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# Breakage of PrP aggregates is essential for efficient autocatalytic propagation of misfolded prion protein

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**Abstract**

The conversion of cellular prion protein ($PrP^C$) to the disease-associated misfolded isoform ($PrP^{Sc}$) is an essential process for prion replication. This structural conversion can be modelled in protein misfolding cyclic amplification (PMCA) reactions in which $PrP^{Sc}$ is inoculated into healthy hamster brain homogenate, followed by cycles of incubation and sonication. In serial transmission PMCA experiments it has recently been shown that the protease-resistant PrP obtained in vitro (PrPres) is generated by an autocatalytic mechanism. Here, serial transmission PMCA experiments were compared with serial transmission reactions lacking the sonication steps. We achieved approximately 200,000-fold PrPres amplification by PMCA. In contrast, although initial amplification was comparable to PMCA reactions, PrPres levels quickly dropped below detection limit when samples were not subjected to ultrasound. These results indicate that aggregate breakage is essential for efficient autocatalytic amplification of misfolded prion protein and suggest an important role of aggregate breakage in prion propagation.


*Keywords:* Aggregation; Amyloid; Breakage; Neurodegeneration; PMCA; Prion; Propagation; Sonication

Transmissible spongiform encephalopathies (TSEs) or prion diseases including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle, and Creutzfeldt–Jakob disease (CJD) in humans are fatal, infectious, neurodegenerative diseases. TSEs appear to be caused by unique proteinaceous infectious agents, termed prions [1]. Prions are considered to be devoid of nucleic acid and consist primarily of a misfolded, protease-resistant isoform of the cellular prion protein ($PrP^C$) designated $PrP^{Sc}$ [2]. The interaction of $PrP^C$ with $PrP^{Sc}$ and the subsequent conversion of $PrP^C$ to its misfolded state are thought to be central events in TSE pathogenesis.

Kocisko et al. [3] were the first to obtain newly formed protease-resistant PrP in vitro using purified $PrP^C$ and $PrP^{Sc}$, their experiments giving support to the 'protein-only' hypothesis. To underline the fact that protease-resistance does not necessarily correlate with infectivity, we refer in this study to protease-resistant PrP generated in vitro as PrPres.

The most recent evidence that prions solely consist of proteins was reported by Legname et al. [4], who were able to induce prion pathology and protease-resistant PrP in mice by inoculating misfolded recombinant prion protein.

In a protein misfolding amplification reaction (PMCA) Saborio et al. [5] reported the conversion of $PrP^C$ to PrPres using brain homogenate from healthy Syrian hamsters mixed with brain homogenate from hamsters infected with 263 K. Amplification of PrPres in this assay is achieved by a cyclic process of alternating incubation and sonication steps. If one assumes a

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hypothetical mechanism of seeded aggregation, the ultrasonic treatment in PMCA experiments would break PrP aggregates into smaller units, providing additional seeds for further aggregate growth during the incubation period [6,7], an effect also observed with purified $PrP^{C}$ and $PrP^{Sc}$ [8].

Using this technique, we recently performed serial transmission experiments reinoculating PMCA products into fresh brain homogenate after 10 PMCA rounds and subjected the samples again to a cycle of 10 alternating sonication and incubation rounds [9]. After 10 cycles (100 rounds of PMCA), including nine dilution steps, we obtained a 300-fold total amplification of PrPres, confirming an autocatalytic self-propagation of misfolded prion protein as postulated by the prion hypothesis.

Although the efficiency of PrPres amplification in PMCA control reactions without sonication appears to be rather poor [5,9], a modified version of the PMCA reaction lacking the laborious ultrasonic treatment has been reported as suitable for PrPres amplification [10] and has already been used as a simplified method for investigating factors that affect prion protein conversion in vitro [11,12].

Considering the ultrasonic treatment as a means of promoting PrP aggregate breakage and referring to mathematical calculations that regard the breakage rate and aggregate size distribution as an important determinant for the infectivity level of a $PrP^{Sc}$ preparation [13], we wanted to investigate if efficient autocatalytic self-propagation of PrPres, as we have shown it in serial transmission PMCA experiments, is also possible without promoting aggregate breakage by ultrasonic treatment. We therefore performed within this study for the first time amplification reactions without sonication using the protocol published by Lucassen et al. [10] with serial dilutions and compared the resulting data with serial transmission PMCA reactions over 15 amplification cycles.

## Materials and methods

*Serial transmission PMCA reactions.* Serial transmission PMCA experiments were performed as described by Bieschke et al. [9]. Briefly, brain homogenate (10% w/v) from 263 K infected Syrian hamster in terminal stage of disease was prepared using the protocol published by Saborio et al. [5], and diluted 1:20 with similarly prepared uninfected hamster brain homogenate. Two hundred microliters of this dilution was subjected to 5× 1 s of sonication, followed by 1 h of incubation at 37 °C completing one round of PMCA. After 10 rounds of sonication and incubation the reaction mixture was passaged by diluting aliquots 2- or 2.5-fold into normal hamster brain homogenate. In total, 15 amplification cycles, including 10 PMCA rounds each, with 14 dilution steps were performed (150 rounds of PMCA). To avoid microbial contaminations all experimental procedures were performed under sterile conditions.

*Serial transmission reactions without sonication.* For the serial transmission experiments without sonication we followed a modified version of the amplification reaction reported by Lucassen et al. [10]. Brain homogenate (10% w/v) from Syrian hamsters infected with 236 K in the terminal stage was prepared and diluted 1:50 with phosphate-buffered saline and 1% Triton X-100 as described [10]. An equal volume of brain homogenate (10% w/v) from uninfected hamsters prepared without detergent was added. The mixture was then incubated for 16 h at 37 °C followed by a 2-fold dilution in brain homogenate (5% w/v) from uninfected hamsters with 0.5 % Triton X-100. Incubation and dilution was alternately repeated, leading to 15 amplification reactions with 14 dilution steps in total. All experimental procedures were performed under sterile conditions.

*Proteinase K digestion, Western blotting, and quantification of PrPres.* For quantification of PrPres during serial transmission reactions samples of 20 µl were taken before and after amplification, frozen for storage, digested with proteinase K (100 µg/ml) for 1 h at 37 °C, and subjected to Western blot analysis using the 3F4 antibody at a dilution of 1:2,000 [14]. Western blots were quantified using Diana II luminescence imaging system (Raytest, Straubenhardt, Germany) and the AIDA software package. Amplification factors after each cycle were calculated by comparing the intensities of un-, mono-, and diglycosylated PrP bands in the molecular mass range between 20 and 30 kDa after ($I_{\text{after Amp}}$) and before ($I_{\text{before Amp}}$) amplification (amplification factor $= I_{\text{after Amp}}/I_{\text{before Amp}}$).

## Results

In serial transmission PMCA experiments after fourteen sequential 2 or 2.5-fold dilutions (Figs. 1B and C) the initial $PrP^{Sc}$ material was diluted by a factor of 16,384 and 372,529, respectively. For the 2.5-fold dilution PrPres quantity obtained after the final passage was about 57% of the initial signal (Fig. 2A). During the course of the experiment the amplification factor varied between 1.3 and 3.0 (Fig. 2B), with an average value of $1.9 \pm 0.2$, resulting in over 200,000-fold total amplification of PrPres after 15 passages (150 rounds of PMCA). In experiments where a 2-fold dilution was applied over 10,000-fold total amplification of PrPres was achieved with an average amplification factor of $1.8 \pm 0.02$.

In order to rule out the possibility that PrPres detected in our PMCA products is generated de novo due to sonication or other experimental conditions apart from the initial $PrP^{Sc}$ that could support protein misfolding, we performed similar transmission reactions with brain homogenate lacking $PrP^{Sc}$. After PK digestion no PrPres could be detected at any time of the experiment (data not shown), proving that PrPres formation under these experimental conditions is strictly dependent on seeds of misfolded protein that catalyze conversion.

If in PMCA experiments as postulated by Saborio et al. [5] PrP aggregates act as seeds that are elongated during incubation and are broken into smaller units by sonication, which in turn provide new catalytic surfaces for further misfolding of $PrP^{C}$, both the sonication and the incubation steps are required for efficient self-propagation of misfolded protein. To verify this, we performed control reactions in which samples were sonicated without incubation (Fig. 1A). No amplification of PrPres