Sonication Induced Intermediate in Prion Protein Conversion

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Abstract: We have observed that hamster prion protein (PrP^{C}) undergoes conformational changes on exposure to heat or sonication. If a sonication induced new conformer is seeded with a small amount of its abnormal pathogenic isoform (PrP^{Sc}) it undergoes a significant conversion to a proteinase-resistant isoform. This suggests the presence of a third stable PrP conformer, which may be intermediate in the conversion of PrP^{C} to PrP^{Sc}.

Keywords: Prion protein, scrapie, PMCA, sonication, protein aggregation.

INTRODUCTION

Transmissible spongiform encephalopathy (TSE) is typically associated with accumulation of amyloid; particularly cerebral accumulation of PrP. Normal PrP is a glycophosphoinositol-linked membrane protein whose function is not well-established. This conformation of the protein is designated PrP^{C}. On the other hand, in TSE much of the PrP undergoes a conformational conversion into a form called PrP^{Sc}, where "Sc" is taken from scrapie, a TSE relatively common in sheep. According to the prion hypothesis, PrP^{Sc} is necessary and sufficient to cause TSE, and presence of PrP^{Sc} is widely used as a diagnostic criterion for TSE.

At the gross level pathogenesis of TSE is unusual and dependent on the host animal and disease strain. After oral challenge with infected brain, misfolded PrP^{Sc} soon appears in the enteric nervous system, from where it slowly migrates to the brain [1]. There a variety of histological changes appear, including spongiform vacuolization and ultimately widespread neuronal death. At the cellular and biochemical levels pathology of TSE remains poorly characterized. The reticuloendothelial system may be involved, and the role of specific cell types (*e.g.*, glia) is unclear. The conformation conversion from PrP^{C} to PrP^{Sc} probably requires accessory molecules, but these are not yet characterized [2-3].

Protein Misfolding Cyclic Amplification (PMCA) is a revolutionary method of amplifying prions *in vitro* that may teach us important principles relevant to the disease-related *in vivo* conversion mechanism [5]. For example PMCA is inhibited by thiol blockers and RNAse [6]. PMCA in a purified system was recently described as requiring polyanion accessory molecules [7].

PMCA has been used to detect 263K in hamster blood and brain at levels that are undetectable by any method other than bioassay [8,9]. The method starts with a homogenate of fresh normal brain tissue, which is diluted with buffer and stabilized by addition of protease inhibitors. A small amount of brain homogenate (BH) from an infected animal is then added as a "seed". Intermittent sonication causes the conformation conversion of PrP^{C} to proceed towards production of PrP^{Sc}.

Compared to PrP^{C}, PrP^{Sc} is relatively resistant to proteases. This property forms the basis for almost all differential diagnoses of TSE. Strictly speaking proteinase-resistant PrP is not the same as PrP^{Sc}, since a fraction of infectious PrP appears to be proteinase-susceptible [8]. Nonetheless, proteinase resistance is a hallmark of converted PrP^{Sc} long associated with prion disease.

Sonication appears to be a critical factor in PMCA, and if sonication is omitted, only a small amount of PrP^{Sc} is produced [10]. Many proteins undergo structural changes upon sonication. Stathopulos and coworkers have reported that sonication of many different proteins result in formation of aggregates with properties similar to those of amyloid [11]. The sonicated proteins are resistant to resolubilization, denaturation, and reducing agents, and are relatively rich in β-sheet content. It has been shown that sonication of a human PrP peptide (residues 113-127) results in kinetically stabilized β-sheet structure, in contrast to initial bulk solution containing large amounts of random coil and small amounts of β-sheet structure [12]. Likewise, sonication of Aβ(25-35) promotes β-sheet structure formation [13].

PrP^{C} probably adopts intermediate conformations on the thermodynamic kinetic pathway to PrP^{Sc}, but little is known about them [14-17]. In this work we show that hamster PrP^{C} undergoes changes with exposure to heat or sonication, in the absence of seeding with PrP^{Sc}. These changes facilitate seeded conversion to PrP^{Sc} during subsequent incubation without further sonication.

EXPERIMENTAL

Chemical Reagents

Syrian golden hamster (*Mesocricetus auratus*; LVG) were obtained from Charles River Laboratories, Wilmington, MA. The brains were harvested from exsanguinated euthanized animals, frozen on dry ice, and stored at -80 °C. Hamster brains infected with the 263K (=Sc237) strain of hamster

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adapted scrapie were obtained from InPro Biotechnology, South San Francisco, CA. Other reagents and suppliers are as follows: Proteinase K and "Complete" proteinase inhibitor cocktail tablets (Roche, Mannheim, Germany), F4-31 MAb (a generous gift of Larry Stanker), 3F4 biotinylated MAb (Signet, Dedham, MA), streptavidin-AP and streptavidin-HRP conjugates (Invitrogen, Carlsbad, CA), "StartingBlock T20" blocking buffer and "SuperSignal ELISA" substrate (Pierce, Rockford, IL).

Equipment

The equipment and suppliers are as follows: Sonicator 3000 (Misonix, Farmingdale, NY), "Spectra Max M5" multimode microplate reader (Molecular Devices, Mississauga, Ontario), "XCell SureLock" mini-cell gel electrophoreses apparatus, NuPAGE Novex 10% bis-Tris gels 1.5 mm 10 well, Nu PAGE MES SDS running buffer, and 0.2 µm PVDF membranes (Invitrogen), Semi-dry blot apparatus (Bio-Rad, Hercules, CA), SigmaFast BCIP/NBT substrate (Sigma-Aldrich, St. Louis, MO), "QuantiScan 3.0" densitometry software (Biosoft, Cambridge, UK), tissue homogenizer GLH-115 (Omni International, Marietta, GA).

Preparation of Brain Homogenate

Healthy or terminally ill hamster BH (10% w/v) were prepared at room temperature in phosphate-buffered saline, pH 7.4 (Fisher BP665-1) containing proteinase inhibitors, Triton X-100 (0.5%), and EDTA (4mM). The homogenates were prepared using a homogenizer, sequentially passed through 24 and 28 gauge needles, aliquoted, and frozen at -80 °C for subsequent experiments.

PMCA

Procedure is as described in literature [9] with following changes. Ill hamster BH (10% w/v) were diluted (1:100) into healthy BH (10% w/v) and subjected to 24 PMCA cycles. One PMCA cycle consisted of samples (50 µl) incubated (1 h, 37 °C) and sonicated (200 W, 5 s pulse) in a sonicator equipped with a microplate horn that was placed inside an incubator.

Detection of PrP^{Sc} (Western Blot)

Samples were digested with proteinase K (PK) (100 µg/ml, 1h, 37 °C,). The digestion was stopped by addition of SDS loading buffer (4x, 33 µl) and boiling for ten minutes. Samples (30 µl) were loaded onto polyacrylamide gels. Following electrophoresis, the proteins were blotted (80 min, 25 V) onto PVDF membranes and blocked with blocking buffer (1 h, 10 ml). The membranes were incubated overnight in blocking buffer (10 ml, 4 °C) containing a pre-made biotinylated 3F4 MAb (4 µl) - streptavidin-AP (2 µl) complex. Western blots were developed with a precipitating substrate and band intensities evaluated by densitometry.

Sonication of PrP^{C}

Healthy hamster BH (120 µl) was subjected to sonication at 37 °C. Samples were removed every minute. Each sample was divided into two aliquots (50 µl). One set of aliquots was analyzed by native ELISA and the matching set by (denaturing) SDS-PAGE/Western blotting without PK digestion Fig. (**2**). This experiment was later repeated with samples subjected to turbidometry at 600 nm Fig. (**4**).

Detection of PrP^{C} (Native ELISA)

The capturing F4-31 MAb was diluted in PBS and loaded (50 µl) on a 96-well plate. The plate was incubated overnight (4 °C), blocked with blocking buffer (2 h, room temperature), and washed three times with PBS (100 µl) containing Tween-20 (0.05% w/v). The sonicated BH aliquots (50 µl) were loaded into wells, incubated overnight (4 °C), and washed three times with the PBS/Tween-20 solution. The captured PrP^{C} was incubated (4 h, 37 °C) with pre-made biotinylated 3F4 MAb-streptavidin-HRP (2:1) complex that was diluted (2 µl/ml) in the PBS/Tween-20 solution. The plates were washed seven times and developed with SuperSignal chemiluminescent substrate according to the manufacture's instructions. The chemiluminescence signal was detected with a microplate reader.

Conversion of PrP^{C} to PrP^{Sc}

Separate BH samples (100 µl) were pretreated either by sonication or heating. Sonication was for six minutes at 200 W. Heating (without sonication) was either prolonged (37 °C, 24 h) or short (80 °C, 5 min). To initiate conversion of PrP^{C} to PrP^{Sc}, infected BH was diluted (1:100) into the pretreated BH samples and incubated (24 h, 37 °C) without further sonication.

RESULTS AND DISCUSSION

TSEs, such as scrapie in sheep and bovine spongiform encephalopathy (BSE) in cattle, are related to posttranslational events that cause misfolding of a normal brain protein PrP^{C} to its abnormal PrP^{Sc} conformation. The PrP^{C} form has high α-helix sheet content, is water-soluble, and relatively sensitive to proteinase degradation. On the other hand, the PrP^{Sc} form is higher in β-helix sheet content, is insoluble in water, partially resistance to proteinase degradation, and tends to form amyloid like aggregates [18].

Very little is known about the mechanism of PrP conformation conversion *in vivo* or *in vitro*. But in converting large amounts of PrP^{C} to its abnormal PrP^{Sc} form using very small amounts of brain-derived PrP^{Sc} seed, PMCA mimics the process of prion protein misfolding that occurs during the development of disease. Indeed, the product of PMCA is reported to be infectious by intracranial inoculation in rodents [19].

In PMCA, PrP^{Sc} seed is mixed with an excess amount of PrP^{C}. The mixture is incubated and PrP^{Sc} grows at the expense of PrP^{C}. The growing aggregate of PrP^{Sc} is then subjected to a short pulse of sonication. By repeating the cycles of incubation and sonication, the amount of PrP^{Sc} rapidly increases. After several cycles the mixture is subjected to PK digestion, which destroys nearly all protein in the mixture except for the 27-30K core fragment of converted PrP^{Sc}. An unseeded reaction typically serves as one negative control, while a sample of infected BH is a positive control. After PK digestion, PrP^{Sc} is measured via ELISA and confirmed by Western blot.

Many labs report difficulty with PMCA, and controlled brain harvest conditions appear to be important for reproducibility. In our hands PMCA works well with the hamster-adapted scrapie strain 263K (also known as strain Sc237) in hamster BH. This strain in the hamster model is well-characterized, at least partly because the latent period is "only" about 70 days, and it is one of the prion strains that exhibits substantial PK resistance.

When we performed the PMCA reaction under conditions described by Saá, *et al*. [9] we observed that the reaction is not limited by the supply of PrP. The conversion of PrP to PrP^{Sc} slowed over time, eventually coming to a stop, even though most of the starting PrP remained unconverted. Addition of fresh BH restored the conversion Fig. (**1**). This suggested that factors necessary for PMCA were consumed or otherwise lost under reaction conditions. These limiting factors might include the native conformation of PrP^{C} or other cofactors (protein X [20]).

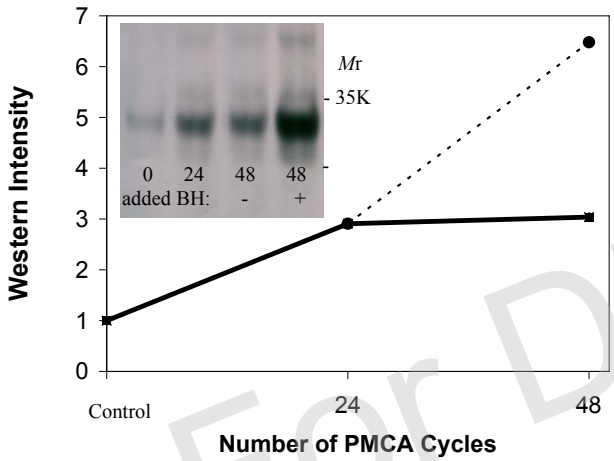

Figure 1. Amplification of PrP^{Sc} by 1st and 2nd PMCA. Infected BH (10% w/v) was diluted 1:100 into healthy BH (10% w/v). A sample (100µl) was subjected to 24 PMCA cycles (one PMCA cycle consisted of samples being incubated (1 h, 37 °C) and sonicated (200 W, 5 s pulse). Half of the sample was further diluted 5-fold into healthy fresh BH, and both were subjected to additional 24 PMCA cycles. PrP^{Sc} was detected by Western blotting after PK digestion. Band intensity was evaluated by densitometry. Intensity was normalized against that of a control sample, which was stored frozen and not heated or sonicated. Insert: Western blot shows raw data.

PMCA typically involves incubation (37 °C) and sonication of BH in the presence of a PrP^{Sc} "seed". With unseeded sonication of normal BH, we observed time-dependent loss of PrP^{C} structure, as reflected by native ELISA assay Fig. (**2**). However, total PrP^{C} protein concentration remained unchanged, as reflected by Western blot. This suggests sonication may have produced an alternative PrP^{C} configuration. To exclude a possibility of PrP^{C} loss from sticking to vial walls, after using them for native ELISA samples, we filled the remaining empty vials with SDS PAGE loading buffer and boiled them (10 min). Analysis of the buffer by Western blotting yielded no PrP^{C} bands (data not shown).

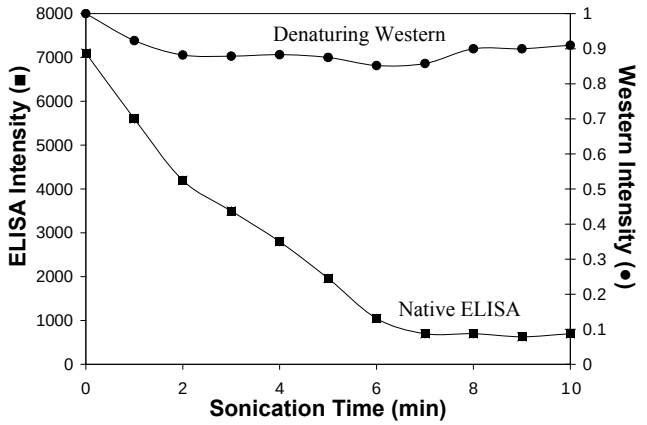

Figure 2. Concentration of PrP^{C} as a function of sonication time as analyzed under native ELISA and denaturing Western blot conditions. Samples were not PK digested. Normal (unseeded) BH samples (100 µl, 10% w/v) were subjected to sonication. Every minute a sample was removed from sonicator and split into two fractions (50 µl). One fraction was analyzed by native ELISA and the other one was subjected to SDS-PAGE and Western blotted. ELISA (■) results reported as raw chemiluminescence data. Western blotting (●) data are from densitometry normalized to intensity at time zero.

Generation of PrP^{Sc} by PMCA depends on sonication. Optimum reaction conditions vary between BH of different species [21]. And while PMCA generates PrP^{Sc} in an autocatalytic fashion [22], incubation without sonication generates very limited conversion [10]. Thus we hypothesized that PMCA conditions might cause PrP^{C} to undergo structural changes and form a stable PrP conformer that is intermediate in the conversion of PrP^{C} to PrP^{Sc} by PMCA. Because sonication for PMCA generates appreciable heat, we compared sonication to a comparable amount of heating performed without sonication.

Fandrich, *et al.* proposed that under appropriate conditions many proteins can adopt amyloid like structures when their native state is destabilized [23]. The native state can be destabilized by many processes including increased temperature, exposure to chaotropes, changes in pH, and sonication. It is known that sonication produces high local temperatures, mechanical forces, and free radicals such as H• and OH•. The free radicals can undergo further reactions and form other reactive molecules, such as H_2O_2 or O_2^-, that can cause proteins to undergo oxidation, crosslinking, and cleavage reactions [24] Furthermore, the relatively high kinetic energy and mechanical forces of sonication can cause protein unfolding and aggregation [25].

We have observed that heat or sonication causes significant structural changes in a homogenized brain material. When we exposed BH samples to heat (37 °C, 24 h; or 80 °C, 5 min) or sonication, and then analyzed them by light scattering spectroscopy, the most notable change was formation of precipitate Fig. (**3**). This may result from conformational changes of proteins present in BH. Formation of aggregates was fastest at 80 °C (5 min). Aggregation at 37 °C was slow and required at least 24 h to generate turbidity comparable to that produced in only 5 minutes at 80 °C.

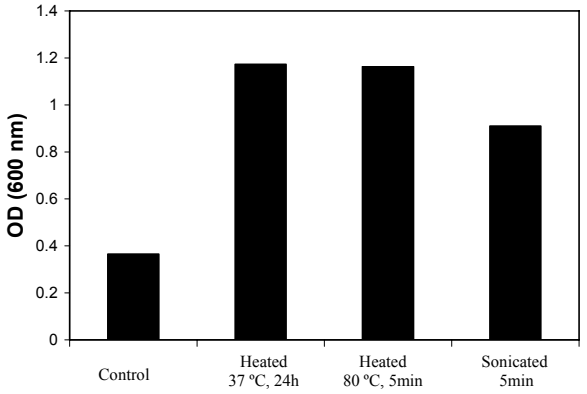

Figure 3. Aggregation of BH. BH samples (100 µl, 10% w/v) were prepared in PMCA buffer. The control sample was stored frozen immediately after its preparation, without heating. Other samples were exposed to heat or sonication as indicated, and formation of precipitate took place. Aggregation/precipitation is reported as light scattering (600 nm).

We next measured light scattering in BH exposed to continuous sonication (200 W, 10 min). Formation of a milky precipitate began immediately with sonication and proceeded for at least 6 minutes Fig. (**4**). After 6 minutes of sonication we observed no additional aggregation up to 10 min at 37 °C. Variation between replicates was relatively poor, although this is typical for sonication experiments, due to uneven distribution and unequal absorbance of energy among samples.

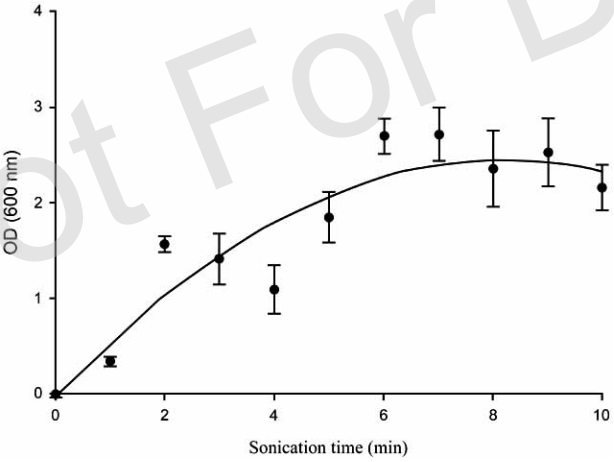

Figure 4. Aggregation of normal brain homogenate (10%) during continuous sonication. Sonicator was set to 170 W, and 3 independent samples were removed every minute over a time course of 10 minutes. Light scattering plateaus with a maximum around 6 minutes, comparable to the minimum ELISA signal reached around 6 minutes (see Figure **2**).

We used MAb 3F4 to characterize structural changes that PrP^C undergoes after exposure of BH to heat or sonication. 3F4 binds to PrP residues 109-112 and has high affinity for native PrP^C and some denatured forms of PrP^C and PrP^{Sc} [26]. Samples from Fig. (**3**) were analyzed by native PAGE/Western (data not shown) and by [native] ELISA Fig. (**5**), both using MAb 3F4. Results were similar for both methods, but ELISA offered better quantitative signal. The data show that the transformation of PrP^C we associated with aggregation is accompanied by loss of 3F4 epitope, implying relatively large change in structure. Relative to the control sample, the ELISA signal decreased approximately 10-fold. When we digested the same samples with PK and subjected them to SDS-PAGE, (data not shown) we observed no significant PK resistance, confirming that PrP^C was not converted to PrP^{Sc} in this experiment.

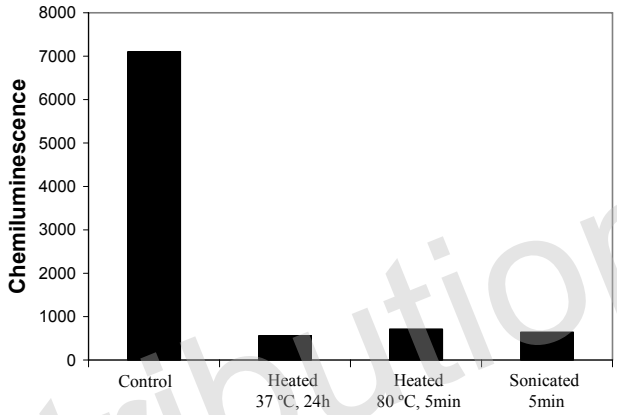

Figure 5. Native ELISA of BH (10% w/v) under different experimental conditions. Samples from Figure 3 were subjected to native ELISA as described in the experimental section. Samples were not PK digested. The data were normalized to frozen control. Background signal 800 arbitrary units.

To initiate conversion of PrP^C to PrP^{Sc} after pretreatment by heat or sonication, we seeded samples (100 µl) from (Fig. **3**) with a small amount (1:100) of infected BH (10% w/v). The samples were then incubated (37 °C, 24 h), PK digested, and analyzed by Western blotting. Samples 1 and 2, which were pretreated with heat (37 °C, 24 h; 80 °C, 5 min) and then seeded with infectious BH without sonication, underwent negligible conversion to PrP^{Sc} Fig. (**6**). The sample subjected to prior sonication and then seeded with infected BH was significantly converted ($p < 0.004$ versus control). And as a positive control, when we applied standard PMCA to an untreated BH sample we observed very strong conversion ($p < 0.0003$ versus sonication pretreatment). These results suggest sonication plays an important role in the conversion process of PrP^C to PrP^{Sc}. The greater conversion achieved through the intermittent incubation and sonication cycles of PMCA suggests conversion requires a few minutes time. It also supports the theory that sonication in PMCA breaks up aggregates and fibers to produce more numerous or perhaps more effective seed material [10].

It is worth noting that we calculate conversion efficiency based on a direct increase in band intensity of Western blots and not on dilution factors as described by Castilla, *et al*. [8] In our laboratory, after five rounds of PMCA we routinely achieve 10^{12}-fold amplification on serially diluted samples, similar to Castilla, *et al*. Fig. (**7**).

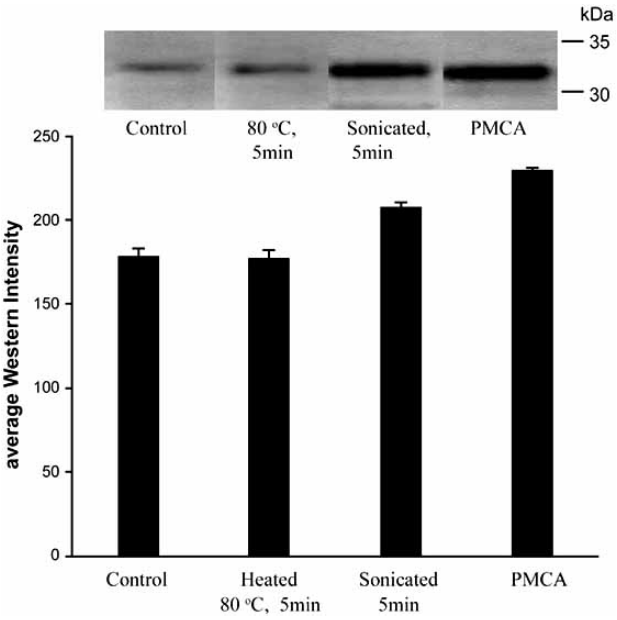

Figure 6. Western blot analysis of conversion efficiency of PrP^{C} to PrP^{Sc}. After treatment as described in Figure 3, all samples were seeded with 1:100 infected BH (10% w/v) and then incubated (37 ºC, 24 h). For comparison, PMCA was performed on a similar untreated sample. All samples were subjected to PK digestion followed by Western blotting and densitometry. The band intensities were evaluated as described in Figure 1. Mean of three trials with error bars representing standard deviation. Although 80 ºC heating did not increase proteinase K resistance and subsequent Western blot signal, sonication significantly increased signal ($p < 0.004$), and standard PMCA generated PrP^{Sc} levels even higher than sonication alone ($p < 0.0003$). Inset: representative Western blot raw data.

CONCLUSIONS

The changes in PrP^{C} induced by sonication, 80 °C, or prolonged exposure to 37 °C affect epitope structure or at least accessibility of the protein Fig. (**5**). Neither sonication nor heat alone produces PrP^{Sc}, in that the products are PK sensitive. But that does not exclude the possibility they are infectious. We plan to inoculate animals with these reaction products to determine whether they are infectious.

When healthy BH is seeded with PrP^{Sc}, sonication becomes the key step in converting PrP^{C} to PrP^{Sc}. Small amounts of PrP^{Sc} are produced by incubation-only in the absence of sonication [5,22]. One of the mechanisms believed to be responsible for sonication-induced conversion is mechanical breakage of growing PrP^{Sc} aggregates to produce more nuclei to facilitate formation of PrP^{Sc} [10]. However, presonication of healthy BH makes it more susceptible to the conversion if the presonicated BH is seeded with a small amount of infected BH Fig. (**6**). Presonication changes PrP^{C} structure or accessibility Fig. (**2**). That suggests that in addition to producing more nuclei, sonication produces intermediate PrP^{C} conformation that subsequently can undergo conversion to PrP^{Sc} in the presence of PrP^{Sc}.

In PMCA, conversion slows over time and stalls Fig. (**1**). Addition of fresh BH restores conversion. This suggests that, in addition to producing an intermediate conformer for conversion to PrP^{Sc}, sonication or heat produces a fraction of dead-end PrP that is not suitable for conversion to PrP^{Sc}. This might be a change in protein conformation to produce an inert structure that is stable on the time scale of the experiment. Or it might be due to the formation of vesicles or precipitation of large membrane fragments. Or perhaps other factors/cofactors necessary for conversion are consumed, destroyed, or compartmentally encrypted by lipid structures during PMCA.

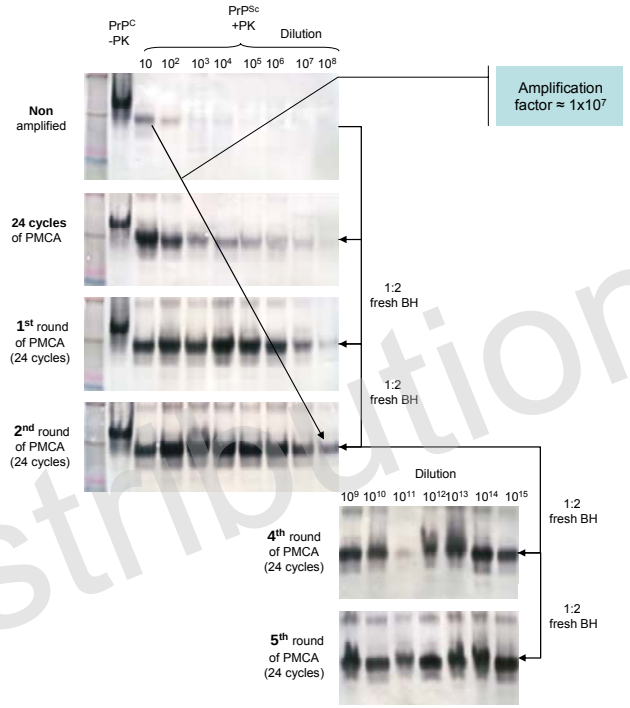

Figure 7. Conversion efficiency of PrP^{C} to PrP^{Sc} of serially diluted samples. Infected BH (10% w/v) were serially diluted into healthy BH (200 µl, 10% w/v) to obtain range from 10 to 10^{15}-fold dilution. The fifteen (200 µl) samples were split into two sets of fractions (100 µl each). One set of fractions was frozen immediately (not amplified). The other set of fractions was subjected to 24 PMCA cycles. After 24 PMCA cycles, the samples (100 µl) were split into two fractions and one set of fractions (50 µl) was frozen for later analysis. The other set of fractions were diluted with fresh BH (10% w/v) to obtain the final volume of 100 µl and subjected to the 1st round of PMCA consisting of 24 cycles. The process was repeated up to the 5th round of PMCA (3rd round is not shown). The samples (50 µl) were PK digested and Western blotted. The blots show detection of 10^{8}-fold dilution after 2nd round of PMCA (10^{7} amplification factor) and 10^{15}-fold dilution after 5th round of PMCA. Samples (–PK) correspond to unseeded control samples.

It seems that several mechanisms compete for facilitation or inhibition of PrP^{Sc} formation in PMCA. Whether sonication changes PrP or unidentified partners required for protein misfolding remains to be clarified. But a better understanding of the molecular processes involved in conversion should help improve robustness of the reaction. Then PMCA might become practical for sensitive TSE detection, ultimately producing a test to determine infectivity in agricultural products

and environmental samples, and identify disease in live animals before they show clinical symptoms.

Note added in proof: While this paper progressed through peer review, Murayama, *et al.*, published data on efficient PMCA in a mouse BH system [27]. They observed formation of an unusual PrP^{C} conformation (interpreted as large aggregates) that prevented progress of the PMCA reaction from PrP^{C} to PrP^{Sc}. They named this PrP^{C} conformation "$PrP^{C\text{-}res}$". Noting that others had used saponin to completely solubilize PrP^{C} in mouse N2A cells [28], Murayama, et al, used 0.05% digitonin to inhibit formation of $PrP^{C\text{-}res}$, thereby greatly facilitating PMCA in mouse BH.

LIST OF ABBREVIATIONS

AP	=	Alkaline phosphatase
BCIP/NBT	=	5-bromo-4-chloro-3-indolyl phosphate, nitro blue tetrazolium
BH	=	Brain homogenate
BSE	=	Bovine spongiform encephalopathy
°C	=	Degrees Celsius
EDTA	=	Ethylenediaminetetraacetic acid
ELISA	=	Enzyme-linked immunosorbent assay
h	=	Hours
HRP	=	Horseradish peroxidase
kDa	=	Kilodaltons
MAb	=	Monoclonal antibody
MES	=	2-(N-morpholino)ethanesulfonic acid
min	=	Minutes
µg	=	Micrograms
µl	=	Microliters
OD	=	Optical density
p	=	Probability
PAGE	=	Polyacrylamide gel electrophoresis
PBS	=	Phosphate-buffered physiological saline
pH	=	-log[H+]
PK	=	Proteinase K
PMCA	=	Protein misfolding cyclic amplification
PrP	=	Prion protein
PVDF	=	Polyvinylidene difluoride
s	=	Seconds
SDS	=	Sodium dodecylsulfate
TSE	=	Transmissible spongiform encephalopathy
W	=	Watts
w/v	=	Weight per volume

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