Amphiblastic fibrils and amorphous aggregates are two types of aberrant aggregates associated with protein misfolding diseases. Although they differ in morphology, the two forms are often treated indiscriminately. β2-microglobulin (β2m), a protein responsible for dialysis-related amylodosis, forms amyloid fibrils or amorphous aggregates depending on the NaCl concentration at pH 2.5. We compared the kinetics of their formation, which was monitored by measuring thioflavin T fluorescence, light scattering, and 8-anilino-1-naphthalenesulfonate fluorescence. Thioflavin T fluorescence specifically monitors amyloid fibrillation, whereas light scattering and 8-anilino-1-naphthalenesulfonate fluorescence monitor both amyloid fibrillation and amorphous aggregation. The amyloid fibrils formed via a nucleation-dependent mechanism in a supersaturated solution, analogous to crystallization. The lag phase of fibrillation was reduced upon agitation with stirring or ultrasonic irradiation, and disappeared by seeding with preformed fibrils. In contrast, the glass-like amorphous aggregates formed rapidly without a lag phase. Neither agitation nor seeding accelerated the amorphous aggregation. Thus, by monitoring the kinetics, we can distinguish between crystal-like amyloid fibrils and glass-like amorphous aggregates. Solubility and supersaturation will be key factors for further understanding the aberrant aggregation of proteins.
a high free energy barrier of nucleation is determined by the ordered structures of amyloid fibrils and that amorphous aggregation occurs promiscuously without a high free energy barrier. Based on an analogy to the crystallization and glass transition of substances, we propose that solubility and supersaturation are key factors for further understanding the aberrant aggregation of proteins.

Results

**NaCl-Dependent Formation of Amyloid Fibrils and Amorphous aggregates.** We first investigated the amyloid fibrillation and amorphous aggregation of β2m at 0, 100, and 1000 mM NaCl by monitoring light scattering at 350 nm and thioflavin T (ThT) fluorescence at 480 nm (Fig. S1). Both amyloid fibrils and amorphous aggregates were expected to show an increase in light scattering, but only amyloid fibrils an increase in ThT fluorescence (30, 31).

The monomeric β2m solutions at a concentration of 0.1 mg/mL at pH 2.5 were subjected to agitation with a stirring magnet at 600 rpm. In the absence of NaCl, neither light scattering nor ThT fluorescence exhibited an increase in intensity, indicating that the acid-unfolded β2m remains monomeric because of the strong intramolecular and intermolecular electrostatic repulsions. Light scattering at 100 mM NaCl increased in intensity after a lag time of 1.5 h (Fig. 1A). ThT fluorescence at 100 mM NaCl also increased after a lag time of 1.3 h (Fig. 1B), consistent with the kinetics monitored by light scattering. It should be noted that, without stirring, the β2m remains monomeric at least for several hours at 100 mM NaCl (see below). In the presence of 1000 mM NaCl, light scattering significantly increased in a few minutes without a lag time, whereas ThT fluorescence did not show an enhancement, representing amorphous aggregation. This reaction was independent of agitation (see below).

To evaluate the dependency of β2m aggregation on the NaCl concentration, we examined the reactions at various NaCl concentrations ranging from 0 to 1000 mM monitored using ThT fluorescence (Fig. S2). The intensity of ThT fluorescence showed a maximum at 300 mM NaCl, but decreased with any further increase in the NaCl concentration, resulting in a bell-shaped profile (Fig. 2A). This NaCl-dependent formation of β2m fibrils monitored with ThT was consistent with that reported previously (29), in which the seed-dependent formation of β2m fibrils was measured at various salt concentrations. Amorphous fibrillation at various NaCl concentrations showed a minimum of the lag time at around 300 mM, while an increase of the lag time was detected at higher NaCl concentrations (Fig. 2B). It is likely that the rapid formation of amorphous aggregates followed by slow reconfiguration to more stable amyloid fibrils occurs above 300 mM NaCl, contributing to the increase in the lag time above 300 mM.

![Fig. 1. Formation of β2m amyloid fibrils and amorphous aggregates at 0 (gray), 100 (red), and 1000 (blue) mM NaCl monitored by measuring light scattering at 350 nm (A) and ThT fluorescence at 480 nm (B). The solution was subjected to agitation with a stirring magnet. The inset in panel A is a close-up view of the early time course at 1000 mM NaCl.](image)

![Fig. 2. Effects of the NaCl concentration on the kinetics and morphology of β2m aggregation. The β2m solutions were subjected to agitation with a stirring magnet at 600 rpm. (A, B) Dependencies on the NaCl concentration of the maximum ThT fluorescence at 480 nm (A) and the lag time of the increase in ThT fluorescence at 480 nm (B). The solid lines are drawn as an eye-guide. In panel B, the lag time at 0, 900, and 1000 mM NaCl was not able to be quantified because ThT fluorescence did not show a significant enhancement (see Fig. S2). (C–F) AFM images of the β2m aggregates at 100 (C), 300 (D), 500 (E), and 1000 (F) mM NaCl. The white bars represent 1 μm. (G–J) TEM images of the β2m aggregates at 100 (G), 300 (H), 500 (I), and 1000 (J) mM NaCl. The black bars represent 250 nm.](image)
at 500 mM NaCl produced a mixture of amyloid fibrils and amorphous aggregates with the former dominant. In the presence of 1000 mM NaCl, amorphous aggregates prevailed without amyloid fibrils. Thus, β2m formed amyloid fibrils and amorphous aggregates depending on the NaCl concentration. Dominant products were amyloid fibrils and amorphous aggregates at the NaCl concentrations below and above 500 mM, respectively.

**Effects of Different Types of Agitation.** Amyloid fibrillation, a nucleation-dependent reaction, is accelerated by agitation of the solution. Stirring as employed in Fig. 1 is one conventional method of agitation. Ultrasonication is another, and more powerful for promoting fibrillation (17–21). We compared the effects of stirring and ultrasonication at 0, 100, and 1000 mM NaCl on the aggregation of β2m by monitoring light scattering at 350 nm (Fig. S1).

The solution in the absence of NaCl did not show an increase in light scattering with stirring or ultrasonic pulses (Fig. S4), indicating it was undersaturated with monomers thermodynamically stable. At 100 mM NaCl, both stirring and ultrasonication produced amyloid fibrils, whereas no amyloid fibril formed under the quiescent conditions (Fig. 3A). The results indicate that the supersaturated solution is metastable in the absence of agitation, that is, kinetically stable, and that stirring and ultrasonication break the metastability. Ultrasonication accelerated the amyloid fibrillation more efficiently than stirring with a lag time of 0.5 h, versus 1.5 h for stirring.

In contrast, in the presence of 1000 mM NaCl, amorphous aggregation monitored by light scattering occurred even without stirring of the solution (Fig. 3B, Fig. S4). The saturating kinetics was independent of stirring or ultrasonication, indicating that no high free energy barrier of nucleation exists in amorphous aggregation (SI Kinetic Barriers of Nucleation, Fig. S5).

The effects of agitation (i.e., stirring or ultrasonication) were also monitored at various concentrations of NaCl by ThT and ANS fluorescence (Fig. 4). When monitored using ThT fluorescence, significant acceleration by ultrasonication in comparison with stirring was evident at 100, 300, and 500 mM NaCl (Fig. 4 A–C). Ultrasonication-dependent acceleration showed a more cooperative kinetics than stirring probably because secondary nucleation (i.e., ultrasonication-dependent fragmentation of the fibrils) is a frequent occurrence with ultrasonic irradiation. The stronger ThT fluorescence exhibited by the amyloid fibrils produced by ultrasonication than by stirring suggests that ultrasonication produces more ordered fibrils with stronger ThT binding. Alternatively, because the fibrils formed by ultrasonication are short and homogeneous, this may provide stronger ThT binding. At 1000 mM NaCl, neither ultrasonication nor stirring increased ThT fluorescence (Fig. 4D), confirming the absence of amyloid fibrils.

When monitored by ANS fluorescence, notable accelerating effects of ultrasonication were observed at 100 and 300 mM NaCl (Fig. 4E and F). As described above, ANS itself is likely to promote additional amorphous aggregation, as revealed by the kinetics without a lag phase even at 300 mM NaCl. However, the marked and cooperative kinetics at 100 and 300 mM NaCl suggest that ultrasonication accelerated predominantly amyloid fibrillation. At 500 and 1000 mM NaCl, ANS fluorescence increased rapidly within a dead time of the measurements, independent of agitation, and subsequent minor change was observed (Fig. 4G and H), revealing that the dominant products are amorphous aggregates.

**Seed-Dependent Growth of β2m Aggregates.** Seeding is an important procedure for evaluating nucleation-dependent mechanisms (22). We investigated the ability of aggregates preformed under ultrasonication to act as seeds. First, the seeding experiments were performed at 100 and 1000 mM NaCl and monitored by measuring light scattering at 350 nm (Fig. 3). With the seeds added, the β2m solution at 100 mM NaCl showed an increase in light scattering without a lag phase (Fig. 3A), consistent with previous reports (33). Although the overall reaction was slower than that induced by ultrasonication, this is explained by the absence of active secondary nucleation promoted by ultrasonication. In contrast, seeding did not accelerate the amorphous aggregation at 1000 mM NaCl monitored using light scattering, as was the case of ultrasonication (Fig. 3B).

The effect of seeding was measured at several salt concentrations with ThT and ANS fluorescence (Fig. 4). Seeding induced an increase in ThT fluorescence and a removal of the lag times at 100, 300, and 500 mM NaCl (Fig. 4A–C). The cooperativity of the reaction was less than that of ultrasonication-induced fibrillation because of the absence of active secondary nucleation. When monitored using ANS fluorescence, seeding effects were evident at 100 and 300 mM NaCl, where amyloid fibrillation was accelerated (Fig. 4E and F). In the presence of 500 and 1000 mM NaCl, where amorphous aggregates are the dominant product, seeding did not change the kinetics, again confirming that the free energy barriers of amorphous aggregation are low (Fig. 4G and H).

**Discussion**

**Crystal-Like Amyloid Fibrils and Glass-Like Amorphous Aggregates.** We compared the amyloid fibrils and amorphous aggregates of β2m at pH 2.5 in their kinetics of formation. The fibrils formed at moderate concentrations of NaCl by a nucleation and growth mechanism in which a high free energy barrier associated with the nucleation rate-limits the overall reaction. In contrast, the amorphous aggregates formed rapidly at high concentrations of NaCl without a high free energy barrier, so that the reaction was independent of seeding or agitation of the solution. It might be possible that, even if the high energy barrier persists for amorphous aggregation, the overall aggregation occurs rapidly without discernible lag phase under the conditions where the growth rate is very fast. However, the simulation with Finke–Watzky mechanism, a two-parameter model for describing protein aggregation kinetics (34), argues that this is unlikely to occur (SI Kinetic Barriers of Nucleation, Fig. S5).

It has been pointed out that amyloid fibrillation is similar to crystallization (22, 23). Both amyloid fibrils and crystals form by a nucleation and growth mechanism. Amyloid fibrils and crystals are also similar in that they grow immediately after the addition of seeds (i.e., preformed crystals or fibrils). The seeds work as a template for crystallization (35) and fibrillation (22), bypassing the high energy barrier of nucleation. As established for crystallization, amyloid fibrillation continues until the solute

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**Fig. 3.** Effects of various forms of agitation on the growth of β2m amyloid fibrils at 100 (A) and 1000 (B) mM NaCl monitored by measuring light scattering at 350 nm. The solutions of β2m were agitated with a stirring magnet or irradiation with ultrasonication pulses. The effects of seeding were also examined. Light scattering intensity at 350 nm was monitored as a function of time. For comparison, the kinetics under quiescent conditions are also shown. When the effects of ultrasonic pulses or seeding were examined, the solutions were stirred.
concentration reaches its thermodynamic solubility (36). This thermodynamic solubility is sometimes called the critical concentration analogous with the critical micelle concentration at which water-excluded amphiphathic solutes form micelles.

The amorphous aggregation observed here is similar to the glass transition. Lattice models suggest that amorphous aggregates of amyloidogenic proteins show a glassy behavior (37) in which the heterogeneous conformations are fixed by strong attractive forces producing various sites of interaction. In the case of native proteins, glass-like states mean amorphous aggregates as observed by the intense salting-out procedure with a high concentration of salt, such as ammonium sulfate and NaCl. Here, it has been suggested that domain swapping leads to amorphous aggregates followed by reconfiguration to more stable species (27). The suggested mechanism explains the rapid formation of amorphous aggregates followed by slow reconfiguration to more stable amyloid fibrils observed for various amyloidogenic fibrils. In fact, this reconfiguration is likely to occur for β2m at pH 2.5 under the transition regions where amyloid fibrils and amorphous aggregates coexist.

Thus, distinguishing between amyloid fibrils and amorphous aggregates in their kinetics of formation is similar to distinguishing between the kinetics of crystallization and glass transition of substances. Because thermodynamic solubility and kinetic supersaturation are key factors in protein crystallization, they are also keys to understanding amyloid fibrillation and amorphous aggregation. Now, we would like to address the roles of solubility and supersaturation in amyloid fibrillation and amorphous aggregation.

Cryystals and glasses of native proteins. First, we illustrate the salt-dependent phase transition of a folded protein (e.g. hen egg white lysozyme at 20 mg/mL with a pH around 4.7) from a soluble state to a crystalline state and finally to a glass state (Fig. 5A). Here, we assume a salting-out experiment starting in the absence of salt, where the protein solution is undersaturated with a thermodynamic solubility higher than 100 mg/mL (Region 1). The solubility decreases with an increase in the concentration of NaCl and becomes equal to the concentration of lysozyme at 0.36 M NaCl (38). However, even at a slightly higher NaCl concentration, the supersaturated solution is apparently soluble, i.e. metastable (Region 2), where spontaneous nucleation does not occur. In the presence of NaCl higher than 0.63 M, a labile region appears where spontaneous nucleation occurs and crystals form by a nucleation-growth mechanism (Region 3) (38). A further increase in the NaCl concentration produces a glass region where too many nuclei lead to amorphous aggregation (Region 4). The schematic representation of phase transitions shown in Fig. 5.4 is generally used to explain the phase transition of such substances as silica and polymers, where the abscissa is usually temperature.

The competition between crystallization and glass transition has been elaborated for understanding the mechanism of protein folding (25–27), in which the folding temperature corresponds to the temperature of crystallization. To achieve cooperative and rapid protein folding, the temperature of glass transition should be much lower than the folding temperature so as to construct a smooth folding funnel with minimal frustration. Here, an analogy seems applicable to proteins self-assembling into amyloid fibrils and amorphous aggregates.

Amyloid fibrils and amorphous aggregates of β2m. The distinct formation of amyloid fibrils and amorphous aggregates of β2m at pH 2.5 can be illustrated with a phase diagram dependent on the NaCl and protein concentrations (Fig. 5B). This type of diagram is often used for representing crystallization and amorphous precipitation depending on the concentrations of protein and precipitant (35). The phase diagram consists of a soluble region (Region 1), metastable region (Region 2), labile region (Region 3), and glass region (Region 4). At pH 2.5 in the absence of salt, β2m is largely unfolded and electrostatic repulsion among positive charges keeps the solubility relatively high. Thus, β2m in the acid-denatured state at 0.1 mg/mL is undersaturated with monomers thermodynamically stable. Addition of NaCl at 100 mM shields the charge repulsion, resulting in a significant reduction of the solubility probably to less than 0.1 mg/mL. This is accompanied by an anion-induced conformational transition often producing a compact molten-globule state (39). However, supersaturation keeps
the unfolded β2m at 0.1 mg/mL apparently soluble in the metastable region. In other words, the metastable state exists because of a high energy barrier of nucleation (40, 41). When seeds are introduced, fibrils form, reducing the monomer concentration to the solubility limit. Upon a further increase in the concentration of NaCl, the labile region, where nucleation occurs spontaneously producing fibrils, starts. The boundary between the metastable and labile regions is shifted downward upon agitation. In the case of crystallization, it has been suggested that ultrasonication decreases the energy barrier of nucleation by reducing the metastable region of the phase diagram (38). In the glass region, where the driving forces of spontaneous nucleation are too strong, too many nuclei result in glassy amorphous aggregates. In other words, interactions between unfolded β2m occur promiscuously and noncooperatively.

As summarized above, the distinct formation of amyloid fibrils and amorphous aggregates can be defined by their locations in the phase diagram (Regions 1–4). The diagram indicates that supersaturation is a critical factor determining conformations under the conditions chosen. While solubility is a thermodynamic property, amyloid fibrillation and amorphous aggregation are kinetically controlled (42).

**Effects of Agitations.** Agitation, including stirring or ultrasonication, is a kinetic factor modifying the apparent phase diagram (Fig. 5B). It is likely that the boundary between the metastable and labile regions is shifted downward upon agitation. Note that the protein concentration of 0.1 mg/mL at 100 mM NaCl is located in the labile region in the presence of agitation, whereas it is located in the metastable region in the absence of agitation (Fig. 5B). A downward shift of the glass region may also occur, introducing transient and local glass regions. Although the real physical events responsible for amyloid nucleation are unclear, it is possible that the local and transient glass conformation provides effective nuclei for fibrillation. In the case of crystallization, it has been suggested that ultrasonication decreases the energy barrier of nucleation by reducing the metastable region of the phase diagram (38).

Practically, one possible mechanism of ultrasonication-dependent nucleation is the formation of amorphous aggregates at the air-water interface. Ultrasonication produces cavitation microbubbles accompanied by local high pressure and high temperature (43). It has been suggested that proteins condense and aggregate at the surface of the microbubbles, producing glassy amorphous aggregates. Amorphous aggregates are likely to contain seed-competent conformations. Thus, the role of ultrasonication might be to induce a transient and local glass state (Region 4) in the labile (Region 3) or metastable (Region 2) region. These effects may also occur with other forms of agitation including stirring and shaking.

**General Phase Diagram for Aggregation of Proteins.** Previously, we proposed a phase diagram for conformational and aggregational states of proteins determined by size and uniqueness of conformation (i.e., folded or unfolded) (44). When peptides are very short, they can form crystals above their solubility limit. For example, some short amyloidogenic peptides form microcrystals (45, 46). As peptides become longer, although still relatively short, they form amyloid fibrils above their solubility limit. When very long and unfolded, they form amorphous aggregates above their solubility limit. However, even extremely long polypeptides can form crystals when they fold into a unique conformation.

Here, we consider that the size of peptides or proteins should be replaced by conformational uniqueness to accommodate the various conformational states of peptides and proteins (Fig. 5C). Native proteins with a unique conformation can form crystals independent of their size. If the conformation is highly flexible and various intermolecular interactions are possible, a glass state may form. Relatively short peptides and proteins in unfolded states can form amyloid fibrils above their solubility limit.

However, one of the most important factors in determining amyloidogenicity is the role of supersaturation. A large number of previous reports as well as the present results argue that amyloid fibrillation starts under supersaturation. On the other hand, the kinetic barriers of glass transition are low compared to those of crystallization. The present results of rapid and saturating kinetics, as well as no effects of seeding or agitation on amorphous aggregation, suggest that the free energy barrier of nucleation is not high in comparison with that of amyloid fibrillation.

**Conclusions.** We have summarized the key characteristics of aggregates formed by denatured proteins. First, amyloid fibrillation and amorphous aggregation are determined by the thermodynamic solubility of the respective peptides and proteins in water. This solubility...
depends on the structure and length of the peptides and proteins. What seems missing in many previous studies is a recognition that amyloidogenicity is a property determined by the concentration of peptides or proteins relative to solubility; that is, critical concentration. Below the solubility limit, even a highly amyloidogenic peptide will remain dissolved. Above the solubility limit, peptides and polypeptides form crystals, amyloid fibrils, or amorphous aggregates depending on the stability and kinetics of the respective aggregated forms. Considering that both thermodynamic and kinetic factors are involved, distinct forms can coexist or apparent conformations can change with time, e.g. rapid formation of amorphous aggregates followed by the formation of amyloid fibrils.

Another important characteristic is the impact of supersaturation, the exact mechanism of which is still unknown. In the metastable region, even strongly amyloidogenic peptides or unfolded proteins will remain soluble. However, the introduction of seeds or various types of agitation modify the phase diagram dramatically, releasing the kinetic trap and establishing the equilibrium determined by the intrinsic solubility of peptides or proteins. Finally, we propose that ultrasonication is a powerful approach to breaking metastability, possibly by introducing transient and local glass or labile states into the metastable region.

Materials and Methods

Self-Assemblies of β2m. Expression and purification of human β2m was described in SI Materials and Methods. Lyophilized β2m was dissolved in 3.2 mM HCl (pH 2.5) at a concentration of 1.0 mg/mL, and then diluted 10-fold in a 1 cm cuvette with 3.2 mM HCl containing a series of NaCl concentrations. The volume of the solution in the cuvette was 2 mL. The solution was incubated at 25 °C. Methods of agitation with a stirring magnet and ultrasonic irradiation and seeding experiments are provided in SI Materials and Methods.

Light Scattering, TTh Fluorescence, and ANS Fluorescence. Light scattering, TTh fluorescence, and ANS fluorescence were measured using a Hitachi fluorescence spectrophotometer F4500 with the excitation wavelengths at 350 nm, 445 nm, and 350 nm, respectively. The lag time for aggregation was defined as the time at which TTh fluorescence or light scattering reach 1/10th of the maximum. Fluorescence of TTh and ANS was monitored by adding ThT at 5 μM and ANS at 50 μM to the solution, respectively.

Microscopic Images. AFM and TEM images were obtained using a Digital Instruments Nanoscope IIIa scanning microscope (Veeco) and a Hitachi H-6560 transmission microscope (Hitachi) respectively, as reported previously (20).

Acknowledgments. We thank Ms. Kyoko Kigawa for the expression and purification of β2m. This work was supported by the Japanese Ministry of Education, Culture, Sports, Science and Technology.