Aggregation-phase diagrams of β₂-microglobulin reveal temperature and salt effects on competitive formation of amyloids versus amorphous aggregates

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Several serious diseases are associated with crystal-like amyloid fibrils or glass-like amorphous aggregates of denatured proteins. However, protein aggregation involving both types of aggregates has not yet been elucidated in much detail. Using a protein associated with dialysis-related amyloidosis, β₂-microglobulin (β₂m), we previously demonstrated that amyloid fibrils and amorphous aggregates form competitively depending on salt (NaCl) concentration. To examine the generality of the underlying competitive mechanisms, we herein investigated the effects of heat on acid-denatured β₂m at pH 2. Using thioflavin fluorescence, CD, and light scattering analysis along with atomic force microscopy imaging, we found that the temperature-dependent aggregation of β₂m markedly depends on NaCl concentration. Stepwise transitions from monomers to amyloids and then back to monomers were observed at low NaCl concentrations. Amorphous aggregates formed rapidly at ambient temperatures at high NaCl concentrations, but the transition from amorphous aggregates to amyloids occurred only as the temperature increased. Combining the data from the temperature- and NaCl-dependent transitions, we constructed a unified phase diagram of conformational states, indicating a parabolic solubility curve with a minimum NaCl concentration at ambient temperatures. Although amyloid fibrils formed above this solubility boundary, amorphous aggregates dominated in regions distant from this boundary. Kinetic competition between supersaturation-limited slow amyloid fibrillation and supersaturation-unlimited fast amorphous aggregation deformed the phase diagram, with amyloid regions disappearing with fast titration rates. We conclude that phase diagrams combining thermodynamics and kinetics data provide a comprehensive view of β₂m aggregation exhibiting severe hysteresis depending on the heat- or salt-titration rates.

There are more than 30 disease-related amyloidogenic proteins, including β-amyloid peptide associated with Alzheimer’s disease and β₂-microglobulin (β₂m)3 with dialysis-related amyloidosis (1–6). Various amyloidogenic proteins form amyloid fibrils with the common properties of highly ordered cross-β structures stabilized by peptide hydrogen bonds (2, 3, 6–8). The kinetics of the formation of amyloid fibrils are also common to various amyloidogenic proteins and may be divided into two steps: the nucleation and elongation phases (9–11). The nucleation phase, the duration of which is a lag time, is long because of a high free-energy barrier. Once amyloid nuclei form, the elongation of fibrils occurs rapidly. Seed-dependent elongation is another common property of amyloid fibril formation, in which the nucleation phase is shortened or avoided by the addition of seed fibrils. Amorphous aggregates are an alternative form of aggregated denatured proteins. Amorphous aggregates are also associated with diseases, such as cataracts (12, 13). The inherent toxicity of aggregates implies a common mechanism for protein misfolding diseases (14). In practice, amorphous aggregates often contaminate the preparation of amyloid fibrils. Amorphous aggregate designation often accommodates the oligomers responsible for the cytotoxicity of amyloidogenic proteins (15, 16). Despite their potential medical importance, the role that amorphous aggregates play in amyloid fibrillation has yet to be clarified.

When amyloid fibrils are regarded as crystal-like precipitates of denatured proteins, amorphous aggregates are presumed to be a glass-like state populated under conditions where the driving forces of precipitation are too strong to retain amyloid fibrils (17, 18). This viewpoint may enable a more comprehensive understanding of aggregation in terms of solubility, super-
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saturation (or supercooling), and competition between the two types of aggregated states (17–19).

By considering solubility and supersaturation, we previously showed with β₂m that amyloid fibril formation and amorphous aggregation competed in a manner that depended on NaCl concentrations, thereby creating a competitive mechanism of aggregation (17, 20). We then examined the heparin-dependent amyloid formation of hen egg white lysozyme, a model amyloidogenic protein, revealing two distinct mechanisms of amyloid formation (21). In both cases, amyloid fibril formation competed against amorphous aggregation, producing a complex heparin concentration-dependent phase diagram.

To establish the generality of the competitive mechanism, we are now focusing on the effects of temperature. A large number of studies have investigated the effects of heat on the formation of amyloid fibrils. Otzen and co-workers (22, 23) reported the stepwise heat-induced formation and degradation (i.e. dissociation or depolymerization) of glucagon amyloid fibrils. We also examined the effects of heat on the fibril formation of β₂m and other proteins (24–30). Moreover, using isothermal titration calorimetry, we performed calorimetric measurements of β₂m amyloid fibrillation and compared thermodynamic parameters with those of protein folding (31–33). The findings obtained showed that amyloid fibrils were in a thermodynamic state of unique calorimetric properties, amyloid fibrils degraded to monomers at higher temperatures, and low-temperature-induced degradation (i.e. cold denaturation) occurred for amyloid fibrils of α-synuclein (32). However, the relationship between amyloid fibrils and amorphous aggregates with respect to temperature has not yet been elucidated.

By combining a series of temperature- and salt-dependent transitions, we constructed a unified phase diagram of the conformational states of β₂m. The phase diagram showed that phase transitions from soluble monomers to crystal-like amyloid fibrils and then glass-like amorphous aggregates are common to three types of variables that decrease the solubility of β₂m (i.e. increasing NaCl concentrations, increasing the temperature at the low temperature region, or decreasing the temperature at the high temperature region), thereby providing a comprehensive view of protein aggregation.

Results

Temperature dependence of amyloid formation and amorphous aggregation

We investigated the dependence of β₂m aggregation on temperature, for which the heating rate was controlled using a Peltier element (Fig. 1A and Figs. S1 and S2). Amyloid fibrillation was measured by thioflavin T (ThT) fluorescence at 485 nm with a heating rate of 0.2 °C/min. We simultaneously measured light scattering at 445 nm to monitor the total amount of aggregates. Based on these measurements, we distinguished amyloid fibrils and amorphous aggregates. Furthermore, to obtain information on secondary structures, CD measurements were performed by removing aliquots of the solution from the fluorometer at the desired time points.

Under standard solvent conditions (8.5 μM (0.1 mg/ml) β₂m, 0.1 M NaCl, 5 μM ThT, and 10 mM HCl under stirring) at a constant temperature of 25 °C, amyloid fibrillation occurred with a lag time of ~3 h and finished at ~5 h (Fig. 1, A and B). Increases in ThT and light scattering intensity occurred simultaneously, and the change in ellipticity was consistent with those in ThT and light scattering intensities (Fig. 1, B and C). These results indicated that the formation of amyloid fibrils occurred cooperatively without the accumulation of amorphous aggregates.

When β₂m solution in the absence of NaCl was heated at 0.2 °C/min, no aggregation occurred when monitored by ThT or light scattering (Fig. 1D). CD spectra showed that β₂m remained unfolded (Fig. 1, E and F). Upon heating in the presence of 0.1 M NaCl, ThT and light scattering intensities both increased at ~40 °C (Fig. 1G), indicating the formation of amyloid fibrils. ThT fluorescence significantly decreased with an increase in temperature. This decrease initially suggested the degradation of amyloid fibrils. However, because subsequent cycles of decreases and increases in temperature reproduced the temperature-dependent change in ThT fluorescence without affecting light scattering and the CD spectrum typical for the β-structure (Fig. S1), the decrease observed in ThT intensity was attributed to a reduction in the efficiency of the fluorescence of ThT at a high temperature.

We previously showed with differential scanning calorimetry (DSC) that β₂m amyloid fibrils at 0.1 mg/ml were completely degraded upon heating and that the transition temperature of degradation increased with elevations in the concentration of NaCl (see Fig. 4 of Ref. (24; see also Figs. 2 and 5). We also reported the complete degradation of β₂m amyloid fibrils at 0.1–0.3 mg/ml upon a 10-min incubation at 99 °C monitored by ThT fluorescence and CD spectroscopy (30). Heat-induced degradation was also reported for various amyloid fibrils (32, 34, 35). The ellipticity value at 200 nm indicative of the β-structure component (Fig. 1, H and I) and the large amount of amyloid fibrils observed in atomic force microscopy (AFM) images taken after cooling (Fig. 1G, inset) suggested that the highest temperature employed under the current experimental conditions (90 °C; Fig. 1, G and H) was not sufficient to completely degrade amyloid fibrils.

When β₂m solution in 1.0 M NaCl at 25 °C was prepared, amorphous aggregation rapidly occurred and was accompanied by an increase in light scattering without any elevations in ThT fluorescence, which is consistent with our previous findings (17, 20) (Fig. 1). Upon increases in temperature, light scattering gradually decreased. A sharp increase in ThT fluorescence occurred beginning at ~45 °C. These results suggested that amyloid fibrils formed and were accompanied by the dissolution of amorphous aggregates. CD measurements also indicated the formation of amyloid fibrils at high temperatures, although these measurements were disturbed by contaminated amorphous aggregates (Fig. 1, K and L). AFM images showed coexisting amyloid fibrils and amorphous-like structures (Fig. 1, inset).

We previously reported the heat-induced conversion of protofibrils to rigid amyloid fibrils in 0.5 M NaCl monitored by DSC, CD, and transmission EM (TEM) (27). In the present study, we assumed that salt-induced protofibrils were categorized into amorphous aggregates and that the heat-induced
Conversion observed was the same transition as that in our previous study (27).

**Degradation of amyloid fibrils upon heating**

To investigate the temperature-dependent stability of amyloid fibrils, amyloid fibrils were prepared under different NaCl concentrations at 25 °C (e.g. Fig. 1B, in 0.1 M NaCl), heated at 0.5 °C/min, and monitored by ellipticity at 220 nm (Fig. 2A). CD measurements confirmed the degradation of preformed amyloid fibrils at a specific temperature and that the midpoint of degradation increased at higher salt concentrations (Fig. 2B), which is consistent with previous findings (27). Furthermore, AFM images showed that the fibrils observed before heating (Fig. 2E) disappeared after heating (Fig. 2F). CD spectra also showed the degradation of preformed amyloid fibrils at 100 °C (Fig. 2, B–D). Collectively, these results showed that amyloid fibrils were degraded at high temperature and that the degradation temperature increased with elevations in the salt concentration.

Amyloid fibrillation was not observed at NaCl concentrations lower than 50 mM, e.g. 25 mM NaCl, even after an incubation at 25 °C for ~12 h (data not shown); however, fibril formation occurred at higher temperatures. On the other hand, NaCl promoted the formation of amyloid fibrils with an increase in its concentration. Taken together, amyloid fibrils formed at moderate NaCl concentrations and at ambient temperature degraded at low and high temperatures. In other words, we observed the “cold denaturation” and “heat denaturation” of β2m amyloid fibrils, as evidently observed for α-synuclein amyloid fibrils (32).

**Temperature-dependent transformation of amorphous aggregates to amyloid fibrils**

We previously reported that amorphous aggregates formed in the presence of high salt concentrations at 37 °C is a thermodynamic state stabilized by the salting-out effect (20). In the present study, we observed the high temperature-triggered dis-
solution of amorphous aggregates, as revealed by a decrease in light scattering and the concomitant formation of amyloid fibrils (Fig. 1). To confirm the validity of these phenomena, we investigated the effects of heat on amorphous aggregates at various salt concentrations.

We prepared amorphous aggregates in 1.0 M NaCl by a 30-min-incubation at 25 °C. The solution was then heated at 1.0 °C/min (Fig. 3A). A gradual decrease initially in light scattering was observed and was followed by a sharp increase in ThT fluorescence at ~60 °C, indicating amyloid fibrillation. AFM images showed that the amorphous aggregates observed after 30 min at 25 °C (Fig. 3B, left panel) transformed to amyloid fibrils upon heating (Fig. 3B, right panel). These results revealed that at high NaCl concentrations, amyloid fibrils formed at high temperatures even if amorphous aggregates populated at low temperatures.

The light scattering curves measured at various NaCl concentrations were analyzed by fitting to a sigmoidal curve to obtain a midpoint temperature of transition ($T_m$) (Fig. 3C and Fig. S2). The temperature for the dissolution of amorphous aggregates increased with elevations in the NaCl concentration between 0.9 and 1.5 M (Fig. 3E). However, at NaCl concentrations greater than 1.6 M, the $T_m$ value was not precisely obtained because of a significant decrease in light scattering (Fig. S2, H–K). We observed large aggregates floating at the water surface under these high salt conditions. We examined differences after the measurement and immediately after stirring the sample with a pipette, revealing the inhomogeneity of the solution (Fig. S3). Therefore, this marked decrease in light scattering (Fig. 3C and Fig. S2) appeared to be caused by the large amorphous aggregates that formed under high salt conditions. On the other hand, we also detected an increase in ThT fluorescence in a NaCl concentration range between 0.9 and 1.5 M (Fig. 3D). These results showed that amorphous aggregates have the potential to transform into amyloid fibrils under high NaCl conditions.

Collectively, these results showed the relationship between NaCl concentrations and the transition temperatures of amorphous aggregates to amyloid fibrils (Fig. 3E). The results also demonstrated that amyloid fibrils are more stable than amor-
phous aggregates at higher temperatures, even though the transition temperature increased with elevations in the NaCl concentration. These results indicate that the amorphous aggregates of denatured proteins do not always persist with increases in temperature.

**Dependence of protein aggregation on the heating rate**

To clarify the kinetics of fibril formation and amorphous aggregation, we changed the heating rate (Fig. 4, A and B). At a high heating rate of 0.5 °C/min in 0.1 M NaCl, we did not observe a marked increase in ThT fluorescence (Fig. 4A). In contrast, amyloid fibrillation was clearly observed at ~40 °C at a heating rate of 0.2 (Fig. 1G) or 0.1 °C/min (Fig. 4B). We then noted the heating rate-dependent transition temperature of monomers to amyloid fibrils at various salt concentrations and heating rates (Fig. S4). The transition temperature was defined as the temperature at which ThT intensity reached 10% of the maximum intensity. Fig. 4C shows the dependence of the transition temperature on the heating rate at various NaCl concentrations. When the heating rate was faster, the transition temperature became higher. The difference observed in the transition temperature between 0.2 and 0.1 °C/min was negligible, suggesting that it equilibrated.

The transition temperature became higher with increases in the NaCl concentration to greater than 0.3 M (Fig. 4C). This was attributed to amorphous aggregates, competing species against amyloid fibrils, accumulating and retarding the formation of amyloid fibrils. These results at various NaCl concentrations were consistent with previous findings on the NaCl concentration dependence of β2m aggregation at 37 °C (17, 20).

**Dependence of aggregation monitored by CD on the heating rate**

We also investigated the dependence of aggregation on the heating rate using CD spectroscopy (Fig. 4, D and F). In the presence of 75 mM NaCl and at heating rate of 0.3 °C/min, amyloid fibrillation occurred at ~45 °C (Fig. 4D). At a higher heating rate of 0.5 °C/min, a symptom of amyloid fibrillation was observed at a higher temperature. However, the unfolded state remained over the temperature range scanned at heating rates of 1 or 2 °C/min, which appeared to be similar to heating in the absence of NaCl, for which no aggregation was observed (Fig. 1, E and F). This feature was also evident from the CD spectra obtained after decreasing the temperature to 25 °C (Fig. 4E).

In the presence of 0.3 M NaCl, amyloid fibrillation occurred at all heating rates (Fig. 4F). The transition temperature and ellipticity value were higher and smaller in magnitude, respectively, with an increase in the heating rate. On the other hand, all of the CD spectra obtained after decreasing the temperature to 25 °C were consistent and typical for β-sheet–rich amyloid fibrils (Fig. 4G). Regarding samples heated at higher heating rates and without strong ellipticity at 100 °C, amyloid fibrillation appeared to have been completed upon cooling to 25 °C.

The transition temperatures monitored by CD were added to the plot constructed from ThT fluorescence, further arguing the importance of kinetic effects (Fig. 4C). Based on these results, under the conditions of relatively low NaCl concentrations, amyloid fibrillation did not occur when the heating rate was fast. The temperature increase was too fast to permit the elongation of amyloid fibrils. The temperature then reached
that of amyloid degradation before completing amyloid elongation.

Fig. 4 (H and I) showed that the amyloid fibrils that formed at 25 °C in the presence of 0.3M NaCl did not degrade, at least within a couple of hours, even at 100 °C. Therefore, under the conditions of a relatively high NaCl concentration, such as 0.3M NaCl, amyloid fibrillation occurred even under a fast heating rate because amyloid fibrils were still stable at high temperatures. Collectively, these results demonstrated that the temperature-dependent formation and degradation were under kinetic control, producing significant hysteresis depending on the heating rate.

**Titration rate dependence of NaCl concentration-dependent aggregation**

Considering the strong dependence of aggregation on the heating rate, we performed NaCl titration at various titration rates under the conditions of 25 μM β2m, 5 μM ThT, and 10 mM HCl at 37 °C under stirring (Fig. S5). In these experiments, an aliquot of a 4 mM NaCl solution was repeatedly injected into the cuvette (Fig. S5A). Although amyloid fibrillation monitored by ThT fluorescence occurred at the slow rate of titration (Fig. S5, C and D), amorphous aggregation dominated at the faster titration rate (Fig. S5, E and F). TEM images were consistent with fluorescence measurements (Fig. S5). Maximal ThT fluorescence at 0.16 μM/h decreased with further incubations and was accompanied by reductions in light scattering, suggesting that the decrease in ThT fluorescence was not caused by the formation of amorphous aggregates at high salt concentrations. Instead, the fragmentation of preformed fibrils appeared to affect ThT fluorescence.

These results indicated that salt-induced aggregation was also controlled by kinetic competition between the supersaturation-limited slow formation of amyloid fibrils and supersaturation-unlimited rapid formation of amorphous aggregates. This led to the kinetic complexity that slow and fast salt titrations mainly produce amyloid fibrils and amorphous aggregates, respectively. These results also suggested that amyloid fibrils once formed at moderate NaCl concentrations did not transform into amorphous aggregates at high NaCl concentrations, at which amorphous aggregates are thermodynamically more stable than fibrils (17, 20).

**Discussion**

**Effects of temperature on protein aggregation**

We investigated the effects of temperature, NaCl, and their relationship on the formation of amyloid fibrils and amorphous aggregates of β2m at pH 2.0. Although protein aggregation is widely considered to occur at high temperatures (36, 37), this
study as well as previous findings (23–25, 29, 30, 32) argue that both types of aggregates may be ultimately degraded to unfolded monomers at high temperatures even if they were initially induced at lower temperatures.

Gibbs free energy change of folding ($\Delta G_{U,N}$) from the monomeric unfolded state (U) to the native state (N) is defined by $\Delta G_{U,N} = -RT\ln K_{U,N}$, where $R$, $T$, and $K_{U,N}$ are the gas constant, temperature, and equilibrium constant between unfolded and folded states, respectively. $\Delta G_{U,N}$ consists of the enthalpy ($\Delta H_{U,N}$) and entropy ($\Delta S_{U,N}$) terms, which are related by the following equation: $\Delta G_{U,N} = \Delta H_{U,N} - T\Delta S_{U,N}$. $\Delta G_{U,N}$ is determined by the balance between various factors stabilizing or destabilizing the native state relative to unfolded state (38–40). One of the most important driving forces of protein folding is hydrophobic interaction. Importantly, hydrophobic interaction exhibits a strong temperature dependence manifested in a positive heat capacity change ($\Delta C_p^n$) of unfolding: the enthalpic contribution increases with temperature, whereas the entropic part decreases. The conformational entropy of the polypeptide chain favors the disordered state increasingly with an increase in temperature. The intricate balance between these stabilizing and destabilizing interactions produces a cold denaturation phenomenon for proteins with a large value for $\Delta C_p^n$, such as apomyoglobin (39, 41).

Although the contributions of the respective factors to amyloid formation appear to differ from those to protein folding, the basic mechanism may be common (31, 33). Thus, the most important driving forces stabilizing amyloid fibrils may be the combined effects of hydrophobic interactions and hydrogen bonds, which is proportional to the $\Delta C_p^n$ value of unfolding or amyloid depolymerization. These combined effects become stronger with an increase in temperature at low temperature regions, showing the temperature-dependent stabilization of fibrils or cold denaturation of fibrils when viewed in an opposite direction (32). On the other hand, the contribution of conformational entropy, which may be larger than that of native folding because of the polymeric nature of fibrils, contributes to the degradation of fibrils at high temperatures.

Amorphous aggregates also take part in amyloid polymerization/degradation. Furthermore, the transition from amorphous aggregates to amyloid fibrils occurs at specific temperature and salt concentration ranges (Fig. 3; see also Refs. 26 and 27). Although apparently intricate, this series of phenomena of protein aggregation dependent on temperature and salt conditions provides insights for understanding protein aggregation based on the thermodynamic mechanism elaborated for monomeric protein folding (38–41).

**Conformational phase diagram**

We summarized the results obtained as phase diagrams showing the relationship between NaCl concentrations and temperatures (Fig. 5). The transition temperature of fibril formation that was obtained from heating experiments followed by ThT fluorescence or CD (Fig. 4) decreased with an increase in the NaCl concentration (squares in Fig. 5B). This indicates a NaCl-dependent stabilization of fibrils to lower temperature regions. On the other hand, the $T_m$ value of amyloid degradation increased with elevations in NaCl concentrations, indicating the NaCl-dependent stabilization of amyloid fibrils to higher temperature regions (triangles in Fig. 5B) (24, 25, 30).

The combination of these adverse salt effects at both sides of the temperature scale shows a U-shaped boundary existing between unfolded monomers and amyloid fibrils (Fig. 5B). At $\sim 0.4 \text{ M NaCl}$ at $37^\circ\text{C}$, amorphous aggregates accumulated as kinetically trapped species before transforming to amyloid fibrils (20). Therefore, the delay in amyloid fibrillation was expected to occur because of the transient amorphous aggregation (red region in Fig. 5B).

Amorphous aggregates that populated under high NaCl concentrations at ambient temperatures showed a melting transition, followed by the formation of amyloid fibrils (Fig. 5A). Melting and amyloid transition temperatures increased with elevations in NaCl concentrations (red and blue circles in Fig. 5A, respectively). Similar transitions of $\beta_2$m at pH 2.5 from aggregated protofibrils to mature fibrils in 0.5 M NaCl were observed by DSC, CD, and AFM (25). The present results combined with our previous findings (24, 25, 29, 30) indicated that the amyloid fibrils that formed at high temperatures were ultimately degraded at a temperature near to or greater than 100 °C.

**Aggregation phase diagrams of $\beta_2$-microglobulin**

**A** Figure 5. Phase transitions of $\beta_2$m dependent on temperature at various NaCl concentrations. A, a transition boundary from amorphous aggregates (red) to amyloid fibrils (blue) at high NaCl concentrations obtained from changes in ThT and light scattering shown in Fig. 3 B, amyloid fibril region (blue) defined by temperature-dependent amyloid fibril formation or degradation at the low NaCl concentration region. Amorphous aggregates in this region (red) may be converted to amyloid fibrils in a prolonged incubation. Transition temperatures obtained from Refs. 24, 29, and 30 are also included. Transition boundaries were drawn manually based on the observed data points without fitting and were also used for the schematic phase diagrams shown in Figs. 6 and 7.
Aggregation phase diagrams of β2-microglobulin

Figure 6. Relationship between the aggregation-phase diagram and free-energy change. A, phase diagram of β2m conformational states dependent on NaCl concentrations and temperature. Conformational states are unfolded monomers (U), amyloid fibrils (F), and amorphous aggregates (AA). B, dependence on temperature of the ΔG values for amyloid fibrils (blue curve) and amorphous aggregates (red curve) in the presence of 100 (bottom), 400 (middle), and 1000 mM (top) NaCl. The level of ΔG = 0 is shown by a dashed line. If the ΔG value is negative, the conformational state potentially accumulates. However, because amorphous aggregates form rapidly and amyloid fibrils form slowly, kinetic and thermodynamic competitions occur as shown in C.

Kinetic and thermodynamic competition

We previously investigated the competitive mechanisms of amyloid formation and amorphous aggregation based on free energy changes and kinetics (20). We assumed equilibria between unfolded monomers and two types of aggregated forms and examined the free energy changes of amyloid fibrillation (ΔG_U,F) or amorphous aggregation (ΔG_U,AA) against unfolded monomers. Here, in the case of the temperature-dependent stability of aggregates under a prolonged incubation or slow rates of titration, the phase diagram with the U-shaped region of amyloid fibrils (Fig. 6A) indicated the U-shaped ΔG profile of amyloid fibrils (Fig. 6B). In other words, heat and cold denaturation are both rationally predicted similar to those of the native state of globular proteins.

In the presence of 100 mM NaCl, only amyloid fibrillation occurred between 40 and 80 °C (Fig. 6A). At this NaCl concentration, amyloid fibrils were the only possible to accumulate under the temperature regions at which ΔG was less than 0 (Fig. 6B, bottom panel). The degradation of amyloid fibrils to monomers occurred above T_m temperature (ΔG_U,F = 0). However, because the formation of amyloid fibrils takes time, fast heating rates did not allow the formation of fibrils (Fig. 7 and Fig. S4).

In the presence of a moderate NaCl concentration (e.g. 0.4 M), ΔG_U,AA was negative, and amorphous aggregates rapidly formed (Fig. 6B, middle panel). However, the ΔG_U,F curve was lower than the ΔG_U,AA curve. This led to maturation from rapidly formed amorphous aggregates to amyloid fibrils upon a prolonged incubation at a fixed temperature. This time-dependent maturing transition was previously observed at moderate NaCl concentrations at 37 °C and was explained by Ostwald ripening (20). The apparent conformational states also depended on the heating rate; therefore, rapid heating at moderate NaCl concentrations did not allow the conversion of amorphous aggregates to amyloid fibrils (Fig. 7 and Fig. S4).

In the presence of very high NaCl concentrations (e.g. 1.0 M NaCl), amorphous aggregates are the most stable state under ambient temperatures (Fig. 6B, top panel). In other words, ΔG_U,AA (red curve) is more negative than ΔG_U,F (blue curve). Upon heating, amorphous aggregates were replaced by amyloid fibrils. This phase transition may be explained by the crossing of ΔG_U,F (blue) and ΔG_U,AA (red) curves (Fig. 6B, top panel).

It is important to note that kinetic effects significantly deform the apparent phase diagram of heating and NaCl-dependent conformational states. Amyloid fibrils form slowly and are limited by supersaturation, whereas amorphous aggregates form rapidly, producing severe hysteresis depending on the heat- or salt-titration rates. This leads to the disappearance of the amyloid phase upon fast titrations either with temperature or NaCl (Fig. 7).

Conclusions

The apparently complex phenomena of the aggregation of β2m at pH 2 dependent on the NaCl concentration and temperature were consistently explained including kinetic and thermodynamic competition between crystal-like amyloid fibril formation and glass-like amorphous aggregation. Amyloid fibril formation proceeds slowly after breaking supersaturation, whereas amorphous aggregation is more likely to occur rapidly, producing kinetic complexity. Although amorphous aggregates are stable at ambient temperatures and high NaCl concentrations, they are replaced by amyloid fibrils upon heat-
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Figure 7. Titration rate-dependent changes in apparent phase diagrams. Because of the fast formation of amorphous aggregates and slow formation of amyloid fibrils, the apparent phase diagram depends on the heating rate and salt-titration rate. Panel i, phase diagram under slow titration rates, which is close to the equilibrium phase diagram. Panels ii and iii, apparent phase diagrams under medium (panel ii) and fast (panel iii) heating rates with the phase of amyloid fibrils fading out. Panels iv and v, apparent phase diagrams under medium (panel iv) and fast (panel v) NaCl-titration rates. In the apparent phase diagrams under medium and fast titration rates, the phase of amyloid fibrils fades out. Schematic models represent dominant structural transitions under slow and fast titrations.

Experimental procedures

Protein and chemicals

Recombinant human β2m protein with an additional methionine residue at the N terminus was expressed in Escherichia coli and purified as previously reported (42). ThT was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other reagents were purchased from Nacalai Tesque (Kyoto, Japan).

Protein aggregation by heating

Lyophilized β2m was dissolved in 10 mM HCl, and pH of the β2m solution was confirmed to be 2.0–2.1. β2m concentrations were measured spectrophotometrically using a molar extinction coefficient of 19,300 M⁻¹ cm⁻¹ at 280 nm based on its amino acid composition. The sample solution of 2.5 ml in a cuvette with 1-cm light path contained 8.5 μM β2m, 5 μM ThT,
The sample composition was the same as those of fluorescence loid fibrils using a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan). Light scattering at 445 nm was also measured to detect amorphous aggregation. All fluorescence measurements were performed under stirring using a magnetic stirring bar at a stirring speed of 800 rpm. The heating rate was controlled using a Peltier element (Nippon Tecmo Co., Ltd.), and the sample temperature was measured by a thermocouple (Anritsu Meter Co., Ltd.).

Monitoring amyloid fibril degradation

To investigate the stability of amyloid fibrils that formed in the presence of various NaCl concentrations, we prepared amyloid fibrils using a Hitachi F4500 fluorescence spectrophotometer (Tokyo, Japan) at 25 °C with stirring using a stirring bar. The sample composition was the same as those of fluorescence measurements of β2m aggregation. 300 µl of preformed fibrils was used for heating measurements using CD with the monitoring of ellipticity at 220 nm.

Monitoring amorphous aggregate dissolution

The dissolution of amorphous aggregates was detected by measuring decreases in light scattering monitored by a Hitachi F4500 fluorescence spectrophotometer and a measuring decreases in light scattering monitored by a Hitachi Far-UV CD measurements were performed using a Jasco J820 spectropolarimeter (Tokyo, Japan). A quartz cuvette with 1-mm path length was used to measure the CD spectra, and the results obtained were expressed as mean residue ellipticity [θ]. A quartz cuvette with 10-mm path length was used to detect ellipticity at 230 nm during heating under stirring with a stirring bar. The stirring speed was 800 rpm. The sample volume was 2 ml, containing 8.5 µM β2m, 10 mM HCl, and various concentrations of NaCl.

AFM measurements were performed using an AFM5100N (Hitachi, Tokyo, Japan). A volume of 20 µl was taken from samples after fluorescence or CD measurements and dropped on to a mica plate. Water was then absorbed using paper to dry the plate, which was then washed away three times using distilled water.

TEM measurements were performed using a Hitachi H-7650 transmission microscope (Tokyo, Japan) at 20 °C with a voltage of 80 kV. The sample solution (5 µl) was spotted onto a colloidal–coated copper grid (Nissin EM, Tokyo, Japan). After 1 min, the solution on the grid was removed with filter paper. Then 5 µl of 2% (w/v) ammonium molybdate was spotted onto the grid. After 1 min, the solution was removed in the same manner as the samples.

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