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Ultrasonication-induced Amyloid Fibril Formation of β_2 -Microglobulin*

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To obtain insight into the mechanism of fibril formation, we examined the effects of ultrasonication, a strong agitator, on β_2 -microglobulin (β_2 -m), a protein responsible for dialysis-related amyloidosis. Upon sonication of an acid-unfolded β_2 -m solution at pH 2.5, thioflavin T fluorescence increased markedly after a lag time of 1–2 h with a simultaneous increase of light scattering. Atomic force microscopy images showed the formation of a large number of short fibrils 3 nm in diameter. When the sonication-induced fibrils were used as seeds in the next seeding experiment at pH 2.5, a rapid and intense formation of long fibrils 3 nm in diameter was observed demonstrating seed-dependent fibril growth. We then examined the effects of sonication on the native β_2 -m at neutral pH, conditions under which amyloid deposits occur in patients. In the presence of 0.5 mM sodium dodecyl sulfate, a model compound of potential trigger and stabilizer of amyloid fibrils in patients, a marked increase of thioflavin T fluorescence was observed after 1 day of sonication at pH 7.0. The products of sonication caused the accelerated fibril formation at pH 7.0. Atomic force microscopy images showed that the fibrils formed at pH 7.0 have a diameter of more than 7 nm, thicker than those prepared at pH 2.5. These results indicate that ultrasonication is one form of agitation triggering the formation of amyloid fibrils of β_2 -m, producing fibrils adapted to the respective pH.

Amyloidosis results from the deposition of normally soluble proteins into insoluble amyloid fibrils: long, unbranched, and often twisted fibrillar structures a few nanometers in diameter and predominantly composed of cross β -sheets (1–4). Among various amyloidogenic proteins, β_2 -microglobulin (β_2 -m)³ is a target of extensive study because of its clinical importance and suitable size for examining the relation between protein folding and amyloid fibril formation (5–12). Dialysis-related amyloidosis is a common and serious complication in patients receiving hemodialysis for more than 10 years (5, 6). β_2 -m, a typical immunoglobulin domain made of 99 residues, is present as the non-polymorphic light chain of the class I major histocompatibility complex (13). As part of its normal catabolic cycle, β_2 -m dissociates from the class I major histocompatibility complex and is transported in serum to the kidneys where the majority (95%) of it is degraded (6). Renal failure disrupts the

clearance of β_2 -m from the serum, and moreover the β_2 -m does not pass through the dialysis membrane, resulting in an increase in the β_2 -m concentration by up to 50-fold in the blood circulation (6). By a mechanism that is currently not well understood, β_2 -m then self-associates to form amyloid fibrils under physiological conditions.

The incubation of β_2 -m *in vitro* under acidic conditions in the presence or absence of seed fibrils results in the formation of high yields of amyloid fibrils with a range of different morphologies (7–11). In contrast, the generation of a substantial amount of amyloid fibrils at neutral pH has been difficult (see below). Recently, Yamamoto *et al.* (14) found that low concentrations of SDS, around the critical micelle concentration (0.7 mM), not only stabilize fibrils but also induce extensive growth of β_2 -m amyloid fibrils at neutral pH. Moreover, Kihara *et al.* (15) found that repeated self-seeding at pH 7.0 with fibrils formed at the same pH results in an acceleration of fibril growth, approaching the phenomenon that occurs under physiological conditions.

It has been considered that amyloid fibril formation consists of nucleation and growth (1–4, 7, 16, 17). The nucleation process, in which a number of monomeric precursor molecules associate producing a minimal fibril unit, does not readily occur. Once the nucleus is formed, however, subsequent growth proceeds rapidly via the incorporation of the monomers into the ends of seed fibrils. These characteristics of amyloid fibril formation are similar to those of the crystal growth of substances, where agitation of solution often accelerates the nucleation process. Among various forms of agitation, shaking or stirring of the solution has been used widely to promote the formation of fibrils. While β_2 -m cannot form amyloid fibrils at pH 2.5 without seeding even after incubation for several days, agitation induced fibrils of β_2 -m to form in the absence of seeds (8). Importantly, before the formation of β_2 -m fibrils, various kinds of amorphous or spherical aggregates were observed (8), suggesting that the agitation accelerates the formation of aggregates which then operate as a scaffold of fibril formation.

Ultrasonication, usually used for preparing seeds from preformed fibrils, is another form of agitation that might affect the nucleation process. Although ultrasonication is widely used in medicine, industry, and research, little is known about its effects on proteins in solution. Recently, Stathopoulos *et al.* (18) reported that, for various proteins, ultrasonication resulted in the formation of amyloid-like aggregates. They proposed that protein unfolding and aggregation are caused by the ultrasonication, leading to the formation of amyloid fibrils. Here, to obtain further insight into the mechanism of fibril formation, in particular the nucleation process, we examined the effects of ultrasonication on the monomeric β_2 -m.

EXPERIMENTAL PROCEDURES

Expression and Purification of β_2 -m—Recombinant human β_2 -m was expressed with an *Escherichia coli* expression system and was puri-

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³ The abbreviations used are: β_2 -m, β_2 -microglobulin; AFM, atomic force microscopy; ThT, thioflavin T.

fied as described previously (19). A Met residue was always present at the N-terminal position of the recombinant protein.

Ultrasonication-induced Fibril Formation—A water bath-type ultrasonic transmitter with temperature controller (ELESTEIN SP070-PG-M, Elekon, Tokyo) was used to induce the formation of β 2-m fibrils. The volume of the water bath was about 12 L. The frequency of the instrument was 17–20 kHz, and the power output was set to deliver a maximum of 350 watts. Reaction mixtures were ultrasonicated from three directions (*i.e.* two sides and bottom) for 1 min and then incubated for 9 min without sonication, a process that was repeated during incubation at 37 °C. The buffers used were 50 mM glycine-HCl (pH 2.5) containing 100 mM NaCl or 50 mM sodium phosphate (pH 7.0) containing 0.5 mM SDS and 100 mM NaCl. The concentration of β 2-m monomer was 25 μ M (0.3 mg/ml).

The effects of ultrasonication were monitored by fluorometric analysis with ThT (7) using a Hitachi fluorescence spectrophotometer F4500 and a cell with a 10 mm light path. They were also monitored by light scattering at 350 nm with excitation at 350 nm at 25 °C using a cell with a 5-mm light path. For the light scattering measurements, the sample solution was diluted 2-fold with the reaction buffer.

In the seeding experiments with the sonication-induced fibrils, sonicated β 2-m fibrils were added at a final monomer concentration of 0.42 and 2.5 μ M to the acidic and neutral pH reaction mixtures containing 25 μ M β 2-m, respectively, and the solutions were incubated at 37 °C *without agitation*.

AFM Measurements—The sample solution was diluted 5-fold with water. A 50- μ l diluted solution was spotted on freshly cleaved mica. After standing on the substrate for 1 min, the residual solution was blown off with compressed air. AFM images were obtained using a Nano Scope IIIa (Digital Instruments). The scanning tip used was a phosphorus (*n*)-doped Si. The scan rate was 0.5 Hz.

CD Measurements—Far-UV CD spectra of β 2-m fibrils were measured with a J-600 Jasco spectropolarimeter at 25 °C as described previously (19) at a monomer concentration of 25 μ M.

RESULTS

Ultrasonication-induced Fibril Formation at Acidic pH—First, the same conditions as used for the standard seed-dependent fibril formation (*i.e.* 25 μ M β 2-m monomer in 50 mM glycine-HCl buffer (pH 2.5) containing 100 mM NaCl) (7) were used to examine the effects of ultrasonication. After preparing the reaction mixture in an Eppendorf tube on ice, ultrasonic treatment was started with the tube placed in the water bath at 37 °C. Repeated ultrasonication of 1 min in every 10 min induced, after a lag time of about 1 h, a sudden and remarkable increase in ThT fluorescence (Fig. 1*a*). The ThT fluorescence value (\sim 300) was much larger than that (\sim 200) of the standard seed-dependent fibril formation. AFM images of the solution at 3 h revealed substantial numbers of short fibrils (10–40 nm) about 3 nm in height (Fig. 1, *b* and *d*).

The sonication-induced ThT increase was reproducible in terms of the overall shape of the kinetics and the final ThT intensity (Fig. 1*a*). However, the lag time varied slightly depending on the experiment and on the volume of the mixture in the tube, suggesting that the formation of fibrils critically depends on the sonication energy. In addition, the lag time depended on the protein concentration: the higher the concentration, the shorter the lag time (data not shown), consistent with the view that the nucleation process is an oligomeric reaction (20, 21).

Seeding Reactions at Acidic pH—The seed-dependent extension of fibrils was examined at pH 2.5 using the sonication-induced short fibrils (*i.e.* the 1st generation of fibrils, F1) as seeds (Fig. 1*a*). It should be noted that the reaction was carried out *without sonication*. The ThT fluorescence increased dramatically without a lag phase, saturating at a mark-

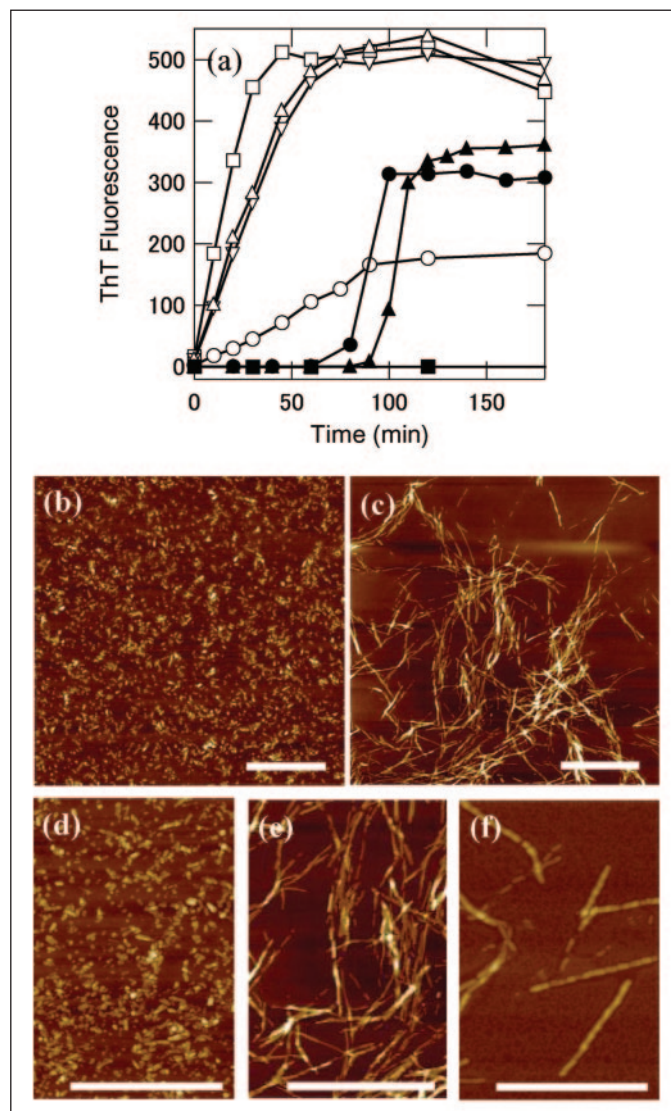


FIGURE 1. Ultrasonication-induced fibril formation of β 2-m at pH 2.5. *a*, kinetics monitored by ThT fluorescence. \bullet and \blacktriangle , ultrasonication-induced F1 fibril formation exhibiting a lag time (60–120 min). The results of two independent experiments are shown, indicating a slight variation of lag time. \square , \triangle , and ∇ , extension reaction producing F2 (\square), F3 (\triangle), and F4 (∇) fibrils, in which ultrasonication-induced F1, F2, and F3 fibrils were used as seeds, respectively. \circ , standard fibril extension reaction with seeds of originally *ex vivo* β 2-m amyloid fibrils. \blacksquare , control reaction without seeds and ultrasonication. *b–f*, AFM images of F1 (*b*, *d*) and F2 (*c*, *e*) fibrils and fibrils extended by the standard reaction at pH 2.5 (*f*). The scale bars represent 1 μ m.

edly high value of about 500. AFM images revealed the formation of long and straight fibrils with a diameter of 3 nm (Fig. 1, *c* and *e*). The result demonstrates that the short F1 fibrils formed by sonication acted as seeds for subsequent growth, producing the second generation of fibrils (F2), a phenomenon characteristic of amyloid fibrils. The repeated seeding reactions produced the third and fourth generations of fibrils (F3 and F4, respectively) with essentially the same kinetics as the F2 fibrils (Fig. 1*a*) and with the same morphology measured by AFM (data not shown).

Compared with the fibrils generated by the standard reaction with seeds originally from patients (22) (Fig. 1*f*), sonication-induced fibrils were thin (about one-fourth the diameter) and had no longitudinal periodicity. However, ThT fluorescence was more than 2-fold stronger in the sonication-induced fibrils than the standard fibrils. The CD spectra of sonication-induced β 2-m fibrils (F1 and F2) exhibited a high content of β -sheet structure, similar to that of the standard fibrils at pH 2.5,

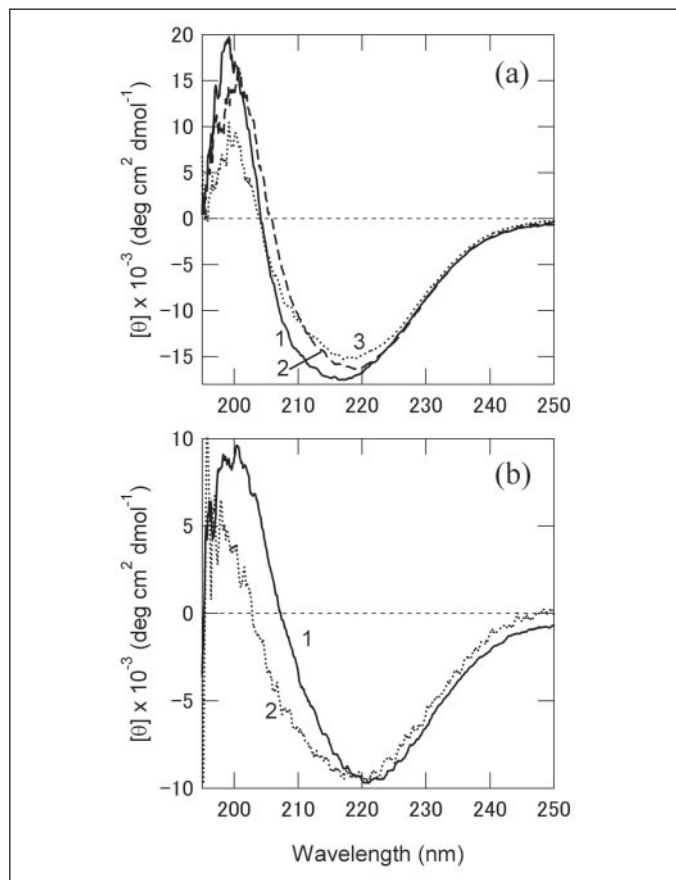


FIGURE 2. CD spectra of ultrasonication-induced β 2-m amyloid fibrils. *a*, ultrasonication-induced F1 (trace 1) and F2 fibrils (trace 2) at pH 2.5 and fibrils extended by the standard reaction at pH 2.5 (trace 3). *b*, Ultrasonication-induced F1 fibrils at pH 7.0 (trace 1) and neutral pH-adapted fibrils prepared by the repeated seeding at pH 7.0 of the acidic amyloid fibrils (trace 2).

indicating that the total amount of β -structure is similar between the two (Fig. 2*a*). These results suggest that thin fibrils have a larger number of ThT-binding sites because of the increased surface area in comparison with thick mature fibrils about 10 nm in diameter. It is conceivable that the sonication-induced fibrils correspond to the protofibrils of mature fibrils.

It has been suggested that the morphology of fibrils can be propagated by seeding (23–26). This was true for the sonication-induced fibrils, since the F3 and F4 fibrils had the same morphology and secondary structure as the F2 fibrils, *i.e.* inheritance of the fibril morphology. The observation that thin fibrils formed spontaneously suggests that the thin fibrils are more adapted to the acidic conditions. In accordance with this, we noticed that repeated seeding under standard conditions using seeds originally from patients tends to reduce the width of fibrils (data not shown).

Absence of Large Aggregates—Kad et al. (8) published AFM images of stirring-induced β 2-m amyloid fibrils under acidic conditions. The agitation made by the shaking or stirring of a solution is known to cause protein aggregation (27, 28). Consistent with this, Kad *et al.* (8) observed the formation of many large amorphous aggregates during a lag phase. Intriguingly, β 2-m fibrils seemed to form using these large aggregates as a scaffold. To examine the possible participation of amorphous or spherical aggregates, the ultrasonication-induced fibril formation was further analyzed by simultaneously monitoring light scattering and AFM images (Fig. 3*a*).

The kinetics measured based on light scattering and ThT fluorescence agreed with each other, indicating that no large aggregate that

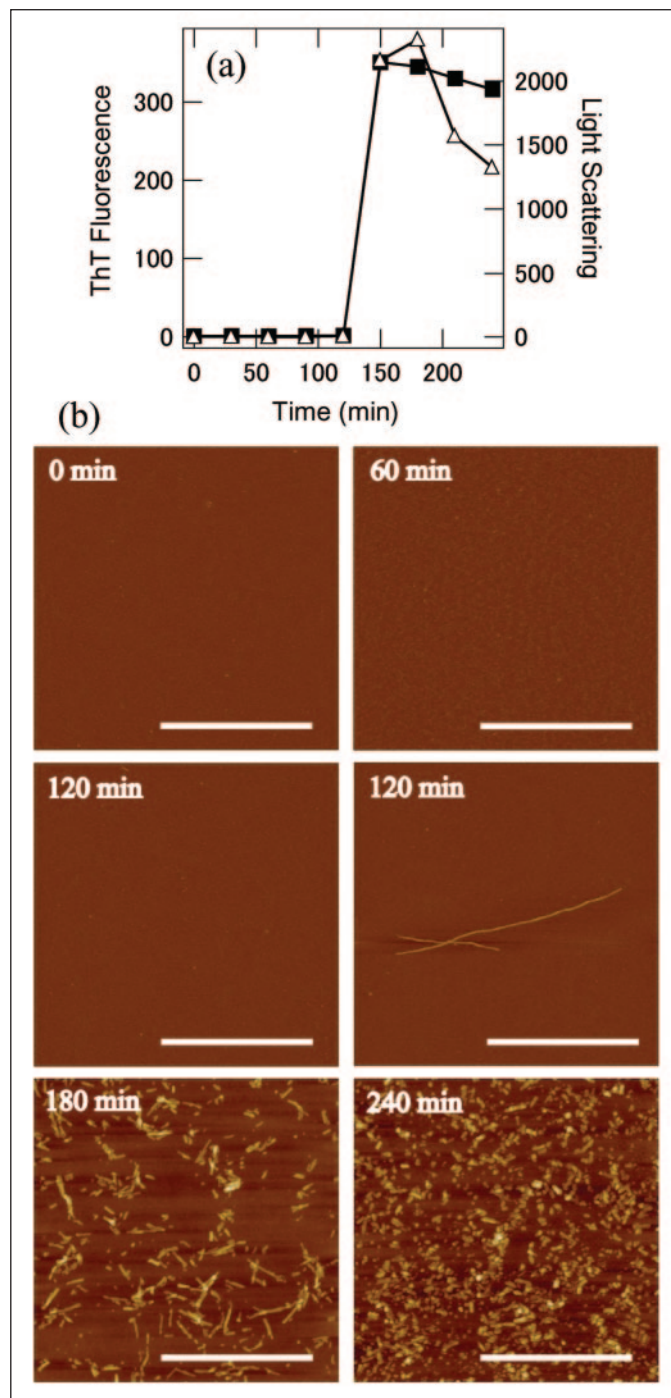


FIGURE 3. Absence of large aggregates before β 2-m fibril formation at pH 2.5 monitored using light scattering and AFM images. *a*, superposition of sonication-induced fibril formation kinetics monitored using ThT fluorescence (solid square) and light scattering (open triangle). *b*, AFM images following the time course of sonication-induced fibril formation. The scale bars represent 1 μm .

could not bind ThT was formed before the explosive fibril formation. The lag time of 120 min was longer than the lag time shown in Fig. 1. This is probably caused by the larger volume (2 ml) of solution used for the experiment in Fig. 3 than that (0.5 ml) for Fig. 1: the larger volume was necessary for a larger number of samples.

Consistent with no increase in light scattering during the lag phase, AFM images showed no aggregates or oligomers until the late stage of the lag phase (Fig. 3*b*). Only dots with a diameter of 1.5–3 nm corresponding to monomeric or dimeric β 2-m were observed at 0 and 60 min. Just before the abrupt increase of ThT fluorescence and light scat-

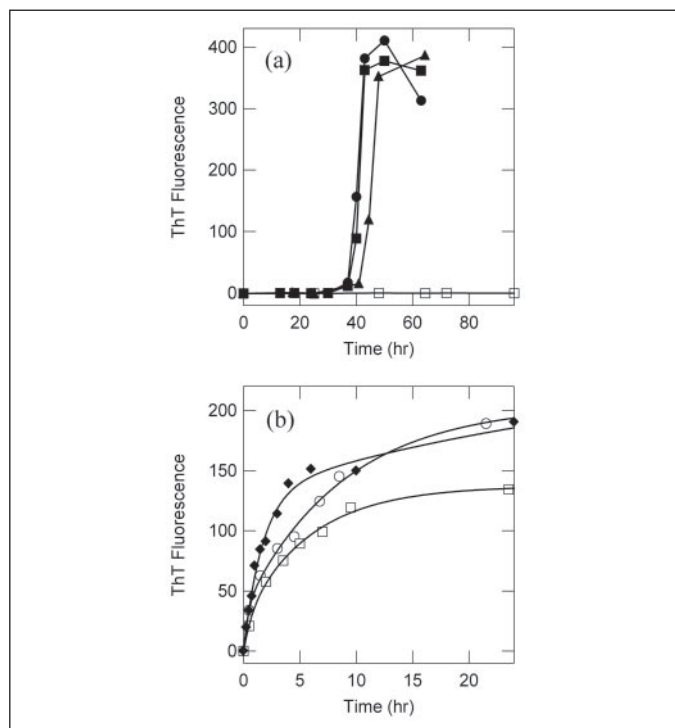


FIGURE 4. Ultrasonication-induced fibril formation of β 2-m in 0.5 mM SDS at pH 7.0 monitored with ThT fluorescence. *a*, sonication-induced F1 fibril formation (●, ▲, ■) and control without sonication (□). The results of three independent experiments are shown, indicating a slight variation of the lag time. *b*, self-seeding experiments forming F2 (○) and F3 (□) fibrils with sonication-induced F1 fibrils at pH 7.0. Time courses of F2 and F3 fibril formation were similar to that of the neutral pH-adapted fibrils at pH 7.0 (◆). The neutral pH-adapted fibrils were prepared by the repeated seeding in 0.5 mM SDS at pH 7.0 of the standard fibrils prepared at pH 2.5.

tering (120 min), long and thin fibrils with a diameter of 3 nm were occasionally found. No large aggregate was observed even at this time point. After the explosive phase, large amounts of relatively long fibrils 100–200 nm in length were found (180 min). Then, the fibrils were fragmented to 10–40 nm by the continued ultrasonication of 1 min in every 10 min (240 min). Simultaneously, the light scattering intensity decreased, consistent with the non-linear relation between the size of aggregates and light scattering. On the other hand, since the value of ThT is independent of the length of fibrils, it did not change significantly after reaching a maximum. Thus, although ultrasonication has the same effect as shaking or stirring with respect to the formation of thin fibrils, the absence of observable aggregates is unique, suggesting that the large aggregates are not essential for amyloid fibril formation.

Ultrasonication-induced Fibril Formation at Neutral pH—Although there are several reports of fibrils forming at neutral pH (29–33), most of them were qualitative and the number of fibrils formed is much smaller than that under acidic conditions. On the other hand, recent findings by Yamamoto *et al.* (14) and Kihara *et al.* (15) suggest that the extensive fibril formation under physiological conditions can be reproduced under certain conditions such as in the presence of 0.5 mM SDS. Here, we examined the effects of ultrasonication on monomeric β 2-m at pH 7.0 in the presence of 0.5 mM SDS. It has been considered that low concentrations of SDS slightly destabilize the native structure of β 2-m, leading to the formation of oligomers with increased amyloidogenicity (15).

At pH 7.0, the ThT fluorescence increased after a long lag time of about 30 h (Fig. 4*a*). The length of the lag phase varied slightly depending on the experiments. The presence of 0.5 mM SDS was essential. Since visible aggregates of proteins were often noted after the increase of ThT fluorescence, the time course of fibril formation was carefully

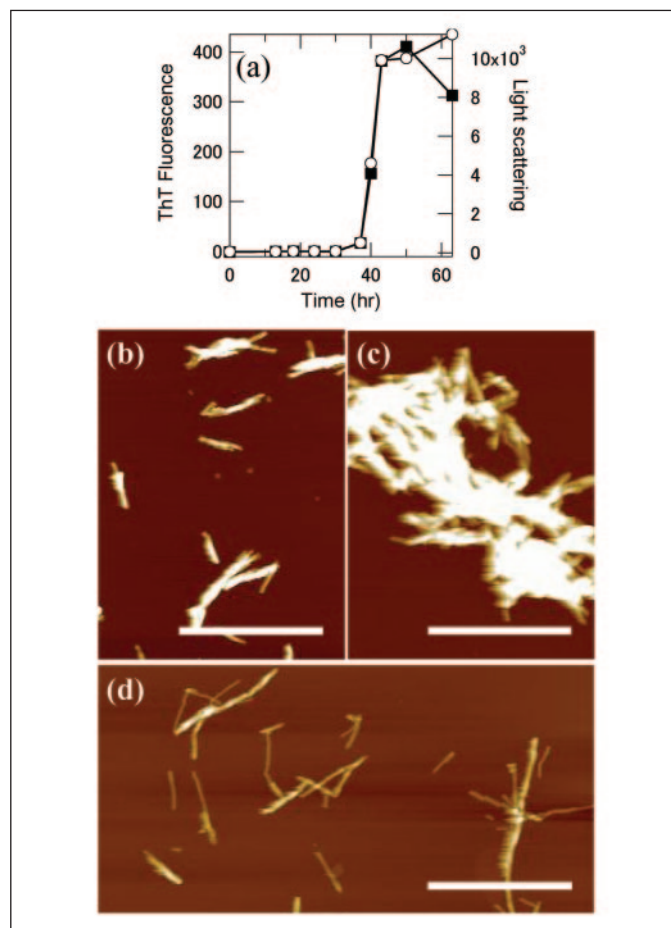


FIGURE 5. Examination of aggregation propensity of ultrasonication-induced β 2-m fibril formation at pH 7.0. *a*, superposition of fibril formation kinetics monitored using ThT fluorescence (open circle) and light scattering (solid square). Although the light scattering intensity of fibrils was greater than that of pH 2.5 fibrils (Fig. 3*a*), no large aggregates were detected during the lag phase, indicating that the strong propensity to aggregate plays a role after the formation of fibrils. *b*, AFM image of the ultrasonication-induced F1 fibrils incubated for 43 h. Thick fibrils with a diameter of more than 7 nm were observed. *c*, AFM image of clumped F1 fibrils formed by continued sonication after the increase of ThT fluorescence. *d*, AFM image of the ultrasonication-induced F2 fibrils at pH 7.0. The scale bars represent 1 μ m.

monitored by light scattering. The time courses monitored by the two methods agreed with each other, and importantly, the light scattering intensities after the explosive phase were much higher than those at acidic pH (Fig. 5*a*). This indicates that, although no large aggregate is formed during the lag time, the sonication-induced fibrils at pH 7.0 have a higher propensity to form aggregates. AFM images right after the extension phase revealed the formation of thick fibrils more than 7 nm in diameter (Fig. 5*b*). There was some variation in the diameter of the fibrils, suggesting that thick fibrils are composed of several thin fibrils. Then, as expected from the light scattering measurements, continuous exposure of extended fibrils to ultrasonication caused further aggregation as monitored by AFM, producing numerous clumps of fibrils (Fig. 5*c*).

When seeding experiments were performed with the sonication-induced F1 fibrils in the presence of 0.5 mM SDS at pH 7.0 *without sonication*, ThT fluorescence increased rapidly without a lag time, reaching a point of saturation within several hours (Fig. 4*b*). The final fluorescence intensity of F2 fibrils was about 200, which is evidently less than that of the sonication-induced F1 fibrils at pH 7.0. The kinetics of fibril formation at pH 7.0 was similar to that of the neutral-pH adapted fibrils as reported by Kihara *et al.* (15): repeated seeding at pH 7.0 starting with low pH-adapted fibrils produced neutral pH-adapted fibrils with a rel-

atively fast rate of fibril growth (Fig. 4b). Further self-seeding producing F2, F3, and F4 fibrils gave similar kinetics of fibril growth. However, the final ThT fluorescence value tended to decrease, suggesting the propensity of fibrils to aggregate under the experimental conditions at pH 7.0 (Fig. 4b). AFM images of F2 fibrils at pH 7.0 often showed associated and clumped fibrils in which the respective fibrils are more than 7 nm in diameter (Fig. 5d), similar to F1 fibrils (Fig. 5b).

The CD spectrum of the sonication-induced F1 fibrils at pH 7.0 was similar to that of the neutral pH-adapted fibrils (Fig. 2b), indicating that the two type of fibrils are indistinguishable with respect to the β -sheet content.

DISCUSSION

Ultrasonication-induced Fibril Formation—There are various effects of ultrasonication on protein solutions, which have been nicely reviewed by Stathopoulos *et al.* (18). Briefly, air bubbles are generated in solution by an effect called cavitation and are then crushed by the surrounding solvent. This causes a violent collision of the solvent, creating an extremely high local temperature. Proteins may be destabilized and unfolded at the air-liquid interface of sonication-induced bubbles. It is likely that partially folded intermediates are induced. Reduction in a size of large molecules, increase of the fluid velocity, and generation of free radicals are also caused by ultrasonication (34–36). Additionally, the aggregation of unfolded or destabilized proteins may be enhanced. There is a report that prion protein fragments compress at the air-water interface and form a stable β -sheet (37). Altogether, ultrasonication exerts two opposing effects on proteins. One is the effect of breaking protein aggregates as occurs during the fragmentation of fibrils to make seeds. This effect has been successfully applied to the replication of infectious prion proteins by dividing infectious prion aggregates into many seeds and extending these seeds via template-dependent growth (38, 39). The other effect is to promote aggregation by increasing the frequency of collisions between unfolded or destabilized protein molecules.

These effects of ultrasonication seem similar to the agitation induced by shaking or stirring of the solution, leading to the formation of amorphous aggregates before fibril form (8). However, an important difference is that the combination of the two effects of sonication minimizes the size of aggregates. In our experiments, no appreciable aggregate was detected by AFM before the abrupt increase in ThT fluorescence and light scattering at pH 2.5 or 7.0. Nevertheless, we believe that small aggregates of denatured proteins detected by neither AFM nor light scattering are induced by ultrasonication. Among these small aggregates, a unique amyloidogenic conformation, which plays the role of a template in fibril growth, may emerge. Once the growth of fibrils has started as shown in Fig. 3b (120 min), ultrasonication-induced fragmentation amplifies the number of seed fibrils. Consequently, after a certain lag time, explosive fibril formation occurs as is evident from the ThT fluorescence and light scattering intensities as well as AFM images (Figs. 1 and 3). Our results suggest that large amorphous or spherical aggregates are not necessarily required for the formation of fibrils if the corresponding conformational templates exist in small oligomers.

Additionally, it has been known that amyloidogenic proteins can form several kinds of fibrils of different morphologies. The β 2-m fibrils formed by the stirring of a solution at pH 2.5 consist of a mixture of fibrils distinct in diameter and morphology (8). In contrast, the fibrils induced by ultrasonication at pH 2.5 were fairly homogeneous with a diameter of 3 nm, suggesting that only the fibrils of the lowest free energy are produced and expanded because ultrasonication destroys unstable fibrils.

Difference between Fibrils at pH 7 and pH 2.5—Amyloid fibril formation under neutral pH conditions is one of the most focused issues for understanding the pathology of dialysis-related amyloidosis. Ultrasonication indeed induced β 2-m fibrils to form at pH 7, where a low concentration (0.5 mM) of SDS was required and the reaction was slower than that at pH 2.5. Although the diameter (\sim 7 nm) of fibrils formed at pH 7 is greater than that (\sim 3 nm) at pH 2.5, approaching the \sim 10 nm fibrils deposited in patients (22), the fibril extension reaction was much slower than that at pH 2.5.

Even at neutral pH, no large aggregate was detected by light scattering before the explosive increase in ThT fluorescence (Fig. 5a). However, unlike under acidic conditions, the fragmentation of fibrils after their formation did not take place at the same level of ultrasonication (Fig. 5, b and c). In contrast, the fibrils aggregated with time. We consider that the difference between pH 2.5 and 7.0 is partly explained by the difference of net charge. The pI value of β 2-m is \sim 6, and the net charge at pH 2.5 and 7.0 is +17 and $-$ 3, respectively; while the positive charge repulsion is large at pH 2.5, it is small at pH 7.0. Because of the decreased net charge at pH 7.0, thick fibrils made of several protofilaments and moreover the clusters of thick fibrils may form at pH 7.0, eventually leading to visible aggregates. The sonication-induced protofibrils at pH 2.5 are well dispersed taking advantage of the high net charge repulsion.

On the other hand, 0.5 mM SDS was necessary at pH 7.5. While β 2-m assumes the monomeric native structure, it is acid-unfolded at pH 2.5. These results indicate that additional factors other than net charge are also responsible for the difference between fibrils at pH 7 and pH 2.5.

Since amyloid fibrils are ordered aggregates, they can be induced to form under conditions which promote general protein aggregation. It has been considered that high hydrophobicity, high β -sheet propensity, and low net charge are important factors for fibril formation (40). High correlations of these factors with the rate of aggregation were revealed in mutation experiments using human muscle acylphosphatase (41, 42). Moreover, partial or global unfolding of protein is an additional important factor (4). β 2-m has comparatively high hydrophobicity and high β -sheet propensity, and its pI value is \sim 6. With respect to these factors, β 2-m has an intrinsic potential to form amyloid fibrils under physiological conditions. However, the native fold definitively prevents the formation (43). Although high hydrophobicity and low net charge are important factors, too much of these factors results in the formation of amorphous aggregates (44).

Potential Risk of Ultrasonication—Finally, in medicine, ultrasonication is widely used for soft tissue imaging because of its perceived safety, non-invasiveness, and low cost (45). It has also been used therapeutically in surgical ophthalmology, physical therapy, and cancer therapy (46). Many studies have been performed on ultrasonication-induced damage to living cells. It was reported that ultrasonication induces apoptosis and the formation of free radicals in cells (45, 47–49). Practical medical treatments using ultrasonication are performed under conditions that suppress such damage.

While it can be envisaged that ultrasonication is useful for dissolving protein aggregates and deposits, the present results as well as those of Stathopoulos *et al.* (18) argue the potential risk when employing sonication for basic research, food, biotechnology, and medical applications, in particular, ultrasonication therapy in the aged or diseased where the ability to clear these protein aggregates is reduced. The relation between medical treatment using ultrasonication and amyloidosis is not reported. Although the high power ultrasonic wave used in this research is not physiologically relevant, it will be important to examine the influence of ultrasonication on proteins in the living body with respect to protein aggregation and possible amyloid formation.

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