Proper calibration of ultrasonic power enabled the quantitative analysis of the ultrasonication-induced amyloid formation process

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Received 2 August 2011; Revised 2 October 2011; Accepted 7 October 2011

DOI: 10.1002/pro.755

Published online 16 November 2011 proteinscience.org

Abstract: To elucidate the mechanisms of ultrasonication on the amyloid fibril formation, we quantitatively determined the ultrasonic power using both calorimetry and potassium iodide (KI) oxidation, and under the properly calibrated ultrasonic power, we investigated the ultrasonication-induced amyloid formation process of the mouse prion protein (mPrP(23–231)). These methods revealed that the ultrasonic power in our system ranged from 0.3 to 2.7 W but entirely dependent on the positions of the ultrasonic stage. Intriguingly, the nucleation time of the amyloid fibrils was found to be shortened almost proportionally to the ultrasonic power, indicating that the probability of the occurrence of nucleus formation increases proportionally to the ultrasonic power. Moreover, mPrP(23–231) formed two types of aggregates: rigid fibrils and short fibrils with disordered aggregates, depending on the ultrasonic power. The nucleation of rigid fibrils required an ultrasonic power larger than 1.5 W. While at the strong ultrasonic power larger than 2.6 W, amyloid fibrils were formed early, but simultaneously fine fragmentation of fibrils occurred. Thus, an ultrasonic power of approximately 2.0 W would be suitable for the formation of rigid mPrP(23–231) fibrils under the conditions utilized (ultrasonication applied for 30 s every 9 min). As ultrasonication has been widely used to amplify the scrapie form of the prion protein, or other amyloids *in vitro*, the calorimetry and KI oxidation methods proposed here might help determining the adequate ultrasonic powers necessary to amplify them efficiently.

Keywords: prion protein; amyloid fibrils; ultrasonic power; calorimetry method; KI oxidation method

Introduction

Prion diseases are a group of fatal neurodegenerative disorders that includes Creutzfeldt–Jakob disease (CJD) in human and scrapie and bovine spongiform encephalopathy (BSE) in animals. Prion diseases have

Abbreviations: CD, circular dichroism; EM, electron microscope; mPrP(23–231), full-length mouse prion protein; PK, proteinase K; PMCA, protein misfolding cyclic amplification; PrP^C, cellular form of prion protein; PrP^{Sc}, scrapie form of prion protein; ThT, thioflavin T.

Additional Supporting Information may be found in the online version of this article.

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Grant sponsors: Program for the Promotion of Fundamental Studies in Health Science of the National Institute of Biomedical Innovation; Research on Measures for Intractable Diseases (Prion Disease and Slow Virus Infections) from the Ministry of Health, Labour and Welfare of Japan; JSPS Research Fellowships for Young Scientists; Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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emerged as a major public health issue following BSE epidemics that have crossed the species barrier, resulting in variant CJD infections in human.^{1,2} In addition, there is a growing concern that the prion agent responsible for chronic wasting disease could spread among elk and deer and eventually across the species barrier into free-range cattle.^{3,4} The pathogenesis of these unusual diseases are associated with the conformational rearrangement of the cellular isoform of the prion protein (PrP^C) to the scrapie isoform (PrP^{Sc}) in the brain.^{5–7} Although PrP^C is monomeric and rich in α -helical structure, PrP^{Sc} conformer is characterized by an increased proportion of β -sheet structure, partial resistance to proteolysis, and a propensity to aggregate into amyloid fibrils or plaques. Although the precise nature of the pathogenic prion agent remains controversial, a growing body of evidence supports the protein-only hypothesis, which states that the prion protein itself is the infectious pathogen.^{8–10}

Substantial efforts had been dedicated to the development of a cell-free conversion system that could reconstitute the infectious PrP^{Sc} from recombinant PrP *in vitro*.^{11–14} The use of ultrasonication to mimic the fragmentation process has been successfully applied to develop an *in vitro* PrP^{Sc} amplification technique, which is known as protein misfolding cyclic amplification (PMCA).^{11,15–19} The population of PrP^{Sc} oligomers, which catalyze the formation of PrP^{Sc}, can be increased by breaking large PrP^{Sc} polymers into smaller units by ultrasonic irradiation. Using this technique, ultrasensitive PrP^{Sc} detection has been achieved in easily accessible specimens, such as blood and urine, in a hamster model that was infected with the hamster-adapted 263K strain of scrapie.^{16,17} In addition, ultrasonication accelerates the nucleation of the amyloid fibrils of several proteins, including β_2 -microglobulin,²⁰ α -synuclein,²¹ lysozyme,²² and others. It is commonly known that ultrasonication triggers the formation of amyloid fibrils. On the other hand, ultrasonication can efficiently break down preformed amyloid fibrils into short ones.^{23,24} Ultrasonic pulses are useful for preparing monodispersed amyloid fibrils of minimal size with an average molecular weight of approximately 1,660,000 (140-mer),²⁴ which are useful for characterizing the structure and dynamics of amyloid fibrils. However, the adequate ultrasonic power required for the stable amplification of amyloid fibrils has not been quantitatively determined yet.

Ultrasonic power is usually expressed as either the electrical input or output power of the generator. However, the conversion efficiency of electrical power into ultrasonic energy depends not only on the models of instruments but also on the conditions of the oscillator (or horn).^{25–29} Several methods are available to estimate the amount of ultrasonic power that enters into a sonochemical reaction.^{25–29} The

most common technique is the calorimetry, where the initial rate of temperature increase are measured when a system is irradiated by ultrasonication.^{25–27} This is based on the assumption that almost all of the mechanical energy produces heat, and thus, the output power can be calculated via calorimetry. Alternative method is the chemical dosimetry, where the sonochemical generation of a chemical species is monitored. A conventional system is the generation of the I_3^- ion from aqueous potassium iodide (KI) solution by ultrasonic irradiation, which is known as the KI oxidation or Weissler reaction.^{25–27} The amount of I_3^- molecules produced after an adequate duration of sonication is regarded as a relative measure of the ultrasonic power. On the other hand, the Fricke reaction is often used in the area of sonochemistry.^{26,28} When a Fricke solution is ultrasonically irradiated, the Fe^{2+} ions in the solution are oxidized to Fe^{3+} ions. Thus, the amount of generated Fe^{3+} is a measure of the ultrasonic strength. These chemical dosimetries are based on the oxidation or reduction reactions that occur in the aqueous solution.

In this report, to elucidate the effects of ultrasonication on the amyloid fibril formation, the ultrasonic power of a sample solution was determined in a 1.5-mL sampling tube using both calorimetry and KI oxidation. As the ultrasonic power was significantly different at positions of the ultrasonic stage, the formation of amyloid fibrils from the full-length mouse prion protein (mPrP(23–231)) was investigated as a function of the ultrasonic power. Intriguingly, amyloid fibrils were formed earlier under stronger ultrasonication. Then, we investigated the morphologies and conformations of the amyloid fibrils that formed at the various ultrasonic powers in detail. The mechanisms of ultrasonic effects that promote the nucleation of amyloid fibrils are also discussed.

Results

Estimation of ultrasonic power and strength using calorimetry and KI oxidation methods, respectively

Sample positions 1–15 of the ultrasonic stage are shown in Figure 1(A). Ultrasonic power and strength were estimated at each position using calorimetry and KI oxidation, respectively.^{25–27} When using the calorimetry method, continuous sonication of the 0.5-mL water sample resulted in the increase of the temperature from 37°C to 42–55°C [Fig. 1(B) and Supporting Information Fig. S1]. Temperatures of these reactions stopped rising while the ultrasonication continued because of the heat exchange between the water and the system. The rate of the temperature increase was entirely dependent on the sample position, because a standing wave was used for

Figure 1. Calorimetry and KI oxidation methods. A: An illustration of the ultrasonic stage that was placed on the surface of water bath. Sampling tubes (1.5 mL) were set at positions 1–15 of the ultrasonic stage. B: For the calorimetry method, temperature increases were measured at positions 2, 5, 7, 9, 12, and 15 of the ultrasonic stage during ultrasonication. The temperature increases measured at the other positions are shown in Supporting Information Figure S1. Each point and bar represents the average and standard deviation of three independent experiments, respectively. C: UV spectra of I_3^- after incubation for 0, 4, 8, 12, 16, and 20 min with 0.1M KI aqueous solution during ultrasonication at position 12 were overlaid; the larger the intensity at 355 nm, the longer the incubation time. D: For the KI oxidation method, the absorbances of I_3^- at 355 nm at positions 2, 5, 7, 9, 12, and 15 of the ultrasonic stage during ultrasonication were fitted using a straight line. Their symbols are the same used in (B). The absorbances at the other positions are shown in Supporting Information Figure S1.

ultrasonication. The response of the thermocouple was dependent on the distance between the surface of the solution and tip of the thermal probe, so the depth of the thermocouple was set to be 2 cm to obtain the reproducible data. Because thermally determined power was essentially independent of the initial temperature,²⁵ the thermal exchange between the coolant and reaction solution was disregarded during the measurement of the initial temperature increase, and then the ultrasonic power was calculated on the basis of the initial temperature increase per second at onset of ultrasonication, as shown in Eq. (1). As a result, the ultrasonic powers at positions 10–14 around the center of the stage were >2.0 W [Fig. 2(A)]. In contrast, the ultrasonic powers at positions 1 and 7–9, which were located on both sides of the stage, were <1.0 W; while, at positions 2–6 and 15, the ultrasonic powers were between 1.0 and 2.0 W.

KI oxidation dosimetry is the measurement of the amount of I_3^- molecules produced per unit of ultrasonic energy.^{25–27} The UV–visible absorption spectrum showed two maxima at 290 and 355 nm after

the sample was ultrasonically irradiated for a period of time [Fig. 1(C)], and the absorbance of I_3^- at 355 nm increased almost linearly with the time as the sample was ultrasonically irradiated [Fig. 1(D) and Supporting Information Fig. S1]. The amount of I_3^- molecules produced per minute differed at each position of the ultrasonic stage because of differences in the ultrasonic irradiation power. The ultrasonic strength determined by KI oxidation was almost in agreement with those determined by calorimetry [Fig. 2(A)], indicating that the rates of KI oxidation are proportional to those of heat production. Subsequently, the sampling positions were reordered according to the ultrasonic power determined by calorimetry [Fig. 2(B)]. Therefore, desired ultrasonic powers ranged from 0.3 to 2.7 W could be prepared at almost regular intervals using this ultrasonic stage.

Ultrasonication-induced amyloid fibrils under various ultrasonic powers

The amyloid fibril formation was achieved at various ultrasonic powers. As a result, thioflavin T (ThT)

Figure 2. Estimation of ultrasonic power and strength using calorimetry and KI oxidation, respectively. A: Ultrasonic power (○) was estimated using the calorimetry method at positions 1–15 of the ultrasonic stage (left ordinate). Each bar represents the standard deviation. The ultrasonic strength (●) was estimated using KI oxidation (right ordinate). B: The positions of the ultrasonic stage were rearranged according to ultrasonic power (○) (left ordinate), and ultrasonic strength was put in the order (●) (right ordinate).

fluorescence, which is largely proportional to the amount of amyloid fibrils, increased with the level of ultrasonication [Fig. 3(A)]. Following powerful ultrasonication (i.e., >2.0 W), ThT fluorescence became increased within 15 h; however, it required 5–65 h of ultrasonication at 1.0–2.0 W and 40–80 h of ultrasonication at <1.0 W. These incubation times required for the rise of ThT fluorescence correspond to the nucleation times of the amyloid fibrils.

Next, the correlation between the ultrasonic power estimated by calorimetry and the nucleation time was examined, and good correlation ($r = -0.82$) was obtained, as shown in Figure 3(B). Similarly, the good correlation ($r = -0.77$) between the ultrasonic strength estimated by KI oxidation and the nucleation time was observed, as shown in Supporting Information Figure S2. Vigorous agitation and cavitation by ultrasonication may increase the probability of the occurrence of nucleus formation. In

addition, aggregates with relative ThT intensities of approximately 250 and 100 were observed [Fig. 3(A)]. The amounts of proteins precipitated by the centrifugation at 30,000g for 1 h at 4°C were 87 and 93% for samples with a high (~ 250) and a low (~ 100) ThT intensities, respectively, indicating that these ThT intensities reflect the conformational difference of produced aggregates rather than the amount of aggregates. These populations also changed depending on the ultrasonic power [Fig. 3(C)]; under a strong ultrasonic power larger than 1.5 W, aggregates with a high ThT fluorescence were formed, while under a weak ultrasonic power smaller than 1.5 W, aggregates with a low ThT fluorescence were easily formed. Taken together, the nucleation time of amyloid fibrils was inversely proportional to the ultrasonic power, and the conformation of the produced aggregates was dependent on the ultrasonic power.

Observation of mPrP(23–231) amyloid fibrils by EM

Electron microscope (EM) observation revealed that the aggregates with a high ThT fluorescence intensity were rigid amyloid fibrils [Fig. 4(A)], but those with a low ThT intensity were short amyloid fibrils with disordered aggregates [Fig. 4(B)]. ThT fluorescence was notably increased upon binding to the ordered β -sheet structure of amyloid fibrils.³⁰ Under powerful ultrasonication (>1.5 W), rigid fibrils were likely to be formed, while under weak ultrasonication (<1.5 W), short fibrils and disordered aggregates were formed simultaneously [Fig. 3(C)]. On the other hand, these aggregates were finely fragmented at the powerful ultrasonication at 2.6 and 2.0 W, respectively [Fig. 4(A,B), right]. Under these conditions, the rigid fibrils were 50–200 nm in length and about 20 nm in width [Fig. 4(A), right] and the short fibrils with disordered aggregates were highly fragmented into small particles of between 20 and 40 nm in length [Fig. 4(B), right]. In contrast, when ultrasonicated at 0.6 W, the rigid fibrils were 0.1–0.3 μm in length and 20 nm in width [Fig. 4(A), left] and the mixture were fully assembled [Fig. 4(B), left]. Thus, ultrasonication promotes not only the fragmentation of preformed fibrils but also the nucleus formation of amyloid fibrils; both of these processes entirely depend on the ultrasonic power.

Conformations of the two types of mPrP(23–231) aggregates

To determine the conformations of the two types of aggregates formed by ultrasonication, the proteinase K (PK)-resistances of these aggregates were investigated. Historically, PK-resistance has been used to distinguish PrP^C from PrP^{Sc}. Treating PrP^{Sc} with PK generates a PK-resistant core that encompasses

Figure 3. Ultrasonication-induced mPrP(23–231) amyloid fibrils. A: The formation of mPrP(23–231) amyloid fibrils at various positions of the ultrasonic stage. ThT fluorescence values at positions 10–14 (red) and 2–6, 15 (black) and 1, 7–9 (blue) correspond to ultrasonic powers of >2.0 W and 1.0–2.0 W and <1.0 W, respectively. The value used to classify the high and low ThT fluorescence is indicated by dashed line. B: Correlation between the nucleation time of amyloid fibrils and the ultrasonic power as estimated by the calorimetry method. Each bar represents the standard deviations of the nucleation times and the ultrasonic power at each position. The curve was fitted using a straight line, and the correlation coefficient (r) was calculated to be -0.82 . C: The two types of aggregates formed by each ultrasonic power. The ThT fluorescence values of 150 or more represent the rigid fibrils and are shown in gray; values of less than 150 represent the short fibrils with disordered aggregates and are shown in black.

residues 90–231, which are referred to as PrP27–30.³¹ The full-length mPrP(23–231) polypeptide displays a 23-kDa band without PK digestion [Fig. 5(A,B), lanes 1]. Upon incubating mPrP(23–231)

aggregates with PK at a molar ratio of 62.5:1, both types of aggregates yielded several partially resistant fragments with molecular masses in the range of 15–18 kDa and 12–13 kDa [Fig. 5(A,B), lanes 3].

Figure 4. EM observations of the aggregates formed at various ultrasonic powers. A: Aggregates with high-intensity ThT fluorescence formed by ultrasonic powers of 0.6 W (left), 1.7 W (middle), and 2.6 W (right). B: Aggregates with low-intensity ThT fluorescence formed by ultrasonic powers of 0.6 W (left), 1.2 W (middle), and 2.0 W (right). The ultrasonic power is shown schematically at the top. The scale bars indicate 200 nm.

Figure 5. PK digestion of two types of mPrP(23–231) aggregates. A, B: Western blotting with the PrP M-20 antibody (epitope of the C-terminus of PrP) after PK digestion of the rigid fibrils (A) formed by ultrasonic powers of 0.6, 1.7, and 2.6 W are shown in left, middle, right panels, respectively. Those of the short fibrils with disordered aggregates (B) formed by ultrasonic powers of 0.6, 1.2, and 2.0 W are shown in left, middle, right panels, respectively. The ultrasonic power is shown schematically at the top. Both isoforms of aggregates (0.12 mg mL^{-1}) were treated with PK for 1 h at 37°C at the following PK:mPrP(23–231) molar ratios: 1:250 (lane 2), 1:62.5 (lane 3), 1:12.5 (lane 4), 1:2.5 (lane 5), 1:0.625 (lane 6), and without PK (lane 1). The apparent molecular masses of the PK-resistant fragments are given in kDa. C: Densitometric quantification of 23 kDa (open symbols) and 11–12 kDa (closed symbols) bands in the Western blots of (A) (left) and those in the Western blots of (B) (right). The rigid fibrils formed by ultrasonic powers of 0.6 W ($\circ$, $\bullet$), 1.7 W ($\square$, $\blacksquare$) and 2.6 W ($\triangle$, $\blacktriangle$) (left), and the short fibrils with disordered aggregates formed by ultrasonic powers of 0.6 W ($\circ$, $\bullet$), 1.2 W ($\square$, $\blacksquare$) and 2.0 W ($\triangle$, $\blacktriangle$) (right). The band intensity at each lane is expressed as a percentage of the full-length mPrP band intensity without PK digestion at lane 1.

However, upon increasing the PK:mPrP(23–231) ratio to 1:12.5, only PK-resistant bands of 11–13 kDa appeared [Fig. 5(A,B), lanes 4]. At PK:mPrP(23–231) ratios of 1:2.5 and 1:0.625, only the rigid fibrils clearly showed PK-resistant bands with molecular weights that ranged from 11 to 12 kDa [Fig. 5(A,B), lanes 5,6]; however, these bands were considerably

small in the short fibrils with disordered aggregates [Fig. 5(C)]. The 11–12-kDa bands, which could be the core of the rigid fibrils, contained epitopes of PrP M-20 (C-terminus of PrP), because they were nonimmunoreactive to SAF32 (residues 59–89 of PrP; Supporting Information Fig. S3); this further suggests that these PK-resistant bands are the C-terminal fragments within residues approximately 90–231 of mPrP, which is consistent with the findings in a previous report.³¹ In contrast, the short fibrils with disordered aggregates have a high sensitivity to PK digestion, as opposed to the rigid fibrils. Thus, disordered aggregates would be neither PK-resistant nor ThT positive. In addition, the rigid fibrils formed under powerful ultrasonication at 2.6 W were more easily digested than those formed under weak ultrasonication [Fig. 5(A,C)], indicating that the amyloid fibrils would be degraded under the powerful ultrasonication, and in general, the number of amyloid fibril ends with which PK could contact increases with fragmentation.³²

The circular dichroism (CD) spectrum of the native form of mPrP(23–231) exhibited an α -helical structure (Fig. 6), but both types of aggregates showed a CD spectrum indicative of β -sheet structure with a minimum at around 216 nm. The ellipticities of the rigid fibrils at 216 nm were much higher than those of the short fibrils with disordered aggregates, indicating that the rigid fibrils are rich in β -sheet and highly ordered, which is consistent with the high PK resistibility of the rigid fibrils [Fig. 5(C)]. In contrast, the formation of disordered aggregates with the short fibrils would decrease their ellipticities considerably. Meanwhile, the ellipticity of the rigid fibrils formed under powerful ultrasonication was slightly lower than those of fibrils formed under weak ultrasonication (Fig. 6), suggesting the degradation of the amyloid fibrils at the ultrasonic power of 2.6 W.

Effects of temperature increase by ultrasonication

It was found that the lag times for nucleus formation of the mPrP(23–231) amyloid fibrils were shortened as the ultrasonic power was increased, but temperature of the solution was simultaneously elevated [Fig. 1(B) and Supporting Information Fig. S1]. Thus, we tried to discriminate between the effect of ultrasonication and that of temperature. Initially, the effect of temperature increase was investigated at 30 s incubation, which corresponds to the length of time required for amyloid fibril formation to occur by continuous irradiation. The temperature of the solution rapidly increased to 42.2, 47.2, and 51.5°C at positions 8, 3, and 12 of the ultrasonic stage, respectively [Fig. 7(A)]. These macroscopic temperature increases can denature the native structure of the amyloidogenic protein,

Figure 6. CD spectra of two types of mPrP(23–231) aggregates. The CD spectra of the rigid fibrils and the short fibrils with disordered aggregates formed in 2M GdnHCl with 50 mM MES (pH 5.5) under various ultrasonic powers are indicated by the solid and dashed lines, respectively, both of which showed a negative minimum at around 216 nm. The rigid fibrils formed by ultrasonic powers of 0.6 W (blue), 1.7 W (black), and 2.6 W (red), and the short fibrils with disordered aggregates formed by ultrasonic power of 0.6 W (blue), 1.2 W (black), and 2.0 W (red). The native spectrum of mPrP(23–231) under 50 mM MES (pH 5.5) is shown by the dotted line, which shows negative minima at around 208 and 222 nm.

leading to the formation of amyloid fibrils.³³ To examine the effect of the temperature independently on the denaturation of the mPrP(23–231) monomer, its thermal stability was measured using the CD spectra. The thermal unfolding curves were monitored using the molar ellipticity at 222 nm, which represents the helical content of mPrP(23–231) [Fig. 7(B)]. Parameters obtained by nonlinear fit, that is, the melting temperature (T_m) and the enthalpy change (ΔH) of the monomer in a solution of pH 5.5 were $70.5 \pm 0.1^\circ\text{C}$ and $51.7 \pm 1.5 \text{ kcal mol}^{-1}$, respectively, and those in a 2M GdnHCl solution of pH 5.5 were $34.1 \pm 0.2^\circ\text{C}$ and $30.3 \pm 0.7 \text{ kcal mol}^{-1}$, respectively. Because about half of the monomer will unfold in a 2M GdnHCl solution at pH 5.5,³⁴ mPrP(23–231) had been almost denatured under the ultrasonic conditions (solution of 2M GdnHCl at 42.2–51.5°C) [Fig. 7(B)].

Next, amyloid fibrils were formed at position 12 of the ultrasonic stage where the ultrasonic power was constantly held at 2.6 W, in which the durations of the ultrasonic irradiation were adjusted to certain lengths to increase the solution temperature up to distinct levels [Fig. 7(C)], and the interval time between the ultrasonic irradiation was adjusted so as to make the total ultrasonic work constant at 78 J during the entire 9.5 min duration of incubation [Fig. 7(D)]. The solution temperature increased up to 51.7°C after ultrasonic irradiation for 30 s [Fig. 7(C)], while the temperature only reached 48.8, 46.6, 43.7, and 39.9°C following irradiation for 20,

15, 10 and 5 s, respectively. Rigid fibrils were formed under all of these ultrasonic conditions [Fig. 7(D)]. As a consequence, after continuous irradiation for 5 s, amyloid fibrils were formed a little bit earlier than after long, consecutive ultrasonication, despite the fact that the solution temperature only reached to 39.9°C. EM observations indicated that the rigid fibrils formed under these conditions were all fragmented into between 50 and 100 nm in length [Fig. 7(E)]. Taken together, the increase in the macroscopic temperature of the solution is not essential for the nucleation process of mPrP(23–231); instead the total work which directly affects the total amount of vigorous agitation and cavitation would have contributed to nucleus formation, as discussed below.

Discussion

Estimation of ultrasonic power using calorimetry and KI oxidation

Although the ultrasonic irradiation has been successfully applied to the *in vitro* amplification of PrP^{Sc},^{11,15–19} the ultrasonic power required for the nucleus formation of PrP amyloid fibrils remains undetermined. Actually, the ultrasonic power estimated in this study (0.3–2.7 W) was considerably less than the real output electric power of about 550 W. Here, the ultrasonic power and strength within the sampling tube were determined using calorimetry and KI oxidation, respectively. The continuous sonication of a volume of 0.5 mL water resulted in an increase in macroscopic temperature [Fig. 1(B)], and the range of ultrasonic power was distributed between 0.3 and 2.7 W depending on the position of the ultrasonic stage [Fig. 2(A)]. Because it is relatively easy to prepare and handle the KI solution, the KI oxidation method is less complicated than the other sonochemical dosimetry methods.^{25–27} Sonochemical effects are caused by a transient microscopic reaction field with several thousand Kelvin and several hundred atmospheres,^{26,35} generated by the quasi-adiabatic collapse of the bubbles produced in solutions, which is known as the cavitation phenomenon. Intriguingly, the efficiency of the sonochemical reaction obtained by the KI oxidation method was in good agreement with the ultrasonic power obtained by the calorimetry method [Fig. 2(A)], indicating that the intense microscopic increases in temperature and pressure caused by cavitation result in macroscopic equilibrium properties, such as macroscopic temperature increases.

Koda *et al.*^{26,27} reported that the sonochemical efficiency (SE-value), which is a convenient parameter for quantifying energy, can be evaluated by the concentration of the reacting molecule (m) divided by the ultrasonic energy density (E): $\text{SE} = m/E \text{ (mol dm}^{-3}\text{)/(J dm}^{-3}\text{)}$. Here, the calorimetrically determined

Figure 7. Effects of temperature increases on the amyloid fibril formation. A: Increases in the temperature of water at positions 3 (□), 8 (△), and 12 (○), and those of the 2M GdnHCl and 50 mM MES solution (pH 5.5) at positions 3 (■), 8 (▲), and 12 (●) following 30 s of ultrasonication. Each point and bar represent the average and standard deviation of three independent experiments, respectively. The times of turning ON and OFF the ultrasonication are indicated by the arrows. B: The thermal unfolding curves of the mPrP(23-231) monomer in 50 mM MES (pH 5.5) (○) and in 2M GdnHCl and 50 mM MES (pH 5.5) (△) when monitored by the molar ellipticity at 222 nm. Each point and bar represent the average and the standard deviation, respectively. The solution temperatures of 42.2, 47.2, and 51.5°C, which were reached after the 30 s of continuous ultrasonication at positions 8, 3, and 12, respectively, are indicated by the closed circles. C: Increases in the water temperature following 30 (○), 20 (□), 15 (△), 10 (▽), and 5 (◇) s of ultrasonication, and those of the 2M GdnHCl and 50 mM MES solution (pH 5.5) following 30 (●), 20 (■), 15 (▲), 10 (▼), and 5 (◆) s of ultrasonication at position 12 of the ultrasonic stage. D: The formation of amyloid fibrils, in which mPrP(23-231) was dissolved to a concentration of 20 μM in 50 mM MES (pH 5.5) containing 2M GdnHCl, following ultrasonication for 30, 20, 15, 10, and 5 s and then incubated for 9, 6, 4.5, 3, and 1.5 min without ultrasonication, respectively; this process was repeated by incubation at position 12. E: EM observations of the amyloid fibrils formed under continuous ultrasonication for 30 s (left), 15 s (middle), and 5 s (right). The scale bars indicate 200 nm.

energy is the amount of ultrasonic energy absorbed (E). The SE-value for KI oxidation, which was obtained by averaging the SE-values of positions 1–15 of the ultrasonic stage, was $0.13 \pm 0.05 \times 10^{-10} \text{ mol J}^{-1}$ at a frequency of 17–20 kHz. Koda *et al.* reported

that the SE-value for KI oxidation is $0.60 \pm 0.02 \times 10^{-10} \text{ mol J}^{-1}$ at 20 kHz.²⁶ The SE-value of this study is smaller than that reported by Koda *et al.*,^{26,27} because the efficiency of the sonochemical reaction depends on the ultrasonic frequency and the height of

liquid level. The formation of bubbles increases the microscopic temperature, which is proportional to the radius ratio of the bubbles before and after the quasi-adiabatic collapse, and the number of bubbles are dependent on the ultrasonic frequency.^{26,27} Basically, the SE-value enables the estimation of the ultrasonic energy induced in an individual reaction system by determining the amount of KI oxidation. The adequate ultrasonic power to form the other amyloid fibrils should be also determined, otherwise the reproducibility of amyloid formation experiments, including PMCA, might be poor. The calorimetry and KI oxidation methods proposed here can be conveniently used for this purpose.

Ultrasonic effects on the nucleation of mPrP(23–231) amyloid fibrils

The nucleation time of mPrP(23–231) amyloid fibrils was accelerated in proportion to the ultrasonic power (correlation coefficient: $r = -0.82$) [Fig. 3(B)]. The quasi-adiabatic collapse of bubbles by ultrasonication induces microscopic field of high temperature and pressure resulting in the vigorous agitation of the solution and a macroscopic increase in temperature,^{26,35} which can be as high as 51.5°C following 30 s of ultrasonication [Fig. 7(A)]. This macroscopic temperature rise can denature the native structure of the protein. Partially unfolding of the amyloidogenic protein by high temperatures or chemical denaturants can induce the formation of amyloid fibrils.³³ Although the effects of ultrasonication are somewhat complicated, the macroscopic temperature increase contributed little to the nucleation of amyloid fibrils as evidenced by this study [Fig. 7(D)]. In fact, the monomer had already been denatured by the amyloid formation buffer at 37°C [Fig. 7(B)]. These results suggest that the nucleation process is independent of the macroscopic increase in temperature up to 51.5°C, but may be entirely dependent on the vigorous agitation and the generation of microscopic reaction fields under ultrasonication.

Nucleus formation requires a series of association steps of monomers, which are thermodynamically unfavorable, constructing the rate-limiting step of amyloid fibril formation. Cavitation accompanies the vigorous agitation and the formation of bubbles, resulting in the drastic increase in the local temperature and pressure, which also increase the probability of the proper association of monomers, thereby promoting the spontaneous nucleation of the aggregates. In fact, the formation of amyloid fibrils has been shown to be accelerated with only agitation at physiological temperature.^{36,37} In addition, Atarashi *et al.*³⁸ showed that the efficiency of shaking at relatively high temperatures (i.e., 37–55°C) is almost equivalent to the *in vitro* ultrasonication for PrP^{Sc} amplification. Thus, ultrasonication is one of the agitation methods that can trigger the nucleation of

amyloid fibrils. In addition, transient microscopic reaction fields at several thousand Kelvin and several hundred atmospheres,³⁵ which are definitely a nonequilibrium dynamical process, may further induce the nucleation of amyloid fibrils. Nucleus formation occurs in the rarely formed reaction field, and the drastic temperature increase would also increase the number of such reaction fields and the probability of nucleus formation, thus shortening the lag time [Fig. 3(A)]. On the other hand, the nucleation process of amyloid fibrils that occurs in a supersaturated solution is similar to that of a crystal, which could be accelerated by irradiation with a femtosecond laser.³⁹ The cavitation bubbles generated by ultrasonic irradiation would temporarily create the regions of high protein concentration around the surface of the bubbles,³⁹ similar to laser irradiation, which could efficiently trigger the nucleation of amyloid fibrils.

Optimum ultrasonic power required to form mPrP(23–231) amyloid fibrils

Although the lag time for the nucleus formation of amyloid fibrils was shortened in proportion to the ultrasonic power [Fig. 3(B)], the rigid fibrils fragmented at a strong ultrasonic power of 2.6 [Fig. 4(A)]. Horn-type ultrasonicators can frequently be used to produce amyloid seeds because its actual power (approximately 10–100 W) is much stronger than the power of water bath-type ultrasonicators.^{25,26} Thus, high power may be not necessary to make amyloid fibrils [Fig. 3(B)]. Meanwhile, the nucleation of rigid fibrils requires an ultrasonic power of >1.5 W [Fig. 3(C)]; moreover, when power is insufficient, mPrP(23–231) can gradually form disordered aggregates if it is incubated at a relatively high temperature for a few days. Therefore, an ultrasonic power of about 2.0 W is suitable for the formation of rigid mPrP(23–231) fibrils under the utilized ultrasonic conditions (i.e., ultrasonication for 30 s every 9 min). Thus, a balance between the extension and the fragmentation of the preformed amyloid fibrils is essential. The formation of amyloid fibrils should be performed at the proper ultrasonic power, otherwise side reactions, such as the excess fragmentation of the preformed amyloid fibrils or the formation of disordered aggregates may occur.

In conclusion, ultrasonic power was estimated using calorimetry and KI oxidation, and the nucleation of mPrP(23–231) amyloid fibrils was shown to be accelerated by the increase of the ultrasonic power. Vigorous agitation, in addition to the accompanying cavitations, contributed to the nucleation of the amyloid fibrils in a solution of 2M GdnHCl at pH 5.5, and the optimum ultrasonic power for the formation of rigid fibrils is about 2.0 W; under these conditions, amyloid fibrils are readily formed but also simultaneously fragmented to some extent.

Thus, proper ultrasonic powers are required for the amplification of amyloid fibrils, and abnormal aggregates, such as PrP^{Sc}, should be individually investigated using convenient methods such as the calorimetry and the KI oxidation described in this report.

Materials and Methods

Recombinant PrP(23–231)

mPrP(23–231) was expressed and purified using an *Escherichia coli* expression system and has been previously described.⁴⁰ The molecular weight of the purified mPrP(23–231) protein was measured by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (Bruker Daltonics, Billerica, MA). The concentration of the monomer was determined from the extinction coefficient ($\epsilon = 63,500 M^{-1} \text{ cm}^{-1}$) at 280 nm based on the amino acid composition.

Calorimetry and KI oxidation methods

In the calorimetric method,^{25–27} the ultrasonic power that dissipated into the liquid was calculated using the following equation:

$$\text{Power (W)} = (dT/dt)c_p M \quad (1)$$

where c_p is the heat capacity of water (4.2 J g^{-1}) and M is the mass of water (g). (dT/dt) is the increase in temperature per second. The initial increase in temperature was measured at 37°C using the R-26 thermocouple data logger (MK Scientific, Kanagawa, Japan), which was immersed in the 0.5-mL sample of water that was placed in the 1.5-mL sampling tube at various positions of the ultrasonic stage. The mean value was determined from three trials. In most cases, the error of the power measurement was estimated to be 5–10% when the temperature increase was small.

In the KI oxidation method,^{25–27} when the aqueous KI solution is ultrasonically irradiated, I^- ions are oxidized to yield I_2 . When excess I^- ions are present in the solution, I_2 reacts with the excess I^- ions to form I_3^- ions.

The concentration of KI was $0.1 M$. The absorbance of I_3^- at 355 nm was measured ($\epsilon = 26,303 M^{-1} \text{ cm}^{-1}$) using a UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan). KI was purchased from Nacalai Tesque (Kyoto, Japan).

Ultrasonication-induced amyloid fibril formation

A water bath-type ultrasonic transmitter with a ELESTEIN 070-GOT temperature controller (Elekon, Chiba, Japan) was used to induce the formation of amyloid fibrils. mPrP(23–231) was dissolved into a 50 mM 2-(*N*-morpholino)ethanesulfonic acid

(MES)-NaOH buffer (pH 5.5) containing $2 M$ GdnHCl to yield a concentration of $20 \mu M$ mPrP(23–231). The volume of the water bath was about 14 L. The frequency of the instrument was 17–20 kHz, and the power output was set to deliver a maximum of approximately 550 W. Reaction mixtures were ultrasonicated from three directions (i.e., two sides and the bottom) for 30 s and then incubated for 9 min without sonication. This process was repeated during incubation at 37°C . For the macroscopic temperature increase experiments, the reaction mixtures were ultrasonicated for 30, 20, 15, 10, and 5 s and then incubated for 9, 6, 4.5, 3, and 1.5 min without sonication, respectively. This process was repeated during incubation at 37°C .

The amount of aggregates was estimated from the fraction of precipitated proteins after the centrifugation of $30,000g$ for 1 h at 4°C .⁴¹ The precipitated fraction was calculated by subtracting the concentration of protein in supernatant after the centrifugation, which was determined by UV absorption, from the initial concentration of monomeric proteins.

ThT fluorescence and CD measurements

From each reaction tube, an aliquot of $5 \mu\text{L}$ was taken and mixed with 0.5 mL of $5 \mu M$ ThT (Wako Pure Chemical Industries, Osaka, Japan) in a 20-mM Tris-HCl buffer (pH 8.0). The fluorescence of ThT was measured using an F-7000 fluorescence spectrophotometer (Hitachi High-Tech, Tokyo, Japan) with a 445 nm excitation wavelength and a 485 nm emission wavelength. Fluorescence was measured immediately after mixing, and the initial 3 s were averaged.

CD spectra were measured at 25°C using an AVIV model 215s spectropolarimeter (AVIV Biomedical, Lakewood, NJ) with a step size of 0.2 nm . The far-UV CD spectra of the monomer and the aggregates were measured in 50 mM MES (pH 5.5) without and with $2 M$ GdnHCl, respectively, using a quartz cell with a light path of 1 mm and a protein concentration of 0.1 mg mL^{-1} . The results are expressed as the mean residue ellipticity $[\theta]$ ($\text{deg cm}^2 \text{ dmol}^{-1}$). Thermal unfolding of the monomer was monitored by measuring the CD signals at 222 nm of the samples that contained 0.1 mg mL^{-1} mPrP(23–231) and 50 mM MES (pH 5.5) with or without $2 M$ GdnHCl with a heating rate of 1°C min^{-1} . The thermal unfolding profiles were fitted to a two-state model⁴²:

$$Y = \frac{m_N T + Y_N}{1 + \exp(-\Delta H(1 - T/T_m)/RT)} + (m_U T + Y_U) \left(1 - \frac{1}{1 + \exp(-\Delta H(1 - T/T_m)/RT)} \right)$$

where R and T are the gas constant and temperature (K), respectively, T_m is the melting temperature, and ΔH is the enthalpy change associated with the thermal unfolding. Y is the CD signal at 222 nm .

Y_N and Y_U are the signals that were contributed by the native and unfolding states, respectively. m_N and m_U indicate the slope of the native and unfolded states, respectively. Nonlinear least-square fitting was performed using Igor Pro software (WaveMetrics Inc., Lake Oswego, OR).

EM observation

A 2- μ L aliquot of the sample solution was placed on a 400-mesh copper grid that was covered with a carbon film for 1 min, and excess solution was removed by blotting with filter paper. The grid was negatively stained with a 5- μ L droplet of 2% uranyl acetate for 1 min. Again, the liquid on the grid was removed by blotting and then dried. Electron micrographs were taken using a JEM-2100F transmission electron microscope (JEOL, Tokyo, Japan) operating at 200 kV acceleration with a magnification of $\times 20,000$.

PK digestion and Western blotting

Following incubation and ultrasonication, the aggregates (0.12 mg mL^{-1}) were treated with PK in 0.1M Tris-HCl (pH 7.2) at 37°C for 1 h. Digestion was stopped by incubation with 4 mM Pefabloc SC, followed by the addition of 10M urea to reach a final concentration of 7.5M urea. PK and Pefabloc SC were purchased from Roche Diagnostics (Rotkreuz, Switzerland). The samples were suspended in a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and then loaded onto a 15% polyacrylamide gel and heated to 95°C for 10 min. For the Western blot experiments, proteins were electroblotted onto the Immobilon-P transfer membrane (Millipore, Billerica, MA), and mPrP was detected using the polyclonal PrP M-20 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the monoclonal SAF32 antibody (SPI-BIO, Montigny le Bretonneux, France) as the primary antibody. Signals were visualized using ECL Plus (GE Healthcare, Buckinghamshire, UK) and scanned using a LAS-1000UVmini analyzer (Fujifilm, Tokyo, Japan). The band intensity in each sample was measured using Multi Gauge software (Fujifilm, Tokyo, Japan). To normalize differences between experiments, the band intensity at each lane was expressed as a percentage of the full-length mPrP band intensity without PK digestion.

Acknowledgment

The authors thank Dr. Junji Hosokawa-Muto for help with the Western blot experiments and the Equipment Center for Common Research at the Graduate School of Medicine at Gifu University for assistance with the EM observations.

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