depolymerized with an increase in temperature similar to our heat-induced depolymerization of β2m fibrils (12).

Here, we demonstrate that amyloid formation of β2m occurs even under neutral pH conditions at high temperatures. Of note, although heating caused unfolding (i.e. denaturation) of β2m, a necessary condition of amyloid formation, it was not sufficient for amyloid formation. Amyloid formation occurred only when heat unfolding was performed under agitation, which forced the breakdown of supersaturated unfolded monomers. In other words, both unfolding and breakdown of supersaturation were required. In this report, we use “unfolding” and “denaturation” interchangeably, although the definitions of the two terms may differ. We also use “formation of amyloid fibrils” and “amyloid misfolding” interchangeably to reflect the differences between protein folding and misfolding. The temperature- and NaCl concentration–dependent conformational phase diagrams revealed that the unfolding temperature of the native state decreases once amyloids form, which was exactly reproduced by the linked function of protein folding/unfolding and amyloid misfolding.

Figure 1. Amyloid formation of β2m at neutral pH requires both heat unfolding and agitation. A–D, CD spectra of 8.5 μM β2m at pH 7.0 with 1.0 M NaCl upon heating in the absence (A) or presence of stirring (C). The values of [θ] at 230 nm in the absence (B) or presence (D) of stirring were plotted against temperature (Temp.). The arrows indicate the values after cooling to 25 °C. E and F, ThT assays of β2m under the same conditions as A–D. The intensities of ThT fluorescence and LS are indicated by blue and red, respectively. Dashed lines, absence of stirring; solid lines, presence of stirring. In E, the time course of temperature change is shown by the black line. In F, the Tm value estimated from B is shown. The measurements shown in E and F were repeated three times, and all the data are shown in Fig. S1. a.u., arbitrary units.

Results

Amyloid formation of β2m upon heat unfolding under agitation

We monitored the conformational change of β2m upon heating at neutral pH by far-UV CD measurements. β2m samples in 20 mM sodium phosphate buffer (pH 7.0) and 1.0 M NaCl were heated from 25 to 90 °C with a heating rate of 0.5 °C/min. Without stirring, the CD spectrum demonstrated reversible heat unfolding, with β2m refolded to the native state after cooling to 25 °C (Fig. 1, A and B). In contrast, under stirring, structural conversion to the pronounced β-sheet conformation occurred at 60 °C, and β2m did not refold to the native conformation (Fig. 1, C and D).

To confirm that the conformational change under stirring led to amyloid formation, we carried out thioflavin T (ThT) assays (Fig. 1, E and F, and Fig. S1). We added 5 μM ThT to the same samples as above and measured the ThT fluorescence at 485 nm at a heating rate of 1 °C/min. We simultaneously measured the light scattering (LS) at 450 nm to monitor the total amount of aggregates. In the absence of stirring, neither ThT fluorescence nor LS changed up to 90 °C. Under stirring, both ThT fluorescence and LS increased markedly beginning at ~66 °C, which was near the midpoint temperature (Tm) for heat unfolding of β2m under these conditions (Fig. 1, E and F, and Fig. S1).
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We investigated the seeding activity of heat-induced β₂m amyloid fibrils prepared with 1.0 mM NaCl. Although no amyloid formation occurred without stirring when monitored by ThT fluorescence or LS at a heating rate of 1 °C/min, it occurred in the presence of seeds even under quiescent conditions (Fig. 4A). After these experiments, we carried out CD measurements at 25 °C. The seeded sample after heat-induced transition exhibited β-structure typical of amyloid fibrils, whereas the non-seeded sample demonstrated refolding to native β₂m (Fig. 4C). Under stirring, we detected no difference between heating-denatured spontaneous amyloid formation and seed-dependent reactions (Fig. 4B). Additionally, we performed differential scanning calorimetry measurements in the presence or absence of seeds, confirming that seeds broke supersaturation when combined with unfolding of the native state (Fig. 4D). These results were confirmed by LED visualization images (Fig. 3, D and E, blue boxes).

With 250 mM NaCl at 70 °C, the seeds shortened the lag time (Fig. 4E). In contrast, with 250 mM NaCl at 90 °C, no reaction occurred even in the presence of seeds (Fig. 4F). TEM images confirmed amyloid fibrils and no fibrous aggregates at 70 and 90 °C, respectively (Fig. 4, E and F, insets). The LED visualization images were consistent with these temperature-dependent seeding reactions (Fig. 3, D and E, green boxes). The results suggested that because the solubility of β₂m with 250 mM NaCl at 90 °C was higher than the protein concentration (8.5 μM), no fibrils formed, even with seeds.

Depolymerization of heat-induced amyloid fibrils

First, β₂m amyloid fibrils were prepared in 0.5 mM NaCl at pH 7.0 and 70 °C. The fibrils were recovered by centrifugation and

Figure 2. Effects of temperature on heat-induced β₂m amyloid formation. A and B, amyloid formation of 8.5 μM β₂m at pH 7.0 with 1.0 mM NaCl under stirring was monitored by ThT fluorescence (A) and LS (B) at varying temperatures with n = 3. C, dependences of final ThT intensity (blue), final LS intensity (red), and lag time monitored by ThT (black) on temperature (Temp.). The final ThT and LS intensities were measured at 25 °C. D, CD spectra of the samples after the assays of amyloid formation. Each measurement was carried out at the reaction temperature indicated by the spectral color. The dashed black line indicates the spectrum of the native state at 25 °C. The CD spectra at various NaCl concentrations are systematically shown in Fig. S5, and D is the same as Fig. S5 at 1.0 mM NaCl. E–J, TEM images of the β₂m samples after the conformational transition. Scale bars, 200 nm. The TEM images at various NaCl concentrations and 70 and 90 °C are shown in Fig. S6. To show fibril formation at low NaCl concentrations, and H and J are the same as those at 1.0 mM NaCl in Fig. S6. a.u., arbitrary units.

Temperature dependence of heat-induced amyloid formation

ThT assays with 0.1 mg/ml β₂m were performed in 20 mM sodium phosphate buffer (pH 7.0), 1.0 mM NaCl, and 5 μM ThT at varying temperatures under stirring. Amyloid formation occurred at all temperatures between 40 and 90 °C, although the reaction accelerated markedly with an increase in temperature (Fig. 2, A and B). After these assays, we measured the final ThT intensity at 25 °C to remove complexity caused by thermal effects on ThT fluorescence. ThT fluorescence at 25 °C did not depend on the reaction temperature, although the final LS value slightly increased with an increase in temperature. In contrast, the lag time became significantly shorter at higher temperatures (Fig. 2C). We also carried out CD measurements at the reaction temperatures. All CD spectra after heat-induced transition exhibited β-structures typical for amyloid fibrils (Fig. 2D). Additionally, we confirmed amyloid formation by TEM images (Fig. 2, E–J).

Effects of NaCl on amyloid formation

To investigate the effects of NaCl on the amyloid formation, we carried out the same ThT assays as those shown in Fig. 2 (A and B) at varying NaCl concentrations (0–3.0 mM) and temperatures (50–90 °C). With 0.5–3.0 mM NaCl, amyloid formation monitored by ThT fluorescence occurred at varying temperatures and NaCl concentrations. However, with 0–0.25 mM NaCl, no reaction occurred at 90 °C, although amyloid fibrils formed at lower temperatures (Fig. 3, A–C, Figs. S2–S4).

We assessed the secondary structures and morphologies after ThT assays. Samples without an increase in ThT fluorescence or LS remained unfolded when monitored by CD (Fig. S5) and exhibited no fibrous aggregates when examined by TEM (Fig. S6). In contrast, the samples with increases in ThT fluorescence and LS had amyloid fibrils even at lower NaCl concentrations (Fig. S6).

We previously reported that visualization using white and blue light-emitting diode (LED) lamps is a useful method for detecting amyloid fibrils (Fig. 3, D and E) (11, 34). The heat-treated β₂m samples at pH 7.0 without agitation exhibited neither turbidity nor ThT fluorescence, consistent with the reversible heat unfolding under these conditions. Under stirring, samples with lower concentrations of NaCl at higher temperatures exhibited neither turbidity nor ThT fluorescence, confirming that they remained soluble below the solubility limit. The remaining samples at pH 7.0 under agitation exhibited slight turbidity and strong ThT fluorescence, suggesting that agitation broke supersaturation and led to amyloid formation. For comparison, we prepared amorphous aggregates of β₂m with 1.0 mM NaCl at pH 2.0 (9, 12). These amorphous aggregates exhibited strong turbidity without ThT fluorescence (Fig. 3, D and E, Amorphous).
then suspended in 0 m NaCl at pH 7.0. When these fibrils were incubated at 90 °C, significant decreases in both LS and ThT intensities were observed (Fig. S7 A). However, the LS intensity remained relatively high, and the CD spectrum did not represent the refolded β2m structure (Fig. S7, B and C), suggesting that fibril depolymerization occurred partially. According to the TEM images, long amyloid fibrils were observed with 0.5 m NaCl at 70 °C, whereas no significant fibrils remained after the incubation with 0 m NaCl at 90 °C (Fig. S7, D and E). We also examined fibril depolymerization by LED images, which demonstrated weakened ThT fluorescence (Fig. 3, D and E, Depolymerized).

**Effects of ultrasonication and other salts**

We examined the effects of ultrasonic irradiation (35, 36) on the amyloid formation of β2m at pH 7.0 and 60 °C with a water bath–type ultrasonicator and microplate reader. As expected, ultrasonication caused amyloid formation with 0.1–2.0 m NaCl in a manner similar to that by stirring (Fig. S8, A and D). We also investigated the effects of Na₂SO₄ (Fig. S8, B and E) and tetraphosphate (tetP) (Fig. S8, C and F), both of which are amyloid-inducing additives. The results confirmed that, when combined with other promoting factors, heating effectively induces amyloid fibrils.

**Discussion**

**Comparison of monomer folding and amyloid misfolding**

To address the mechanism underlying the temperature-dependent amyloid formation/depolymerization, we recalled the thermodynamics of monomer folding/unfolding (Figs. 5 and 6 and Movie S1) (30, 31). The stability of the native state of globular proteins depends on temperature, exhibiting “heat denaturation” and occasionally “cold denaturation.” We assumed folding transition from the denatured (D) to native (N) states (D ⇔ N) with the equilibrium constant of folding (Kₙ) to compare with amyloid “misfolding”:

\[
\text{D} \rightleftharpoons \text{N}
\]

**Mechanism 1**

\[
K_n = [\text{N}]/[\text{D}]
\]

(Geq. 1)

Gibbs free energy change of folding to the native state (ΔGₙ(T) = −RT ln Kₙ) at temperature T in Kelvin is determined by the enthalpy (ΔHₙ(T)) and entropy changes (ΔSₙ(T)),

\[
\Delta G_n(T) = -RT \ln K_n = \Delta H_n(T) - T \Delta S_n(T) - a[\text{NaCl}]
\]

(Eq. 2)

\[
\Delta H_n(T) = \Delta H_n(T_0) + \Delta C_{p,N} (T - T_0)
\]

(Eq. 3)

\[
\Delta S_n(T) = \Delta S_n(T_0) + \Delta C_{p,N} \ln(T/T_0)
\]

(Eq. 4)

where \(\Delta H_n(T_0), \Delta S_n(T_0), \Delta C_{p,N},\) and \(a[\text{NaCl}]\) are the enthalpy and entropy changes at a reference temperature \(T_0\), heat capacity change of folding, and a contribution of NaCl at [NaCl] with a coefficient \(a\), respectively. The temperature dependences of \(\Delta G_n(T), \Delta H_n(T),\) and \(T \Delta S_n(T)\), assuming \(T_0 = 310 \text{ K}, \Delta H_n(T_0) = -174.7 \text{ kJ mol}^{-1}, \Delta S_n(T_0) = -0.5 \text{ kJ mol}^{-1} \text{ K}^{-1}, \Delta C_{p,N} = -5.6 \text{ kJ mol}^{-1} \text{ K}^{-1}\), which were taken from Kardos et al. (29), and \(a = 2.5 \text{ kJ mol}^{-1} \text{ M}^{-1} \text{ NaCl}^{-1}\), which was estimated from the \(T_m\) measurements (Fig. 6B, panel i), are shown in panel i of Fig. 6A.

**Figure 3. Effects of NaCl concentration on heat-induced β2m amyloid formation.** A–C, comparisons of the final ThT intensities (A), final LS intensities (B), and ThT-monitored lag times (C) at varying NaCl concentrations and temperatures (Temp.), \(n = 3\). D and E, direct LED visualization images of β2m samples used in this study and the amorphous aggregates of acid-denatured β2m. Turbidimetry (D) and ThT fluorescence (E) images were obtained using white and blue LED lamps, respectively. The real size of images is 6.5 cm × 6.5 cm. a.u., arbitrary units.

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Figure 4. Seeding activities of the heat-induced β2m amyloid fibrils. A and B, ThT assays with or without 5% preformed seed fibrils under the same conditions as (Fig. 1C) with (B) or without (A) stirring. Time courses of ThT and LS are represented by the blue and red lines, respectively. C, CD spectra of the samples after assays shown in A and the native β2m. The measurements were performed at 25 °C. D, DSC measurements of 42.5 μm β2m with 1.0 μM NaCl at pH 7.0 in the absence or presence of 1% seeds. The lines represent the C1 values for the nonseeded (blue) and seeded (red) reactions. E and F, seeding experiments under the conditions of spontaneous amyloid formation (i.e. 250 μM NaCl and 70 °C) and of no amyloid formation (i.e. 250 μM NaCl and 90 °C). Insets show the TEM images of seeded samples. Scale bars, 200 nm. a.u., arbitrary units; Temp., temperature.

The fraction of the N state (F_N) was defined by the following.

\[
F_N = \frac{[N]}{[N] + [D]} = \frac{K_{N1}}{K_{N1} + 1} \exp \left( -\frac{\Delta G_{N}(T)/RT}{RT} + 1 \right)
\]  
(Eq. 5)

The temperature dependences of F_N and F_D (1 - F_N) at 1.0 M NaCl are shown in panel iii of Fig. 6A. We constructed the temperature- and NaCl concentration-dependent folding phase diagram drawn using the following,

\[
[\text{NaCl}]_{0.5}(T) = \frac{\Delta H_{N0}(T) - T\Delta S_{N0}(T)}{\alpha}
\]  
(Eq. 6)

where [NaCl]_{0.5}(T) is the midpoint NaCl concentration of folding (ΔG_{N0}(T) = 0, F_N = 0.5) at T (Fig. 6B, panel i).

The ΔH_N(T) value, which is “negative” above 20 °C, decreases with an increase in temperature because of a negative heat capacity change of folding (ΔC_{p,N}). The ΔC_{p,N} value, and thus temperature dependence of ΔH_N(T), largely result from hydrophobic interactions that stabilize the native state. On the other hand, -TΔS_{N}(T) term, which includes the decrease in conformational entropy upon folding, increases in magnitude with an increase in temperature more than that of the enthalpy term. The combined effects of destabilizing entropy and stabilizing enthalpy terms are the “negative” ΔG_N(T) (folding) around room temperature and “positive” ΔG_N(T) (unfolding) at high temperatures, leading to heat denaturation. Regarding cold denaturation, which was not evident for β2m, the decrease in ΔH_N(T) in magnitude directly explains the conversion of negative ΔG_N(T) (folding) to positive ΔG_N(T) (unfolding) upon cooling (30, 31).

**Thermodynamics of amyloid formation**

Although the detailed mechanisms of amyloid formation remain elusive (37), a simplified model (mechanism 2) is valid for describing the equilibrium between monomers (M) and polymeric fibrils (P) (15, 29, 38, 39).

\[
P + M \rightleftharpoons P
\]  
(Mechanism 2)

The elongation of fibrils is defined by the equilibrium association constant (K_{pol}) as follows.

\[
K_{pol} = \frac{[P]}{[P][M]}
\]  
(Eq. 7)

The equilibrium is independent of the molar concentration of amyloid fibrils, [P]. Hence, we obtained the equilibrium monomer concentration [M]_C as follows.

\[
[M]_C = 1/K_{pol}
\]  
(Eq. 8)

[M]_C is referred to as the “critical concentration” (28, 29, 38, 39) because amyloid fibrils form when the concentration of monomers exceeds [M]_C. By determining [M]_C, we can calculate the apparent free energy change of amyloid formation (ΔG_{pol}(T)) by the following.

\[
\Delta G_{pol}(T) = -RT\ln K_{pol} = RT\ln[M]_C
\]  
(Eq. 9)

We assumed that Gibbs free energy equations (i.e. ΔH_{pol}(T) and ΔS_{pol}(T)) also hold true for polymeric amyloid fibrils (28, 29) (Fig. 6A, panel ii),

\[
\Delta G_{pol}(T) = \Delta H_{pol}(T) - T\Delta S_{pol}(T) = -b[\text{NaCl}]
\]  
(Eq. 10)

\[
\Delta H_{pol}(T) = \Delta H_{pol}(T_0) + \Delta C_{p, pol}(T - T_0)
\]  
(Eq. 11)

\[
\Delta S_{pol}(T) = \Delta S_{pol}(T_0) + \Delta C_{p, pol}\ln(T/T_0)
\]  
(Eq. 12)

where b[NaCl] represents the contribution of NaCl at [NaCl] with a coefficient b. We previously reported that ΔH_{pol}(T) value for β2m at pH 2 was linearly dependent on temperature, giving a ΔC_{p, pol} value of -5.0 kJ mol^{-1} K^{-1} (28), a slightly smaller value than that of the native state: -5.6 kJ mol^{-1} K^{-1} (29). We assumed the same ΔC_{p, pol} value for the amyloid fibrils at pH 7.

If the total protein concentration, [Prot]_T, is higher than [M]_C, the fraction of the polymeric P state is defined by the following.
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![Figure 5. Schematic representations of $\beta_2$m before and after the linkage of folding and misfolding transitions. Schematic (upper panel) and free-energy (lower panel) representations of the linkage of $\beta_2$m unfolding with two types of aggregates, in which amyloid misfolding occurs after agitation-dependent breakdown of supersaturation or by seeding.](Image)

\[ F_{\text{Pol}} = \frac{[\text{Prot}] - [M]_C}{[\text{Prot}]_{\text{T}}} = \frac{[\text{Prot}] - \exp(\Delta G_{\text{pol}}(T)/RT)}{[\text{Prot}]_{\text{T}}} \]  

(Eq. 13)

Temperature dependences of fractions of D and P states for amyloid formation are shown in panel iv of Fig. 6A. If $[\text{Prot}]_{\text{T}}$ is smaller than $[M]_C$, $F_{\text{Pol}} = 0$. The NaCl concentration, $[\text{NaCl}]_{\text{Pol,0.5}}(T)$, where $[\text{Prot}]_{\text{T}} = [M]_C$, is obtained by the following equation.

\[ [\text{NaCl}]_{\text{Pol,0.5}}(T) = \frac{-RT\ln[\text{Prot}]_{\text{T}} + \Delta H_{\text{pol}}(T) - T\Delta S_{\text{pol}}(T)}{b} \]  

(Eq. 14)

Based on these considerations, we constructed the temperature- and NaCl concentration-dependent phase diagram of amyloid formation at $[\text{Prot}]_{\text{T}} = 0.1 \text{ mg/ml}$ drawn by the following,

\[ [\text{NaCl}]_{\text{Pol,0.5}}(T) = \frac{-RT\ln[\text{Prot}]_{\text{T}} + \Delta H_{\text{pol}}(T) - T\Delta S_{\text{pol}}(T) - \exp((\Delta G_{\text{pol}}(T)/RT)}{b} \]  

(Eq. 15)

where $[\text{NaCl}]_{\text{Pol,0.5}}(T)$ is the midpoint NaCl concentration of amyloid formation ($F_{\text{Pol}} = 0.5$), assuming $b = 25.0 \text{ kJ mol}^{-1} \text{ M}_\text{NaCl}^{-1}$ (Fig. 6B, panel ii).

The previous results (28, 38, 39) and current considerations argue that the solubility of monomers ([M]C) and thus the stability of amyloid fibrils are determined by a mechanism similar to that of protein folding. This is not surprising considering that intramolecular protein folding and intermolecular amyloid misfolding are driven by similar forces, including hydrophobic interactions, hydrogen bonds, and van der Waals interactions. It is well-known that the solubility in water of hydrophobic substances against temperature exhibits a U-shaped dependence, representing the large positive $\Delta C_p$ value for “dissolution” (40). Thus, the simplified mechanism of amyloid dissolution resembles the dissolution equilibrium of a hydrophobic solute in which the equilibrium solute concentration (i.e. solubility) represents the free energy change of dissolution.

Assuming the similarity of protein folding and amyloid misfolding, heat-induced depolymerization of amyloid fibrils is expected because of the increased conformational entropy. On the other hand, the low temperature-induced depolymerization (41) or decreased amyloid formation at low temperatures is expected to occur because of the decreased hydrophobic interactions. Although the rigid tight packing of side chains producing steric zippers and more extensive cross-$\beta$ network (1) may provide distinct properties, including supersaturation-limited amyloid formation, the overall calorimetric properties of amyloid formation resemble those of protein folding.

**Linkage between reversible unfolding and supersaturation-limited misfolding**

Three conformational states are linked after the breakdown of supersaturation ($N \rightleftharpoons D \rightleftharpoons P$) and are under thermodynamic equilibrium (mechanism 3 and Movie S1):

$$ N \rightleftharpoons D \rightleftharpoons P $$

= Mechanism 3

Although equations for the processes of $N \rightleftharpoons D$ and $D \rightleftharpoons P$ did not change, the fractions of $N$ ($F_N$), $D$ ($F_D$), and $P$ ($F_{\text{Pol}}$) were newly defined as follows,

$$ F_N = \frac{[N]}{[N] + [D] + [P]} = \frac{K_{N[D]}[\text{Prot}]}{[\text{Prot}]_{\text{T}}} \exp\left((-\Delta G_{\text{pro}}(T)/RT)\right) $$  

(Eq. 16)

$$ F_D = \frac{[D]}{[N] + [D] + [P]} = \frac{\exp(\Delta G_{\text{pro}}(T)/RT)}{[\text{Prot}]_{\text{T}}} $$  

(Eq. 17)

and $F_{\text{Pol}} = 1 - F_N - F_D$. These equations are valid when $[\text{Prot}]_{\text{T}} > (1 + K_{N[D]}[M]_C)$, where the right side indicates the maximal equilib-
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When [Prot]₀ < (1 + K_N)[M]₀, the fractions of respective species are the same as those described under supersaturation (i.e. without amyloid fibrils).

The temperature dependences of the three conformational states with 1.0 M NaCl shown in Fig. 6 (A, panel v, and B, panel iii) were distinct from those under supersaturation. Most notably, the unfolding transition started at significantly lower temperatures than that under supersaturation, indicating a significant decrease in $T_m$ for thermal denaturation. This can be simply explained by the law of mass action: the N $\rightleftharpoons$ D equilibrium shifts to D because the newly participating amyloid fibrils (P) drive the overall equilibrium to P. Indeed, with 1.0 M NaCl, amyloid fibrils formed at 40 °C, even though the lag time was as long as 30 h (Fig. 2C), consistent with the consideration addressed here. Although the law of mass action has not been addressed previously in the formation of amyloid fibrils starting from the native state, it may have an important role in explaining the propagation of amyloid fibrils even under conditions where the precursor amyloidogenic states are negligibly small.

We constructed the temperature- and NaCl concentration–dependent conformational phase diagrams of β₂m before and after the linkage of folding and misfolding transitions. A, temperature dependences of thermodynamic parameters ($\Delta G(T)$, $\Delta H(T)$, and $T\Delta S(T)$) for folding (mechanism 1, panel i) and amyloid formation (mechanism 2, panel ii). Fractions of N, D, and P states for folding (panel iii), amyloid formation (panel iv), and their linked conditions (mechanism 3, panel v) are also shown. The plots were made using 0.1 mg/ml β₂m, 1.0 M NaCl, pH 7.0. B, phase diagrams for folding/unfolding (mechanism 1, panel i), amyloid misfolding (mechanism 2, panel ii), and their linked conditions (mechanism 3, panel iii). Lines show the simulated phase boundaries and dots show the experimental data at 0.1 mg/ml β₂m and pH 7.0. Temp., temperature.

Figure 6. Temperature- and NaCl concentration–dependent conformational phase diagrams of β₂m before and after the linkage of folding and misfolding transitions. A, temperature dependences of thermodynamic parameters ($\Delta G(T)$, $\Delta H(T)$, and $T\Delta S(T)$) for folding (mechanism 1, panel i) and amyloid formation (mechanism 2, panel ii). Fractions of N, D, and P states for folding (panel iii), amyloid formation (panel iv), and their linked conditions (mechanism 3, panel v) are also shown. The plots were made using 0.1 mg/ml β₂m, 1.0 M NaCl, pH 7.0. B, phase diagrams for folding/unfolding (mechanism 1, panel i), amyloid misfolding (mechanism 2, panel ii), and their linked conditions (mechanism 3, panel iii). Lines show the simulated phase boundaries and dots show the experimental data at 0.1 mg/ml β₂m and pH 7.0. Temp., temperature.
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Dependent “three states” phase diagram defined by the midpoint NaCl concentrations for the native state ([NaCl]_{N,0.5}(T)) and polymeric amyloid ([NaCl]_{Pol,0.5}(T)).

\[
[\text{NaCl}]_{N,0.5}(T) = \frac{\Delta H_{\text{Prot}}(T) - \Delta H_n(T) - T\Delta S_{\text{Prot}}(T) + T\Delta S_n(T) - RT \ln([\text{Prot}]_r/2)}{b - a} 
\]

(Eq. 18)

\[
[\text{NaCl}]_{Pol,0.5}(T) = \frac{RT \ln([\text{Prot}]_r/2) - 2\Delta H_{\text{Prot}}(T) + \Delta H_n(T) + 2T\Delta S_{\text{Prot}}(T) - T\Delta S_n(T)}{a - 2b} 
\]

(Eq. 19)

The calculated and experimental phase diagrams are shown in Fig. 6B (panel iii). It is noted that at NaCl concentrations below 0.1 M, where the three states coexisted, we calculated the boundaries by estimating the points where the concentrations of two species are equal. This type of linkage between protein unfolding and aggregation will occur for any type of aggregate, and the consideration presented here provides a quantitative explanation as to how the linkage affects the stability of the native state.

Implications for amyloid formation under physiological conditions

Although we demonstrated amyloid formation of β2m at pH 7.0 and temperatures higher than 40 °C, similar amyloid formation likely effectively occurs even at physiological temperatures once helped by additional amyloid-promoting factors. These include various biological factors (heparins, lysophosphatidic acids, and polyphosphates) and mechanical agitation, which will cause the onset of DRA (6, 14, 21, 42, 43). Localized unfolding and/or enhanced aggregation at air–liquid interface are likely to be additional important factors breaking supersaturation. Recently, the effects of polyphosphate on amyloid formation have been focused on (6, 22). We found that tetP markedly accelerated amyloid formation at 60 °C, although the exact dependence on the tetP concentration was complicated. We further argue that because of the linkage of protein unfolding with amyloid misfolding, local high temperatures, even if moderate (e.g., 40 °C), are an important risk factor.

Another important parameter determining the phase diagram is the protein concentration. As protein aggregates form above the solubility of their monomers, high protein concentrations enhance protein aggregation (32, 44). We carried out heat-induced amyloid formation at 25.5 μM (0.3 mg/ml) β2m under neutral pH conditions (Fig. S9). β2m at 25.5 μM formed amyloid fibrils under several conditions, whereas β2m at 8.5 μM did not form fibrils with lower NaCl concentrations at higher temperatures. Of note, 25.5 μM β2m with 1.0 μM Na2SO4 formed a mixture of amyloid fibrils and amorphous aggregates, although 8.5 μM β2m formed only amyloid fibrils under the same conditions (Fig. S10).

Conclusions

We demonstrated that heat unfolding under agitation effectively caused amyloid formation of intact β2m even at neutral pH and temperatures slightly higher than physiological. Importantly, heat-induced amyloid formation of β2m occurred only under agitation or seeding, which suggested that the breakdown of supersaturation is necessary for amyloid formation, in addition to protein unfolding. We formulated a relationship between the reversible protein unfolding and supersaturation-limited amyloid misfolding. Upon the breakdown of supersaturation, the law of mass action shifts the monomer equilibrium to the direction of the unfolded state, destabilizing the native state and thereby enabling amyloid formation even under physiological conditions with a negligible amount of the unfolded precursor (Movie S1). It is possible that the linked function under seeding conditions plays a role in the pathology of various amyloidoses, including the marked infectious behavior observed for prion seeds.

Finally, we compared heat-dependent protein unfolding and amyloid formation to obtain a unified mechanism of protein folding and misfolding. In the past decades, studies on protein folding/unfolding often employed heat denaturation at non-physiological high temperatures (30, 31). The thermodynamic parameters elucidated under these extreme conditions have been valuable for clarifying the mechanism of protein folding under physiological conditions, because they can be extrapolated back to the physiological conditions. The same was true for amyloid misfolding. The properties elucidated under non-physiological high temperatures could be extrapolated to the physiological conditions. Most importantly, we elucidated that the linkage of protein unfolding and misfolding promotes amyloid formation even at physiological temperatures. Taken together, the heating and agitation used in this paper, although they were nonphysiological, will be powerful for further addressing the mechanism of amyloid formation under physiological conditions.
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under quiescence. The sample temperature was controlled using a Peltier element (Nippon Tecmo Co., Ltd., Fukuoka, Japan) and measured by a thermocouple Compact Thermog-ger AM-8000K (Anritsu Meter Co., Ltd., Tokyo, Japan).

CD and TEM measurements

The sample solutions were the same as above, except for the volume. Far-UV CD spectra (212–250 nm) were obtained with a J-720 spectropolarimeter (Jasco Co., Ltd., Tokyo, Japan) using a quartz cell with a 1-mm or 1-cm path length. CD data were expressed as mean residue ellipticity.

The sample solution (5 $\mu$L) was spotted onto a collodion-coated copper grid (Nissin EM Co., Ltd., Tokyo, Japan). After 1 min, the solution on the grid was removed with filter paper. Then 5 $\mu$L of 0.5% (w/v) hafnium chloride was spotted onto the grid. After 1 min, the solution was removed in the same manner.

TEM images were obtained using a H-7650 transmission microscope (Hitachi High-Technologies Corporation, Tokyo, Japan) at 20 °C with a voltage of 80 kV.

Seeding reactions

Seeds were obtained from the preformed fibrils of heat-induced spontaneous amyloid formation under stirring and sonicated moderately before seeding experiments. The conditions for the sample preparation and measurements were the same as for standard ThT assays, except for the addition of 5% (v/v) seeds.

Fibril depolymerization

Heat-induced amyloid fibrils of $\beta2m$ were formed under the same conditions used for the ThT assays with 500 mM NaCl at 70 °C. These fibrils were centrifuged at 15,000 rpm and 25 °C for 20 min and then suspended at 5 $\mu$m ThT in 20 mM phosphate buffer (pH 7.0). The CD measurement and ThT assay were carried out at 25 °C, and the solution was then incubated at 90 °C under stirring at 600 rpm. Intermittent ThT assays were performed every 10 min during the incubation. After the incubation, the CD measurement and ThT assay were performed at 25 °C.

Microplate ThT assays

Lyophilized $\beta2m$ was dissolved in deionized water. The sample solution contained 8.5 $\mu$m $\beta2m$, 5 $\mu$m ThT, 20 mM phosphate buffer (pH 7.0), and variable concentrations of NaCl, Na$_2$SO$_4$, or sodium tetP. The sample solutions of 0.2 ml were distributed into the wells of a 96-well microplate (catalog no. 675076; Greiner Bio-one Co., Ltd., Frickenhausen, Germany). The microplate was set on a Elestein SP070-PG-M water bath–temperature and salt effects on competitive formation of amyloids versus amyloidization.

Heating-induced amyloid fibrils of $\beta2m$ were formed under the same conditions used for the ThT assays with 500 mM NaCl at 70 °C. These fibrils were centrifuged at 15,000 rpm and 25 °C for 20 min and then suspended at 5 $\mu$m ThT in 20 mM phosphate buffer (pH 7.0). The CD measurement and ThT assay were carried out at 25 °C, and the solution was then incubated at 90 °C under stirring at 600 rpm. Intermittent ThT assays were performed every 10 min during the incubation. After the incubation, the CD measurement and ThT assay were performed at 25 °C.

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