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Early stage prion assembly involves two subpopulations with different quaternary structures and a secondary templating pathway

Angélique Igel-Egalon, Florent Laferrière, Mohammed Moudjou, Jan Bohl, Mathieu Mezache, Tina Knäpple, Laetitia Herzog, Fabienne Reine, Christelle Jas-Duval, Marie Doumic, Human Rezaei, and Vincent Béringue

The dynamics of aggregation and structural diversification of misfolded, host-encoded proteins in neurodegenerative diseases are poorly understood. In many of these disorders, including Alzheimer’s, Parkinson’s and prion diseases, the misfolded proteins are self-organized into conformationally distinct assemblies or strains. The existence of intrastrain structural heterogeneity is increasingly recognized. However, the underlying processes of emergence and coevolution of structurally distinct assemblies are not mechanistically understood. Here, we show that early prion replication generates two subsets of structurally different assemblies by two sequential processes of formation, regardless of the strain considered. The first process corresponds to a quaternary structural convergence, by reducing the parental strain polydispersity to generate small oligomers. The second process transforms these oligomers into larger ones, by a secondary autocatalytic templating pathway requiring the prion protein. This pathway provides mechanistic insights into prion structural diversification, a key determinant for prion adaptation and toxicity.
I n terms of pathogenic mechanisms, the prion paradigm unifies a number of neurodegenerative disorders that are caused by protein misfolding and aggregation1–4. These disorders include Alzheimer’s, Parkinson’s, Huntington’s, and prion diseases. In principle, host-encoded monomeric proteins are converted into a misfolded, β-sheet-rich conformer termed PrPSc5,7. In susceptible hosts, PrPSc assemblies form stable, structurally distinct PrPSc conformers termed prion strains6,9, which encode stereotypical biological phenotypes10–13. The strain-specific differences can be observed at the secondary and tertiary structural level in terms of local structural variation but also at the quaternary level with strain-specific size distribution signature1,14,15. A large body of evidence supports the view for structural diversity within specific prion populations and strains: first, prion substrains can be preferentially selected during prion transmission16–19 with a species/transmission barrier; second, size- or density-fractionation studies support the existence of a heterogeneous spectrum of PrPSc assemblies with distinct tertiary/quaternary structures14,15,20–25 and biological activity (templating activity and infectivity)14,15,20, and third, kinetic studies of prion pathogenesis suggest that the formation of neurotoxic PrPSc species26 occurs at the late stage of prion infection when replicative PrPSc assemblies are formed at earlier stages27,28. The prion replication process thus intrinsically allows the structural diversification of PrPSc assemblies.

While the kinetic aspects of prion replication “as a whole” have been comprehensively described by measuring infectivity or PrPSc levels in the brain (e.g., references29,30), PrPSc structural diversification processes remain undescribed and are not mechanistically supported by prion paradigm frameworks. The autocatalytic conversion model proposed by Griffith in 196731, the nucleated-polymerization model described by Lansbury and Caughey in 199532 and other derived models (e.g.33) merely assume the existence of structurally homogenous assemblies that have absolutely identical propensity to replicate throughout disease progression. These mechanisms intrinsically reduce PrPSc heterogeneity due to the best replicator selection process34,35,36. A recent high-resolution structural analysis of the N-terminal domain of the yeast prion Sup35 suggests that conformational fluctuations in natively disordered monomeric Sup35 are responsible for the stochastic, structural diversification of Sup35 aggregates37. While this idea may be extrapolated to mammalian prion PrP to explain intrastrain structural diversification and strain mutation38, it does not explain the co-propagation of structurally distinct PrPSc subassemblies in the same environment16,38.

To examine the molecular mechanisms of PrPSc replication and structural diversification, we explore, by sedimentation velocity (SV)-based methods, the quaternary structure evolution of PrPSc assemblies during the early stage of prion conversion in vivo and in a cell-free system by protein misfolding cyclic amplification (PMCA). By using several prion strains as templates, we demonstrate that early prion replication invariably generates two subsets of assemblies, termed A1 and B0, which differ in proportion, size, the architecture of their elementary bricks and structure. Analyzing their kinetics of formation during PMCA together with kinetic data assimilation reveals the existence of two sequential processes of formation. The first process corresponds to a quaternary structural convergence, as it tends to reduce the parental quaternary structure polydispersity to generate mostly small-sized assemblies, namely A1. The second process transforms the A1 into structurally different assemblies, namely B0, according to a secondary auto-catalytic pathway requiring PrPC, whereby B0 facilitates its own formation.

**Results**

Small PrPSc oligomers are formed at early replication stage. The early phases of prion replication are commonly thought to consist of an elongation process40, with the PrPSc template serving as a base. We studied the size distribution of proteinase K (PK)-resistant PrPSc (PrP(Res)) assemblies at the early step of prion replication in the brain by SV in an iodixanol gradient using a previously published methodology15,20,41. The PrP(Res) size distribution at the disease end-stage served as control. Three different host-PrP/strain combinations were studied: the 127S cloned scrapie prion strain in ovine PrP tg338 transgenic mice29, the 139A cloned mouse scrapie prion strain in murine PrP tg20 mice42, and the vCJD cloned human prion strain in human PrP tg650 mice43,44. As shown in Fig. 1a–c (Supplementary Fig. 1a–c for the corresponding immunoblots), small oligomers sedimenting between fractions 1 and 4 were preferentially detected at the early stage of pathogenesis, regardless of the strain considered. A second population of oligomers with a larger size distribution and peaking in fractions 8–10 and 18 was observed for 127S prions. The contribution of incompletely digested PrPC or remnant PrPSc inoculum to the PrP signal detected in the top fractions was discarded. No PrPC signal was detected after PK treatment of uninfected tg338 brain (Supplementary Fig. 2a–b). No PrP(Res) was detected in the brain of vCJD inoculated PrP knock-out mice (PrP(−/−) analyzed for PrP(Res) content at early time points (Supplementary Fig. 3). At the disease end stage and for the three strains, larger assemblies were observed (Fig. 1a–c, Supplementary Fig. 1a–c). These observations suggest that the replication process follows two phases that are common to the three strains: (i) a first phase generating mainly small oligomers equivalent in size; (ii) a second phase of quaternary structural diversification during the disease evolution.

We next determined whether these phases can be reproduced by an in vitro bona fide amplification method. We used a high-throughput variant of PMCA (termed mb-PMCA41,45,46). mb-PMCA generates in one unique round of 48 h as much infectivity as in the brain at the terminal stage of the disease, with high reproducibility in terms of limiting dilution and amplification yield45,46. When the size distribution of the amplified products was analysed by SV, two discrete distributions were observed for the three strains, a major set of small PrP(Res) assemblies sedimenting between fractions 1 and 3 (named peak P1) and a minor set of larger assemblies with a well-defined Gaussian distribution centered on fraction 15 (named peak P2) (Fig. 1d; Supplementary Fig. 1d). The relative proportions of P1 and P2 varied among the three strains; P2 was barely detected in the 139A amiplicons. When the mb-PMCA reaction was seeded with healthy tg338 brain homogenate, there was no evidence of spontaneous formation of P1 and P2 PrP(Res) in the amplified products (Supplementary Fig. 2c–d). These data indicate that mb-PMCA generates two populations of PrPSc assemblies that differ according to their quaternary structures, with a predominance of small assemblies.

The bimodal (i.e., generation of two peaks) and discrete behavior of the size distribution as well as the formation of predominantly small assemblies in P1 may originate from the mb-PMCA conditions (i.e. shearing forces during the sonication step47–49) rather than from the replication process itself. To discriminate between these two possibilities, undiluted 127S seeds (i.e., 20% brain homogenate) were incubated and sonicated in identical mb-PMCA conditions, but without the PrPSc substrate (i.e., 1:1 dilution in PrP(0/0) brain lysate). The samples were then SV-fractionated and analysed for PrP(Res) content by western blot. For comparison, the same brain was diluted in the PMCA buffer immediately before the mb-PMCA reaction, as we reported previously that a simple dilution affects the size distribution of
PrPSc assemblies, by displacing the equilibrium between PrPSc assemblies and their suPrP elementary subunit (PrPSc \( \rightleftharpoons \) PrPSc + suPrP)\(^{41,50}\). While the dilution of 127S seed indeed drastically affected the size distribution of 127S PrPSc assemblies, sonication of concentrated 127S seeds in the Prp0/0 substrate revealed mostly the presence of large-sized assemblies (Fig. 1e, Supplementary Fig. 1), peaking in fraction 12–16, as for 127S fractionated brain material solubilized at 37 °C\(^{20}\). The absence of sonication effect on PrPSc assemblies' size distribution rules out a fragmentation process during the mb-PMCA reaction.

Altogether, these observations suggest that (i) in vivo, the early phase of replication generates mainly small-sized assemblies, which diversify with respect to quaternary structure during the disease pathogenesis; (ii) similar to in vivo replication, the mb-PMCA amplification conditions generate two sets of PrP assemblies that differ in their quaternary structures. The formation of these two groups of assemblies is common to the 127S, 139A, and vCJD strains.

**Bimodal and autocatalytic evolution of PrPres from P1 to P2.** We next asked whether P2 formation resulted from a simple condensation of P1 peak assemblies (Oswald ripening\(^{31}\) or coagulation\(^{32,33}\) process) or from an alternative templating pathway. We first examined the influence of the amplification rate on the formation of these two species by varying the concentration of the seed used as template for the mb-PMCA reaction. We generated mb-PMCA products seeded with 10\(^{-5}\)–10\(^{-10}\) dilutions of 127S brain homogenate. The amounts of PrPres amongst the amplified products were similar, whatever the seed dilution (Supplementary Fig. 4), as previously observed\(^{46}\). The SV-sedimentograms of the mb-PMCA products are shown in Fig. 2a. The relative amounts of assemblies in P1 decreased as the concentration of the seed used as template for the mb-PMCA reaction increased, whereas the amounts of those from P2 increased as a function of the seed concentration. The variation in the P1 and P2 peak maximum as a function of the logarithm of the dilution factor revealed a quasi-linear decrease in P1 when the P2 peak maximum followed a sigmoidal increase (Fig. 2b). Such non-linear correlation in P1 and P2 peak variation indicates that (i) P2 peak formation does not result from the simple condensation of assemblies present in P1, (ii) the formation of PrPres assemblies in P2 follows a seed concentration-dependent cooperative process. This strongly suggests that the assemblies forming the P1 and P2 peaks result from distinct polymerization pathways and should therefore be structurally distinct.

To further explore the entanglement between the assemblies forming P1 and P2, we set the mb-PMCA regime to favor the
formation of the P1 peak by using high dilutions of the inoculum seed, followed by quiescent incubations (i.e., without sonication) at 37 °C for increasing periods. As shown with 127S prions, the SV analysis at defined incubation time points post-PMCA revealed a time-dependent, drastic decrease in the population of P1 in favor of P2 (Fig. 2c).

Comparing the distribution in isopycnic gradients20 of the PrPres populations at 0 h and 24 h of quiescent incubation revealed a quasi-similar density for PrPres assemblies composing the P1 and the P2 peaks (Fig. 2d). Thus, the SV increase of P2 compared to P1 results strictly from a quaternary structure rearrangement through size increase.

During the quiescent incubations, the formation of assemblies sedimenting in P2 exhibited a bimodal behavior (i.e., absence of assemblies of intermediate size), without any noticeable shift in the P2 peak position (Fig. 2c). This suggests that the formation of
these assemblies resulted from the association with a specific number of assemblies present in P1. Analyzing the time-dependent surface variation of P1 and P2 showed that the formation of P2 assemblies started slowly, increased steadily from ~2–3 h up to ~7 h and finally reached a plateau. This sigmoidal variation is hallmark of an autocatalytic reaction and indicates that the assemblies present in P2 enhance their own formation. Similarly, the 139A and vCJD prions showed a bimodal evolution of P1 to P2 during a 24-h quiescent phase (Fig. 2f), arguing in favor of a generic process of transformation.

To determine whether the P2 peak assemblies could further evolve, we extended the quiescent phase up to 30 days. For the three prion strains, the sedimentogram curves at 7 and 30 days showed a translational shift in the P2 peak to higher fractions, indicative of an isokinetic increase in their mean average sizes (Fig. 3a, left curves). This size translation deeply contrasts with the bimodal phase observed during the 0 to 7-day quiescent incubation and highlights a change in the kinetic regime. This new regime would be compatible with a coalescence process whereby assemblies would grow by end-to-end or lateral association rather than by incorporation of monomers (Supplementary Fig. 5).

Altogether, the quaternary structure variation of PrPres assemblies as a function of seed-concentration or time followed two distinct kinetic regimes. The first regime, occurring during the early steps of the conversion process, leads to a bimodal and cooperative size increase, which indicates the existence of an autocatalytic transformation of PrPres assemblies present in P1 to P2. The bimodal aspect of the size distribution tends to indicate that the PrPres assemblies forming P1 structurally differ from those forming P2. The second regime, occurring on long-term quiescence is more compatible with a coalescence process.

**Quasi-reversible transformation of PrPres from P1 to P2.** The bimodal and cooperative transformation of P1 to P2 reported in Fig. 2c, e is incompatible with the existence of an equilibrium between the assemblies populating these peaks and a coalescence or coagulation process (Supplementary Fig. 5). To further disprove the existence of such an equilibrium (or detailed-balance), we first set a mb-PMCA regime favoring the formation of the P1 peak together with the P2 peak (low dilution of the inoculum seed, Fig. 4a), isolated by SV the assemblies forming P1 and P2 and studied their quaternary structural evolution on isolation during quiescent incubation for 7 days at 37 °C. Almost all P1 in isolation was transformed into P2 (Fig. 4b). The P2 peak in isolation did not lead to the retro-transformation of the P1 peak by depolymerization (Fig. 4c). These observations reflect an irreversible transformation process and underly the absence of an equilibrium between P2 and P1. The irreversible character of the transformation of P1 to P2 argues in favor of the existence of a thermodynamically driven “locking” process. This implies structural rearrangements of P1 assemblies and formation of higher stable P2 objects.

P1 and P2 contain structurally distinct PrPres assemblies. To further confirm the structural rearrangement in the PrPres assemblies accompanying the P1 to P2 transformation, we determined the specific infectivity of the P1 and P2 assemblies. A 127S-PMCA product was fractionated at the end of the reaction or after 48 h of quiescent incubation. Pools of fractions corresponding to the P1 and P2 peaks were inoculated into reporter tgs338 mice. The specific infectivity (infectivity per PrP molecule), which is mostly associated to PrPres assemblies (i.e., negligible contribution of PK-sensitive PrPres species to 127S infectivity), was calculated from the mean survival time using 127S dose-response curves. The specific infectivity of the P1 peak assemblies was 50–100-fold higher than that of the P2 peak assemblies (Fig. 5). This indicates that the P1 and P2 peaks contain structurally distinct sets of PrPres assemblies, named A1 and B1 (the i index referring to the number of monomer/subunit in the assembly).

The specific infectivity of P1 did not change over a longer period of quiescent incubation (7 days), suggesting that the transformation of the assemblies present in the P2 peak into larger assemblies was not associated with a structural change measurable by their specific activity.

Architectural characterization of A1 and B1 assemblies. To characterize the structural difference between A1 and B1 assemblies at the level of their elementary subunit, we used a size exclusion chromatography (SEC) method in native condition, allowing hydrodynamic radius-based analyses. To determine if the hydrodynamic radius from B1 elementary subunit (suPrPB) differ from that of A1 (suPrPA), 127S-PMCA products generated at high-seed dilution (10−8) were analysed by SEC immediately at the end of the reaction (defined at t0) or after a 7-day quiescent incubation. At t0, the SEC profile showed the existence of a unique peak eluting at 14.7 ml (Fig. 6a). As PMCA products generated at 10−8 seed dilution mostly contain A1 assemblies in the P1 peak (Fig. 6b), one can attribute the SEC peak at t0 to suPrPA. After the 7-day quiescence, the chromatogram revealed the emergence of an additional peak eluting at 15.5 ml (Fig. 6a), which correlates with the transformation of A1 to B1, observed by SV (Fig. 6b). This new peak was thus attributed to suPrPB. The
tiny difference observed in the elution volume between suPrPA and suPrPB suggests a difference in their hydrodynamic radius (suPrPB assemblies being more compact than suPrPA assemblies), and therefore a difference in their structure.

To gain further insight into the mechanism of suPrPB formation, 127S-PMCA products generated with different seed concentrations as in Fig. 2a were analysed by SEC. At high-seed dilution (10⁻⁸ dilution factor), the chromatogram revealed the existence of suPrPA (Fig. 6c). Lower seed dilutions led to the emergence of a new peak with an elution volume of 15.5 ml corresponding to the emergence of suPrPB, and a shift toward lower elution volume of suPrPA (Fig. 6c). This last phenomenon could be the chromatographic echo of a dissociation/association equilibrium displacement between different species during the separation on the SEC column⁵⁵,⁵⁶.

Collectively, the SEC analysis of the P₁ to P₂ transformation demonstrates that the formation of B₃ species is concerted with the emergence of a new elementary subunit (suPrPB). suPrPB differs from suPrPA by its hydrodynamic radius and therefore its...
PrP-dependent generation of B, assemblies from A, assemblies. a PMCA products from 127S, 139A and vCJD prions (10^5, 10^4, and 10^4 diluted seeds, respectively) were treated with or without PK to eliminate PrP^C before quiescent incubation at 37 °C for 2 days, 7 days or 30 days, as indicated. At each time point, the collected products were frozen. All collected samples were then thawed, SV-fractionated in parallel and analysed by immunoblotting (mean ± SEM values from n = 3 independent experiments). b, c Relative percentage of P1 versus P2 peaks in SV-sedimentograms from ±PK-treated PMCA products reconstituted in PrP0/0 or PrP^C containing tg338 mouse brain homogenates, and incubated in quiescent conditions for 2 or 7 days. b PMCA products were generated with a 10^6-diluted 127S prion seed. At the end of the PMCA reaction (10), the products were mixed, and eventually treated with high concentration of PK to remove residual PrP^C. After PK inhibition, the products were then diluted 1:1 in either PrP0/0 or PrP^C containing tg338 brain homogenate and incubated for 2 days or 7 days at 37 °C in quiescent conditions. c The PMCA products were then fractionated by sedimentation velocity and analysed for PrPSc content by immunoblot. The amount of PrPSc in the fractions corresponding to P1 and P2 populations was quantified. The results shown are the mean ± SEM values from n = 3 independent experiments.

PrP-dependent generation of B, assemblies from A, assemblies. a PMCA products from 127S, 139A and vCJD prions (10^5, 10^4, and 10^4 diluted seeds, respectively) were treated with or without PK to eliminate PrP^C before quiescent incubation at 37 °C for 2 days, 7 days or 30 days, as indicated. At each time point, the collected products were frozen. All collected samples were then thawed, SV-fractionated in parallel and analysed by immunoblotting (mean ± SEM values from n = 3 independent experiments). b, c Relative percentage of P1 versus P2 peaks in SV-sedimentograms from ±PK-treated PMCA products reconstituted in PrP0/0 or PrP^C containing tg338 mouse brain homogenates, and incubated in quiescent conditions for 2 or 7 days. b PMCA products were generated with a 10^6-diluted 127S prion seed. At the end of the PMCA reaction (10), the products were mixed, and eventually treated with high concentration of PK to remove residual PrP^C. After PK inhibition, the products were then diluted 1:1 in either PrP0/0 or PrP^C containing tg338 brain homogenate and incubated for 2 days or 7 days at 37 °C in quiescent conditions. c The PMCA products were then fractionated by sedimentation velocity and analysed for PrPSc content by immunoblot. The amount of PrPSc in the fractions corresponding to P1 and P2 populations was quantified. The results shown are the mean ± SEM values from n = 3 independent experiments.

The formation of B, from A, assemblies requires PrP^C. Our previous studies revealed that only ~30% of the PrP^C substrate is converted into PrPSc after a complete round of mb-PMCA. To determine whether the remaining 70% still participated in the transformation of A, to B, assemblies during the quiescent phase, PMCA products from the 139A, 127S and vCJD prions were treated with PK to eliminate PrP^C before quiescent incubation at 37 °C. As shown in Fig. 3a (right panel), the amount of B, assemblies generated during the 48-h quiescent incubation was drastically and invariably decreased. Further quiescent incubation for 7 and 30 days in the absence of PrP^C allowed the formation of comparatively low amounts of B, assemblies for 127S and 139A prions.

To determine if the drastic decrease of A, to B, transformation was specific to depletion of PrP^C or of cofactors, we performed reconstitution experiments of ±PK-treated 127S-PMCA products (e.g., without potential, PK-susceptible co-factors) with either PrP^000 brain homogenate (e.g., media containing all brain cofactors except PrP^C) or tg338 brain homogenate (e.g., media containing all cofactors and PrP^C) before 48 h or 7 days of quiescent incubation (Fig. 3b). The quiescent products were then SV-fractionated and the amount of PrPSc in the fractions corresponding to P1 and P2 peaks was quantified. As shown in Fig. 3c, reconstitution of the PK-treated PMCA amplicons with PrP^000 brain homogenate did not allow B, neoformation as compared with reconstitution in tg338 media. A depolymerization of B, assemblies was even observed when the reconstitution was done in PrP^000 brain homogenate. Thus, the contribution of PK-sensitive PrP conformers and protein cofactors appeared
Fig. 6 Characterization of the elementary subunit of PMCA-generated PrPSc assemblies by size exclusion chromatography (SEC) under native conditions. 

**a** SEC analysis (n ≥ 3 independent fractions) of mb-PMCA products generated with 127S prions (10⁻⁸ dilution) immediately after the PMCA phase (day 0) or after 7 days of quiescent incubation (day 7). A representative immunoblot corresponding to elution volumes 12 ml to 18 ml is shown (electrophoretic concentration on loading as in Supplementary Fig. 1). The column calibration was performed using standard MW calibrants under identical conditions as for PMCA products analysis. 

**b** Representative sedimentogram of mb-PMCA products generated with 127 S prions (10⁻⁸ dilution) post-PMCA reaction (day 0) and after a 7-day quiescent incubation, highlighting the P₁ to P₂ evolution of PrPSc assemblies. 

**c** SEC profiles of mb-PMCA products generated with 127S seeds at different dilution factors, as indicated. Thirty minutes after the last sonication, the amplified products were solubilized and SEC-fractionated. The mean relative levels of PK-resistant PrPSc per fraction were obtained from the immunoblot analysis of a number of elementary steps was identified based on experimental observations and used as unavoidable constraints⁵⁷. The first constraint is the existence of two structurally distinct PrPSc subassemblies, namely Aᵢ and Bᵢ, with distinct dynamics. Indeed, structurally equivalent assemblies would fail to present a bimodal size distribution, cooperative seed concentration and kinetic evolution or distinct specific infectivity, as mathematically demonstrated in Supplementary Fig. 5. The second constraint is the existence of a detailed-balance between the PrPSc assemblies and their elementary subunit, as previously shown⁴¹, making the size distribution of the PrPSc assemblies highly dynamic and dependent on the assembly concentration (Fig. 1e). The 3rd constraint is that Aᵢ and Bᵢ assemblies are in detailed-balance with their respective suPrPs (Fig. 6), denoted suPrPᴬ and suPrPᴮ but with distinct equilibrium constants Kᴬ and Kᴮ. Thus, at any moment of the process of assembly transformation of Aᵢ to Bᵢ, the following equilibrium should be respected:

\[ Aᵢ ⇌ Aᵢ₋₁ + suPrPᴬ \]  

\[ Bᵢ ⇌ Bᵢ₋₁ + suPrPᴮ \]  

The equilibrium constants Kᴬ and Kᴮ govern the respective size distribution of the Aᵢ and Bᵢ assemblies and, thus, the bimodal aspect of the curve. According to our previous SV calibrations with PrP oligomers and globular mass markers¹⁵, the size distribution of the Aᵢ and Bᵢ subassemblies were fixed: i_A < 5 and i_B centered around 20 PrP-mers. Due to the limited resolution of SV fractionation for small assemblies, we assumed that Aᵢ and suPrPᴮ cosedimented. The fourth constraint relies on the fact that A to B transformation requires PrP⁴ and that the kinetic is cooperative (Figs. 1e and 2). This cooperativity implies that B subassemblies facilitate their own formation according to an autocatalytic process. This can be resumed by the following minimalistic autocatalytic process:

\[ suPrPᴬ + suPrPᴮ ⇌ C \]  

\[ C + PrP⁴ → 2suPrPᴮ \]  

where C is an active complex reacting with PrP⁴ that generates B assemblies. Considering that suPrPᴮ can condense into Bⱼ⁴¹ and according to detailed-balance (2), one can establish the reaction model describing the formation of Bᵢ assemblies from the neo-formed suPrPᴮ:

\[ 2suPrPᴮ ⇌ Bᵢ \]  

Altogether, these five elementary steps constitute the reaction mechanism that describes the transformation of Aᵢ into Bᵢ subassembly species.

To validate the designed mechanism, we translated these elementary reactions into time-dependent differential equations (for more details, see Supplementary Note) and performed kinetic simulations using the size distribution of the PrPSc assemblies immediately after cyclic amplification as the initial condition (blue curve in Fig. 2a). According to the model, the simulated size
distribution variation as a function of time showed bimodal behavior, as was experimentally observed (Fig. 7a). Furthermore, the theoretical size distribution centroid presented similar sigmoidal patterns to those of the experimental data (Fig. 7b), arguing in favor of an autocatalytic kinetic model describing the overall quaternary structure evolution of PrPSc assemblies during the quiescent phase. The analysis of the model (for more details, see Supplementary Note) revealed that the autocatalytic formation of B species occurs at the expense of A species and with PrPc consumption (Fig. 7c). According to this model, when PrPc is in large excess, A constitutes the limiting compound for the formation of B assemblies. Therefore, during the quiescent phase, the PrPc to PrPSc conversion rate is directly proportional to the amount of A assemblies (Fig. 7c).

**Discussion**

The mechanisms of prion replication and the dynamics responsible for prion structural diversification in the infected host remain unclear and rarely addressed. In the actual framework of the prion paradigm, the templating process is considered at the prion assembly interface, leading to an increased size of the complex formed by the template:substrate, out of the fragmentation/denaturation context. The atypical size distribution observed here at the early replication stage for three distinct prion strains, where accumulation of small-sized assemblies dominates, contrasts with this canonical templating model and requires an additional process that considers the replication dynamics.

As shown in vivo for the vCJD, 127S and 139A strains, the early stage of the replication process in the brain is dominated by the accumulation of small assemblies, whereas higher-size subsets are detected at the terminal stage of pathogenesis. Such quaternary structural diversity, and beyond the existence of structurally distinct types of assemblies, as defined by their specific infectivity (refs. 13,20 and Supplementary Fig. 6), can be exclusively explained by the balance between at least two kinetic modes taking place at different stages of the pathogenesis. Both can be governed by evolution or a fluctuation in the replication microenvironment due to the physio-pathological state of the infected animal and/or to the sequential involvement of specific prion-replicating cell types. However, another possibility lies in the intrinsic and deterministic properties of the PrP replication process to generate structurally distinct types of assemblies. Discriminating between these two non-mutually exclusive hypotheses is technically difficult in vivo. The mb-PMCA as a bona fide amplification method in a more simplified and kinetically controlled context constitutes a relevant method for investigating the intrinsic propensity of the replication process to generate structurally distinct assemblies. Interestingly, and against common belief, the size distribution of the PrPSc assemblies used as seeds was relatively insensitive to repeated sonication cycles when a simple dilution displaced the assemblies towards a smaller size (Fig. 1e and ref. 41). These observations exclude the contribution of the fragmentation process during the mb-PMCA sonication cycles to the size distribution pattern of PrPSc assemblies and emphasize the existence of a constitutional dynamic between the PrPSc subpopulation41, which should be considered during the replication process. We showed that two sets of PrPSc assemblies, A and B, were generated during the mb-PMCA reaction. The A and B assemblies constitute two structurally distinct PrPSc subpopulations. Beside the fact that the bimodal size distribution instead of a continuum constitutes an indirect but solid argument for structural differences in the PrP assemblies populating the P1 and P2 peaks, the best arguments are undoubtedly their distinct specific infectivity and the existence of two distinct assembly subunits. The irreversibility of the P1 to P2 transformation (Fig. 4) physically demonstrates a PrP structural rearrangement associated to the A1→B1 transformation process. Therefore, the prion replication process per se intrinsically generates structurally diverse PrPSc subassemblies in a deterministic process.

According to our SV experiments, small-sized PrPSc assemblies were mainly formed at the early stage of prion replication in the brain and during the mb-PMCA reaction. This was observed with three distinct prion strains on three different PrP genetic backgrounds. Considering that the PrPSc assemblies that constitute each strain are structurally distinct, one can ask how distinct PrPSc assemblies can all generate A1 assemblies that harbor strain

![Figure 7](https://example.com/fig7.png)

**Figure 7** Mathematical modeling of the time-dependent dynamic evolution of the PMCA-generated PrPSc assemblies. **a** The size distribution evolution of a structurally distinct set of assemblies A1 and B1 dimensioned on gradient fraction numbers was simulated based on the kinetic scheme described in the results section (Eq. 1 to 5) and the Supplementary note. **b** The time dependency evolution of the simulated centroid (black line) and centroid calculated from experimental sedimentograms of Fig. 2d (red circle) show a similar shape, supporting the cooperativity hypothesis of the transformation of A1 into B1. **c** The simulation of time dependency evolution of the total amount of A assemblies (ΣA, in blue) and B assemblies (ΣB, in blue) and the monomer (in red) revealed that A1 assemblies constitute the limiting species for the conversion of PrPc during the quiescent phase. In the present simulation framework (for more details, see Supplementary note), only 14% of PrPc is consumed.
The origin of the generic formation of a small-size subset in the 
that Ai have the intrinsic propensity to generate Bi assemblies in 
the transformation of Ai to Bi (reactions 3 and 4). This last 
formation of Bi assemblies. Furthermore, there is compelling 
existence of a common narrow subpopulation of PrPSc (with 
10
structural phenomenon can explain why, for certain prion strains, the most 
critical prion replication process32. It can be reasonably envisaged 
by the combined addition of 4 mM Pefabloc and 2x EDTA-
activity was inactivated by the combined addition of 4 mM Pefabloc and 2x EDTA-
the seeds, input PrPSc is not detected in the mb-PMCA products. PMCA was per-
formed in a 96-well microplate format (Asxgen, Corning) using a Q700 sonicator 
(Qsonica, USA, Delta Labo, Colombelles, France). For quiescent incubation, the 
samples were left in the incubator at 37 °C for the indicated period of time, without 
any sonication. To eliminate residual PrPSc present in the PMCA products before 
quotient incubation, the samples were treated with PK (80 μg/ml final concentra-
the treatment was stopped by adding 2 mM Pefabloc and 1x EDTA-free 
protease inhibitor cocktail. All final products were kept for PrPSc size fractionation, 
and aliquots were PK-digested (115 μg/ml final concentration, 0.6% SDS, 1 h, 37 °C) 
prion prior to immunoblot analyses, as described below.

For reconstitution experiments, mb-PMCA products were generated with a 105-
diluted 127S prion seed. At the end of the mb-PMCA reaction, the products were 
mixed, eventually treated with PK (80 μg/ml final concentration). PK activity was inactivated by the combined addition of 4 mM Pefabloc and 2x EDTA-
protease inhibitor cocktail. The products were then diluted 1:1 in either PrP0/0 
or in tg338 brain homogenate and incubated for 48 h or 7 days at 37 °C in 
quiescent conditions. The PMCA products were then fractionated by 
sedimentation velocity and analysed for PrPSc content by immunoblot.

Sedimentation velocity fractionation. SV experiments were performed as 
described previously15,20,41. Mouse brain homogenates or PMCA products were 
solubilized by adding an equal volume of solubilization buffer (50 mM HEPES pH 7.4, 300 mM NaCl, 10 mM EDTA, 4% wt/vol dodecyl-β-D-maltoside (Sigma)) and 
inactivated for 45 min on ice. Sarkosyl (N-lauryl sarcosine Fluka) was added to a 
final concentration of 2% wt/vol, and the incubation continued for an additional 
30 min on ice. A total of 150 μl of solubilized samples was loaded atop a 4.8-ml 
continuous 10–25% iodixanol gradient (Optiprep, Asysshield), with a final con-
centration of 150 mM HEPES pH 7.4, 300 mM NaCl, 2 mM EDTA, 0.5% Sarkosyl. 
The gradients were centrifuged at 285,000 g for 45 min in a swinging-bucket SW-55 
rotor using an Optima LE-80K ultracentrifuge (Beckman Coulter). Gradients were 
then manually segregated into 30 equal fractions of 165 μl from the bottom using a 
peristaltic pump and analysed by immunoblotting or bioassay for PrPSc or infect-
ity, respectively. To avoid any cross-contamination, each piece of equipment was 
thermally deactivated with 5 N NaOH followed by several rinses in deionized 
water after each gradient collection40.

Isopycnic sedimentation. The entire procedure was performed as described 
previously20. Mouse brain homogenates or PMCA products were solubilized as

Methods

Ethics. Animal experiments were conducted in strict compliance with ECC and EU 
directives 86/099 and 2010/63 and were approved by the local ethics committee of 
the author’s institution (Cornellae; permit numbers 12/034 and 15/045).

Transgenic mouse lines and prion strains. The ovine (tg338 line; Vall136-
Arg154-Gln171 VRQ allele), human (tg650 line; Met129 allele) and mouse (tg20) 
PrP transgenic lines have been described previously25,42,43. The mouse lines were 
found to be homozygous and overexpressed approximately 8-, 6-, and 10-fold amounts 
of heterologous PrPSc on a mouse PrP wild background. PrP0/0 mice were the 
so-called Zürich-1 mice58. Cloned 127S scrapie, human vCJD and mouse 139A 
prion strains were serially passaged in tg338, tg650, and tg20 mice, respectively53,54. 
These strains were used as pools of mouse-infected brains and prepared as 20% wt/ 
vol homogenates in 5% glucose by use of a tissue homogenizer (Precellys 24 
RiboLyzer; Ozyme, Orsay, France).

Time course analysis of prion accumulation. Eight-week-old female tg338, tg650 
and tg20 mice were inoculated intracerebrally in the right cerebral hemisphere with 
127S, vCJD or 139A prions (20 μl of a 10% brain homogenate dose). Infected 
animals were euthanized by cervical column disruption in triplicate at regular time 
points and at the terminal stage of disease. Brains were removed and kept for PrPSc 
size fractionation.

Miniaturized bead-PMCA assay. The miniaturized bead-PMCA (mb-PMCA) 
assay45,46 was used to amplify prions. Briefly, serial ten-fold dilutions of 127S, 
vCJD, and 139A prions (mouse brain homogenates diluted in PMCA buffer) were 
mixed with brain lysates (10% wt/vol) from healthy tg338, tg650 and tg20 mice as 
respectively substrates and subjected to one round of 96 cycles of 30-s sonications 
(220–240 Watts) followed by 29.5 min of incubation at 37 °C. With a >104 dilution 
of the seeds, input PrPSc is not detected in the mb-PMCA products. PMCA was per-
formed in a 96-well microplate format (Asxgen, Corning) using a Q700 sonicator 
(Qsonica, USA, Delta Labo, Colombelles, France). For quiescent incubation, the 
samples were left in the incubator at 37 °C for the indicated period of time, without 
any sonication. To eliminate residual PrPSc present in the PMCA products before 
quiescent incubation, the samples were treated with PK (80 μg/ml final concentra-
the treatment was stopped by adding 2 mM Pefabloc and 1x EDTA-free protease inhibitor cocktail. All final products were kept for PrPSc size fractionation, and aliquots were PK-digested (115 μg/ml final concentration, 0.6% SDS, 1 h, 37 °C) prior to immunoblot analyses, as described below.

For reconstitution experiments, mb-PMCA products were generated with a 105-
diluted 127S prion seed. At the end of the mb-PMCA reaction, the products were 
mixed, eventually treated with PK (150 μg/ml final concentration, 1 h, 37 °C). PK 
activity was inactivated by the combined addition of 4 mM Pefabloc and 2x EDTA-
protease inhibitor cocktail. The products were then diluted 1:1 in either PrP0/0 
or in tg338 brain homogenate and incubated for 48 h or 7 days at 37 °C in 
quiescent conditions. The PMCA products were then fractionated by 
sedimentation velocity and analysed for PrPSc content by immunoblot.
S2, and S3) give rise to the formation of common oligomeric assemblies, termed Ai, with a narrowed size distribution during mb-PMCA reactions. This suPrPB (B1) is assisted by suPrPB, making the process autocatalytic suPrPA and suPrPB (step III). The formation of this complex is at the origin of a secondary templating pathway where the transformation of suPrPA (A1) to information41,50, represented in red).

**Fig. 8** Quaternary structural convergence and secondary autocatalytic pathway at the root of the formation of B1 assemblies. a Different prion strains (S1, S2, and S3) give rise to the formation of common oligomeric assemblies, termed Ai, with a narrowed size distribution during mb-PMCA reactions. This common quaternary structural convergence at the early stage of the replication process suggests the existence of a common conversion pathway and a common oligomerization domain that is independent of the strain structural determinant (SSD, i.e., the PrP domain(s) harboring the replicative and strain information41,50, represented in red). B Ai and B1 assemblies are in an equilibrium/detailed-balance with their respective suPrP (step I and II) as was previously showed41 and also demonstrated by the dilution experiment (see Fig. 1e). Based on the constraints imposed by the experimental observations, the best model to account for the cooperative and PrPC dependency transformation of Ai into B1 assemblies implicates the formation of complex between suPrPA and suPrPB (step III). The formation of this complex is at the origin of a secondary templating pathway where the transformation of suPrPA (A1) to suPrPB (B1) is assisted by suPrPB, making the process autocatalytic.

**Size exclusion chromatography.** SEC analysis was performed using an AKTA-100 purifier FPLC (GE Healthcare). 200 μl of the PMCA products were mixed with an equal volume of 2X-buffer to reach 25 mM HEPES pH 7.4, 150 mM NaCl, 10 mM EDTA, 5 mM n-Dodecyl β-D-Maltoside, 2 % w/w Sarkosyl and 0.5% Triton-X100 final concentration. After centrifugation at 10 000 g for 3 min (no visible pellet), the solution was loaded on Superdex 200 10/300 GL column (24 ml, GE healthcare). The chromatography running buffer was HEPES 25 mM pH 7.2, 200 mM NaCl, without detergents to avoid the formation of micellar structure. The flow rate was fixed at 0.35 ml/min. After sample injection, the flow-through of the column was fractionated every 250 μl. The PrP levels per fraction were estimated by western blotting, as for SV. For molecular weight estimation, the Superdex 200 was calibrated with blue dextran molecules with varying molecular weight.

**Analysis of PrPSc content by immunoblotting.** Aliquots of the SV-fractionated samples were treated with PK (50 μg/ml final concentration, 1 h, 37 °C) before mixing in Laemmli buffer and denaturation at 100 °C for 5 min. The samples were run on 12% Bis-Tris Criterion gels (Bio-Rad, Marne la Vallée, France) and electrottransferred onto nitrocellulose membranes. In some instances, denatured samples were spotted onto nitrocellulose membranes using a dot-blot apparatus (Schleicher & Schuell BioScience (Whatman)). Nitrocellulose membranes were probed for PrP with 0.1 μg/ml biotinylated anti-PrP monoclonal antibody Sha3159. Immunoreactivity was visualized by chemiluminescence (ECL, Pierce or Clarity, Bio-Rad). The protein levels were quantified with ImageLab software after acquisition of chemiluminescent signals with a Chemidoc digital imager (Bio-Rad, Marnes-la-Coquette, France). For all SDS-PAGE analyses, a fixed quantity of human recombinant PrP was employed for consistent calibration of the PrP signals in different gels.

**To improve the sensitivity of the western blot detection method for the samples containing low levels of PrPSc (e.g., samples at early stage of replication and SEC fractions) a double-deposit was made to electro-concentrate the sample. Typically, after a first round of sample loading in SDS-PAGE wells, a short migration time was performed to allow running within the acrylamide gel for 2 mm. Then, a second round of sample loading was done identically to the first one and the migration was continued until the front reached 3 cm within the gel. The electrotransfer and detection was then identical as above.**

**Bioassays.** The pool of fractions of interest was extemporarily diluted ten-fold in 5% glucose and immediately inoculated via the intracerebral route into reporter tg338 mice (20 μl per pool of fraction, n = 5 mice per pool). Mice showing prion-specific neurological signs were euthanized at the end stage. To confirm prion disease, brains were removed and analysed for PrPSc content using the Bio-Rad...
TsE512 detection kit12 prior to immunoblotting, as described above. The survival time was defined as the number of days from inoculation to euthanasia. To estimate what the difference in mean survival times means in terms of infectivity, strain-specific curves correlating the relative infectious dose to survival times were used, as previously described15.

Kinetic simulation. The details of the kinetic simulation are reported in the Supplementary note. Briefly, two distinct sets of assemblies were considered (A, and B). Based on experimental observations, a set of constraints was retained to build biochemical reactions describing the evolution of the quaternary structure of PrPres assemblies. The ordinary differential equations of the biochemical reactions 1–6 (in the manuscript) were established and coded in MATLAB for simulations.

Statistics and reproducibility. The SV and SEC experiments reported here result from at least three independent experiments (biological repeats). For SV fractionation of in vivo brain material, pools of three brains collected at different time points were used to minimize individual brain-to-brain variations. At least three independent fractionations were done using these pools. For fractionation of in vitro material, a new batch of mb-PMCA amplified products was prepared for each fractionation. The 127S mouse biosassy was done with n = 5 mice per group at two different dilutions to ensure statistical significance with this fast prion model, while keeping the number of mice as low as possible for ethical reason. The results are reported as mean values ± standard error of the mean (SEM).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

The SV and SEC raw data set have been deposited to the DRYAD repository https://doi.org/10.5061/dryad.f28b4m660. All other data are available from the corresponding authors on request.

Code availability

The MATLAB code associated with this study is available from authors upon request.

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Author contributions
A.I.E., F.I., Mo.M., J.B., M.D., H.R. and V.B. conceived and designed the experiments. A.I.E., F.I., Mo.M., J.B., Ma.M., T.K., L.H., F.R., C.J.D., M.D., H.R. and V.B. performed the experiments and analysed the data. A.I.E., H.R. and V.B. wrote the paper. All authors reviewed and edited the paper.

Competing interests
The authors declare no competing interests.

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