

De novo mammalian prion synthesis

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Abbreviations: PrP, prion protein; PrP^C, cellular form of prion protein; PrP^{Sc}, scrapie prion protein or prion; Tg, transgenic; Mo, mouse; Hu, human; SHa, syrian hamster; rec, recombinant; PrP^{res}, proteinase-resistant form of prion protein; PK, proteinase K; DPI, days post inoculation; ERC, endosomal recycling compartment

Prions are responsible for a heterogeneous group of fatal neurodegenerative diseases. They can be sporadic, genetic, or infectious disorders involving post-translational modifications of the cellular prion protein (PrP^C). Prions (PrP^{Sc}) are characterized by their infectious property and intrinsic ability to convert the physiological PrP^C into the pathological form, acting as a template. The “protein-only” hypothesis, postulated by Stanley B. Prusiner, implies the possibility to generate de novo prions in vivo and in vitro. Here we describe major milestones towards proving this hypothesis, taking into account physiological environment/s, biochemical properties and interactors of the PrP^C.

Prions are responsible for a heterogeneous group of fatal neurodegenerative diseases (Table 1).¹ They can be sporadic, genetic or infectious disorders involving post-translational modifications of the cellular prion protein (PrP^C).² Prions are characterized by their infectious properties and by their intrinsic ability to encipher distinct biochemical properties through their secondary, tertiary and quaternary protein structures. In particular, the transmission of the disease is due to the ability of a prion to convert the physiological PrP^C into the pathological form (PrP^{Sc}), acting as a template.³ The two isoforms of PrP appear to be different in terms of protein structures, as revealed by optical spectroscopy experiments such as Fourier-transform infrared and circular dichroism.⁴ PrP^C contains 40% α -helix and 3% β -sheet, while the pathological isoform, PrP^{Sc}, presents approximately 30% α -helix and 45% β -sheet.^{4,5} PrP^{Sc} differs from PrP^C because of its altered physical-chemical properties such as insolubility in non-denaturing detergents and proteinases resistance.^{2,6,7}

The prion conversion occurring in prion diseases seems to involve only conformational changes instead of covalent modifications. However, Mehlhorn et al. demonstrated the importance of a disulfide bond between the two cysteine residues at position

179 and 214 (human (Hu) PrP numbering) to preserve PrP into its physiological form. In the presence of reducing conditions and pH higher than 7, recombinant (rec) PrP tends to assume high β -sheet content and relatively low solubility like PrP^{Sc}.⁸

De novo Prion Generation: In vivo Systems

PrP^{Sc} propagation and de novo prions generation were extensively studied using transgenic (Tg) animals. In particular, an homologue point mutation of the Hu Gerstmann-Sträussler-Scheinker syndrome (GSS), P102L, has been used to produce Tg mice and study both genetic and infectious illness.^{9,10} Tg mice—Tg174 and Tg87—with approx. 60 copies of the transgene and overexpressing 8 folds mouse (Mo) PrP-P101L compared to wild-type (as mentioned, corresponding to P102L in HuPrP sequence) (Tg(MoPrP-P101L))—developed ataxia, lethargy, bradykinesia and rigidity at an early age (150–200 days).^{9,11} Both Tg174 and Tg87 mice showed vacuolar degeneration in the neocortex and hippocampus. Brain homogenates extracted from symptomatic Tg174 and Tg87 mice were intracerebrally inoculated into Tg196 mice. These latter mice are characterized by a low expression level (~2 folds) of the P101L mutant transgene, and apparently do not develop central nervous system (CNS) alterations spontaneously. Sixty-two out of 138 Tg196 mice developed neurological signs 226–712 days post inoculation (DPI).⁹ None of 26 Tg196 mice inoculated with brain homogenates from pre-symptomatic Tg174 and Tg87 showed pathological signs. One out of 88 Tg196 mice inoculated with CD1 and non-Tg mice brain homogenates developed neurological signs after 476 DPI. De novo infectious prions, generated in Tg(MoPrP-P101L), revealed different properties and neurological signs from RML scrapie isolates. RML-infected mice presented spongiosis in hypothalamus, basal forebrain and rostral cingulate gyrus, whilst Tg196 inoculated with Tg174 brain homogenates showed degeneration in hippocampus.

Telling and coworkers studied the effect of the genetic background, *Prnp*^{0/0} or *Prnp*^{+/-} (FVB), on the onset of the neurological disease in Tg(MoPrP-P101L). Tg(MoPrP-P101L)/*Prnp*^{0/0} spontaneously developed CNS dysfunctions earlier than that Tg(MoPrP-P101L)/FVB. Inoculated Tg196/*Prnp*^{0/0} were more

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Table 1. The prion diseases

Prion disease	Host	Mechanism
iCJD	humans	infection
vCJD	humans	infection
fCJD	humans	genetic: octarepeat insertion, D178N-I29V, V180I, T183A, T188K, T188R-I29V, E196K, E200K, V203I, R208H, V210I, E211Q, M232R
sCJD	humans	?
GSS	humans	genetic: octarepeat insertion, P102L-I29M, P105-I29M, A117V-I29V, G131V-I29M, Y145*-I29M, H197R-I29V, F198S-I29V, D202N-I29V, Q212P, Q217R-I29M, M232T
FFI	humans	genetic: D178-I29M
Kuru	fore people	infection
sFI	humans	?
Scrapie	sheep	infection
BSE	cattle	infection
TME	mink	infection
CWD	mule deer, elk	contaminated soils?
FSE	cats	infection
Exotic ungulate encephalopathy	greater kudu, nyala, oryx	infection

i, infective form; v, variant; f, familial; s, sporadic; CJD, Creutzfeldt-Jakob disease; GSS, Gerstmann-Sträussler-Sheinker disease; FFI, fatal familial insomnia; sFI, sporadic fatal insomnia; BSE, bovine spongiform encephalopathy; TME, transmissible mink encephalopathy; CWD, chronic wasting disease; FSE, feline spongiform encephalopathy.^{73,78}

susceptible to develop illness than Tg196/FVB, 200 vs. 350 DPI, revealing an inhibitory effect of endogenous MoPrP^C on prion transmission.¹⁰ PrP deposits, obtained using Tg(MoPrP-P101L) mice, were not made from proteinase-resistant PrP (PrP^{res}). In order to verify the presence of the pathological PrP isoform, Nazor et al. tested the prototypic PrP^{Sc}-specific Mab 15B3 on the brain extracts of Tg(GSS) mice.¹² They showed that only MoPrP-P101L aggregates from symptomatic Tg and RML-infected FVB mice were immunoprecipitated with 15B3,¹² while PrP^C was detected with Mab 6H4 from both symptomatic and asymptomatic Tg mice. Since Tg196 mice were afterwards found to develop CNS disorders after more than 600 days, the transmission of prions from Tg(MoPrP-P101L) to Tg196 needed to be re-interpreted as disease acceleration.¹²

The expression of HuPrP point mutations on a murine background presumes no conformational alterations of the expressed chimeric protein. Two independent studies performed by Wildegger, Collinge and coworkers demonstrated that, the substitution of the native phenylalanine 175 with a tryptophan residue for studying the folding dynamics caused no measurable effects on the stability of rec MoPrP but destabilization on HuPrP.^{13,14} To study the biological properties of occurring mutations in HuPrP without any conformational alteration, Asante et al. produced two Tg Mo lines homozygote for HuPrP(P102L, 129M) and HuPrP(E200K, 129M) on a homozygous PrP gene null background.¹⁴ These Tg mice did not spontaneously develop CNS dysfunctions but, inoculated with CJD and inherited prion disease isolates (P102L and E200K), revealed high susceptibility to the infection. Transmission of prion strains to recipients was dependent on the polymorphism 129 M/V.¹⁴ The presence of a mismatch at the position 129 reduced the transmission

efficiency and increased the incubation time. Hu *PRNP* 102L and 200K-expressing Tg mice affected the PrP^{Sc} glycoform ratio. In particular, typical PrP^{Sc} glycoform ratio of CJD was altered on passage in Tg HuPrP(P102L, 129M) and Tg HuPrP(E200K, 129M) mice suggesting that PrP point mutations influence the stoichiometry and packing order of the three PrP glycoforms.

Many experimental evidences such as the infectious N-terminally truncated HuPrP(90-231),¹⁵ the shorter version PrP106,¹⁶ and the Japanese patient carrying the mutation Y145STOP¹⁷ suggested an infective role for the fragment MoPrP(89-143). Tg196 mice, intracerebrally inoculated with the β -folded MoPrP(89-143), exhibited ataxia and other neurological signs—spongiform degeneration in the neocortex, hippocampus, caudate nucleus, cerebellar cortex and corpus callosum and loss of granule cells in the cerebellar cortex—360 $\pm$ 30 DPI.¹⁸ These neurological changes were similar to those present in GSS patients.¹⁰ Brain homogenates extracted from β -folded MoPrP(89-143)-infected Tg196 mice were serially transmitted to other Tg196 mice. All these mice developed disease after an incubation time of approximately 300 DPI suggesting that the β -folded MoPrP(89-143) caused the de novo GSS prion formation or was itself a synthetic prion.¹⁹

One of the major features that distinguish PrP^C from the pathological isoform is its resistance to proteolytic cleavage. Tg(MoPrP-P101L) mice, associate with a familial form of prion disease, do not produce detectable proteinase-resistant PrP in the brain. Tg mice expressing a nine-octapeptide insertional mutation, Tg(PG14), spontaneously produced proteinase-resistant form of PrP.²⁰ Tg(PG14) showed progressive neurological disorders characterized by ataxia, gliosis, apoptosis of cerebellar granule cells and PrP deposition in a synaptic-like pattern. These

mice resemble patients with nine- and six-octapeptide PrP insertion.^{21,22} In Tg(PG14), the time course of the pathology is profoundly affected by zygosity, as demonstrated by clinical data. Homozygous mice developed disease symptoms at 68 days of age, while hemizygous showed clinical signs only after 235 days. The duration of the illness is also shorter for homozygous than hemizygous mice, 49 and 154 days respectively. Conversely to the clinical illness appearance in Tg(PG14), the PrP^{Sc}-like molecules are formed at the first month of life. Therefore, these observations suggested that PrP^{Sc} represents a causative factor in the development of neuropathology and neurological symptoms.

More recently, Sigurdson and coworkers developed a new Tg Mo overexpressing two folds the PrP S170N and N174T variant (rigid-loop PrP, RL-PrP).²³ These point mutations are located in the loop of residue 166–175 that connects the β 2-strand with the α 2-helix. Asparagine170 and threonine174 are normally present in elk PrP primary sequence conferring to the loop 166–175 a well defined structure, as revealed by sharp resonance lines in NMR spectra.²⁴ This feature contrasts with other mammalian PrPs that are characterized by a pronounced structural disorder of this loop.^{25–28} TgRL-PrP mice developed neurological dysfunctions at 145 to 637 days of age, with a 50% of incidence by 364 days. Sick TgRL-PrP mice showed ataxia, weight loss, lethargy, kyphosis and hind limb paralysis, while their brains showed PrP deposits in the stratum lacunosum-moleculare of the hippocampus, within the corpus callosum and in the cingulum. Conversely to Tg196,¹⁰ the pathology induced by RL-PrP was not suppressed by co-expression of wild-type PrP. Tga20 mice, which overexpress wild-type *Prnp*, inoculated with brain homogenates from sick TgRL-PrP mice developed neurologic signs by 481 DPI. Serial passages into Tga20 mice brains homogenates from RL-PrP-infected Tga20 mice caused similar symptoms with a shorter lag-phase. Moreover, both the proteinase K (PK) resistance and the conformational stability increased after each passage.²³ De novo generated prions were also transmissible to wild-type mice.

De novo Prion Generation: In vitro Systems

The “protein-only” hypothesis for prion propagation implies that it should be possible to generate prions in vitro from highly purified rec PrP. Many attempts have been carried out to confirm this hypothesis, showing no correlation between infectivity and proteinase resistance.^{9,11,29–31} Kocisko et al. reported the conversion of ³⁵S-labelled rec Syrian hamster (SHa) PrP^C, purified from uninfected tissue culture cells, by PrP^{Sc} in a cell-free system.³² In this system, PrP^{Sc} concentration was 50 folds higher than that of ³⁵S-labelled rec SHaPrP^C. Therefore, the preexisting scrapie infectivity prevented the possibility to test any new scrapie infectivity associated with the conversion of rec PrP to a PK resistant form. Some years later, Hill and coworkers used the chimeric MH2M PrP³³ to study the conversion caused by SHaPrP^{Sc} extracted from animals infected with the Sc237 strain. Since the “species barrier” prevents the infection of mice with SHa prions, the MH2M PrP presented the central region of MoPrP (residues 94–188) replaced with the corresponding SHaPrP sequence. ³⁵S-MH2M PrP, purified from murine erythroleukaemia (MEL) cells, was used for the

conversion reaction in the presence of 3 M guanidine. Also in this case, the authors found a PK resistant form of MH2M PrP. Bioassay performed inoculating intracerebrally Swiss CD-1 mice with the in vitro converted products, revealed no scrapie-like disease suggesting that acquisition of proteinase resistance by PrP^C is not sufficient for the propagation of infectivity.⁶

The proteinase-resistant core of PrP^{Sc} has an apparent molecular mass of 27–30 kDa corresponding to a N-terminally truncated form. Legname, Baskakov et al. produced fibrils with truncated rec MoPrP, also designed as rec MoPrP(89–230), using both seeded and unseeded protocols.³⁴ Amyloid fibrils were prepared in the presence of the chaotropic agent urea. Seeded and unseeded amyloid fibrils were intracerebrally inoculated into Tg mice overexpressing the truncated form MoPrP(89–231). These mice, called Tg(MoPrP, Δ 23–88)9949/*Prnp*^{0/0} or Tg9949, express MoPrP(89–231) 16 times more than that of normal PrP^C expressed in wild-type SHa brain. Both preparations caused neurological defects between 380 and 660 DPI but Tg9949 mice inoculated with seeded preparation exhibited extensive vacuolation associated with gliosis in cerebellum, hippocampus, brainstem and white matter. The different incubation time and the different distribution, density and morphology of vacuoles, suggested that seeded (MoSP1) and unseeded (MoSP2) fibrils belonged to different prion strains.³⁵ Brain homogenates of inoculated mice with both preparations, showed the presence of a PK resistant form. Serial transmissions of the seeded amyloid fibrils (MoSP1) to Tg9949/FVB and Tg(MoPrP-A)4053 mice gave a mean incubation time of 154 and 90 days, respectively. Synthetic prions, generated using truncated rec MoPrP, demonstrated that PrP is necessary and sufficient for infectivity.³⁴

Recently, Prusiner and colleagues have tried to elucidate the molecular mechanisms underlying the formation of different prion strains. They produced an array of rec PrP amyloids with varying conformational stability. Tg4053 mice inoculated with these amyloids revealed an inverse relationship between conformational stability and incubation time. More stable structures exhibited longer incubation times, while labile prions accumulated more rapidly and killed the host faster.^{36,37}

Castilla and coworkers generated infectious scrapie prions using the so-called Protein Misfolding Cyclic Amplification (PMCA) in vitro system.^{38–40} 263K-infected SHa brain homogenates were subjected to several PMCA cycles in the presence of PrP^C. Although after 20 PMCA cycles no molecules of the original scrapie brain homogenate were present in samples, biochemical and structural properties of the in vitro-amplified samples still revealed the same features of the 263K scrapie. In particular, the amplified sample was PK resistant (PrP^{res}), highly insoluble in non-denaturant detergent such as 10% Sarkosyl and enriched in β -sheet content. The newly PrP^{res} was inoculated into SHa animals at 10⁻¹⁰ and 10⁻²⁰ dilutions corresponding to 0.000001% and 0% of the brain-derived PrP^{res}. PrP^{res}-inoculated SHa died at around 170 DPI showing the typical signs of scrapie: hyperactivity, motor impairment, head wobbling, muscle weakness and weight loss. To verify the PrP^{res} in vitro-generated stability over time, serial transmission were performed. All animals inoculated

with this material died after 136.5 DPI exhibiting neurological dysfunctions.

To investigate the mechanism of prion formation, Deleault et al. conducted a series of experiments using the PMCA in vitro system with a preparation containing purified PrP^C, copurified lipids and polyanions.^{41,42} Substrate mixtures containing PrP^C and poly(A)RNA propagated PrP^{res} molecules in the presence of Sc237 and 139H PrP27-30 as seeds. PMCA performed using various polyanions such as Mo brain RNA, poly(dT)ssDNA, poly(dA)·poly(dT)dsDNA and the polycation poly-L-lysine highlighted that only single-stranded polyanions sustained this process. During unseeded PMCA-propagation experiments using PrP^C and poly(A)RNA only, PrP^{res} molecules appeared. De novo generated-PrP^{res} molecules presented the same electrophoretic mobility than the brain-derived Sc237 PrP27-30 and were able to propagate them during the cyclic amplification. Wild-type SHa, intracerebrally inoculated with the de novo generated PrP^{res}, showed spongiform degeneration, astrogliosis and PrP^{Sc} deposition similar to that produced by Sc237 or 139H prions. De novo-generated PrP^{Sc} was efficiently transmitted to normal SHa upon serial passages.⁴²

PMCA in vitro system was also used to propagate prion strains.⁴³⁻⁴⁵ Many PMCA amplifications were carried out using different Mo-adapted as a seed (RML, ME7, 139A and 79A), Hu (vCJD), bovine (301C), SHa (263K) and cervid (CWD) prion strains. Biochemical analyses—PK digestion, glycosylation ratio and electrophoresis mobility—of end products revealed the same features of the seeds.⁴³⁻⁴⁵ This technique has been also employed to study the prion species barrier as in the case of cervid/RML prion.⁴⁴

The most common form of Hu prion disease is sporadic and affects one new case per million people each year.^{46,47} De novo in vitro-generation of PrP^{Sc} from PrP^C and poly(A)RNA, using PMCA system, opened new ways to investigate the molecular mechanisms underlying the spontaneous misfolding of the PrP and then its propagation. Barria and colleagues modified the PMCA conditions to study the de novo conversion of PrP^C into its infectious form.⁴⁸ After 240 PMCA cycles, corresponding to 5 days of sonications every 30 minutes each, a PrP^{res} was obtained from healthy SHa and Mo brain homogenates. The infectivity of the new PrP^{res} was assessed inoculating them into wild-type SHa. All animals inoculated with de novo generated PrP^{res} showed behavioral abnormalities after an incubation period of 112.6 days. SHa animals inoculated with 263K, Hyper (HY) and Drowsy (DY) showed neurological signs after 89.8, 86.5 and 218.8 days respectively.⁴⁸ A detailed study of the brains revealed spongiform degeneration, astroglyosis and PrP accumulation.

Transition metals—copper, zinc, iron and manganese—are involved in many physiological processes such as energy production, redox reactions, synapses regulation etc., but also in pathological events as in the case of neurodegenerative disorders.⁴⁹⁻⁵⁴ PrP^C seems to be involved in transition metals homeostasis and its physiological function/s could be affected by the presence of these elements.

Therefore, some authors have studied the PrP^C conversion into its pathological form in the presence of metals.^{55,56} Independently,

Basu and Treiber reported the formation of a proteinase-resistant PrP form in the presence of ferrous or cupric and manganous ions respectively.^{55,56} In the presence of ferrous ions (0.1–0.3 mM), there was the formation of a *PrP^{Sc} PK-resistant form able to propagate itself in Hu neuroblastoma (M17) in vitro system.⁵⁶ Similar effects were produced by the addition of copper (II) (1–5 mM) and manganese(II) (1–5 mM) in *P. pastoris* yeast cells.⁵⁵

Conclusions and Future Perspective

Prions are pathological agents causing sporadic, genetic and infectious neurodegenerative disorders.^{1,2} Although the molecular mechanisms of these diseases are not well understood, the “protein-only” hypothesis suggests a misfolded PrP (PrP^{Sc}) as the etiologic agent. Many attempts have been done to de novo generate prions in the in vivo and in vitro systems. Tg mice over-expressing PrP mutants⁹⁻²⁷ spontaneously developed neurological symptoms similar to that of prion disorders, and their brain homogenates resulted to be infectious. In vitro systems revealed the possibility to generate infectious prions starting from rec MoPrP(89-230) only or from healthy brain homogenates with and without seeds.^{29-35,38-49} Basu and Treiber have also demonstrated the effects of transition metals, such as iron and copper, on the conversion of PrP^C into its pathological form.

To understand the molecular mechanisms of prion diseases, in light of the “protein-only” hypothesis, one should also comprehend the action, mechanism/s, physiological environment/s and function/s of PrP^C. Many studies have been performed to discover the functional domains of PrP^C involved in protein-protein interactions. The lack of these domains, and in particular of the central domain that comprises a charge cluster (CC, amino acid residues 95–110) and a hydrophobic core (HC, amino acid residues 112–134), causes neurotoxic effects.⁵⁷⁻⁶³ A PrP variant lacking 40 central residues (amino acids residues 94–134) induces a rapid and progressive lethal phenotype with extensive central and peripheral myelin degeneration.⁵⁸ The expression of a PrP variant lacking of the region comprised by amino acids 105–125 causes a severe neurodegenerative illness that is lethal within one week of birth. All these phenotypes were rescued by the presence of endogenous PrP^C, in a dose-dependent manner. The central domain contains two copper (II) binding sites at histidines 96 and 111 or a high affinity, K_d around 10⁻¹⁵–10⁻¹² M, copper (I) site comprising histidine 96 and 111, methionine 109 and 112.⁶⁴⁻⁶⁷ This unstructured region (particularly amino acid residues 106–126) seems to be involved in the PrP^C misfolding and PK-resistance. The copper binding sites could affect the structural and functional properties of this area.^{68,69}

PrP^C is also able to chelate other transition metals such as zinc, manganese, nickel and cobalt. Iron, zinc and copper were found in the gray matter in the same order of magnitude as magnesium (0.1–0.5 mM),⁴⁹ ten times less abundant than that used by Basu and Treiber in their experiments.^{55,56} In particular, zinc is released together with glutamate vesicles from pre-synaptic compartment, while copper during the synaptic transmission from post-synaptic

area.^{54,70-72} Recently, it has been shown that the endosomal recycling compartment (ERC) is the likely site for prion conversion.⁷³ Since copper stimulates PrP^C endocytosis from cell surface,⁷⁴ its dysmetabolism could affect both the synapse and the conversion in ERC thus contributing to the sporadic prion diseases formation. The disulfide bond seems to have a crucial role for stabilizing PrP^C.⁸ Altered redox phenomena can reduce it and favor the conformational transition into the pathological isoform.

Moreover, PrP^C interacts with many other proteins or macromolecules involved in signal transduction such as Neuronal-Cell Adhesion Molecule (N-CAM) (through amino acid residues 141–176) and laminin,⁷⁵⁻⁷⁷ in cell adhesion such as vitronectin (amino acid residues 105–119), laminin receptor precursor (amino acid residues 144–179)⁷⁸⁻⁸⁵ and glycosaminoglycans (amino acid residues 23–35; 23–52; 53–93; 110–128),⁸⁶⁻⁸⁸ and in synaptic vesicle formation and modulation of neurotransmitter release such as synapsin Ib (amino acid residues 23–100; 90–231).^{89,90} Interactions of PrP^C with laminin and N-CAM play a role on neuritogenesis and neurite outgrowth through the activation of p59Fyn kinase and mitogen-activated protein kinases (MAPK) signaling.^{76,77,91,92} PrP^C interacts with the stress-inducible protein 1 enhancing both the short-term memory formation and long-term memory consolidation.⁹³ This interaction induces neuroprotective signals against apoptosis.⁹⁴ The pathological conversion of PrP^C into PrP^{Sc} may cause alterations in signal transduction, cell adhesion and synapses enhancing the severity of illness. MAPK pathways are dysregulated in prion disease, indicating an active role for this pathway in the pathogenesis. SHa brains infected with 263K scrapie isolates, revealed an increase of activation of c-Jun N-terminal kinases, p38 MAPK and extracellular signal-regulated kinases.⁹⁵

The coexisting PrP isoforms challenge the Anfinsen's principle. This principle foresees that a protein, with a well-established primary sequence, can adopt only one native conformation.⁹⁶ To date, many other proteins have been found to adopt two or more conformations.⁹⁷ Every conformation is characterized by a specific free energy and it is separated from each other by energetic barriers. In vitro-generated synthetic prions were performed in the presence of guanidine or urea, two chaotropic agents that destabilize intra-molecular interactions mediated by non-covalent forces.⁹⁸ Chaotropic agents allow proteins to better cross the energetic barriers, explore the conformational space and reach

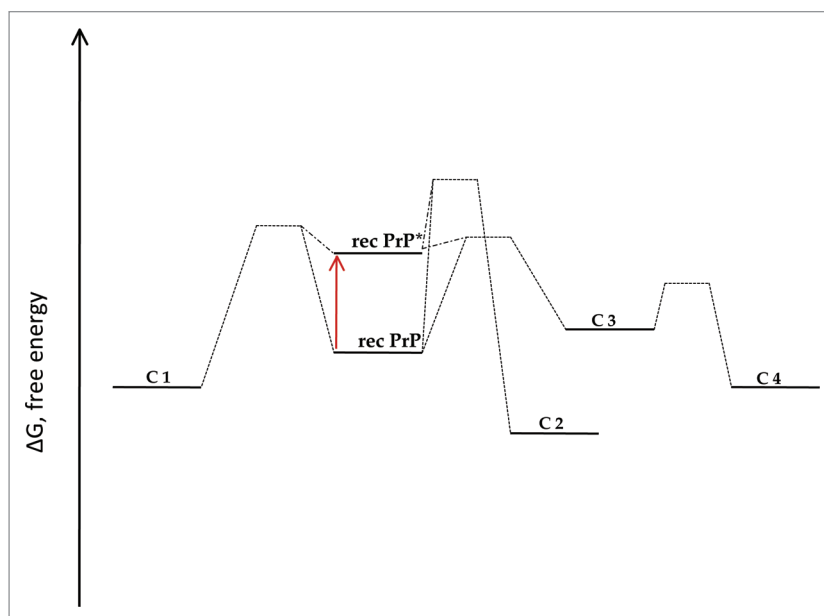


Figure 1. Qualitative representation of the thermodynamics between plausible PrP conformers (C1 to C4) in the presence and absence of chaotropic agents. Rec PrP, α -folded protein; rec PrP*, more unfolded protein due to chaotropic agents or other triggers (e.g., point mutations or seeds); red arrow, energy barrier decrease.

the more stable structure/s.⁹⁹ In particular, these agents loosen the intra-molecular forces of the α -folded rec PrP causing a more unfolded and unstable protein, rec PrP*. The lower energy barriers allow to rec PrP* to reach different local minima, each of them representing a conformer (C) (Fig. 1).

While point mutations and seeds represent a trigger to cross the energetic barriers for the genetic and infectious forms, what happens in the case of sporadic forms? Which are the environmental changes responsible for the transition to PrP^{Sc}? Could they be due to altered redox reactions, pH and/or ionic strength values or protein-protein interactions?

Taking into account the variability of the cellular environment, in vitro prion conversion may become the most powerful tool towards deciphering the physiological role of PrP^C and the pathological molecular mechanisms underlying its conformational changes leading to the formation of various prion strains.

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References

1. Prusiner SB. Novel proteinaceous infectious particles cause scrapie. *Science* 1982; 216:136-44.
2. Prusiner SB. Prions. *Proc Natl Acad Sci USA* 1998; 95:13363-83.
3. Supattapone S. Prion protein conversion in vitro. *J Mol Med* 2004; 82:348-56.
4. Pan KM, Baldwin M, Nguyen J, Gasset M, Serban A, Groth D, et al. Conversion of alpha-helices into beta-sheets features in the formation of the scrapie prion proteins. *Proc Natl Acad Sci USA* 1993; 90:10962-6.
5. Pergami P, Jaffe H, Safar J. Semipreparative chromatographic method to purify the normal cellular isoform of the prion protein in nondenatured form. *Anal Biochem* 1996; 236:63-73.
6. Hill AF, Antoniou M, Collinge J. Protease-resistant prion protein produced in vitro lacks detectable infectivity. *J Gen Virol* 1999; 80:11-4.
7. Cohen FE, Prusiner SB. Pathologic conformations of prion proteins. *Annu Rev Biochem* 1998; 67:793-819.
8. Mehlhorn I, Groth D, Stockel J, Moffat B, Reilly D, Yansura D, et al. High-level expression and characterization of a purified 142-residue polypeptide of the prion protein. *Biochemistry* 1996; 35:5528-37.

9. Hsiao KK, Groth D, Scott M, Yang SL, Serban H, Rapp D, et al. Serial transmission in rodents of neurodegeneration from transgenic mice expressing mutant prion protein. *Proc Natl Acad Sci USA* 1994; 91:9126-30.
10. Telling GC, Haga T, Torchia M, Tremblay P, DeArmond SJ, Prusiner SB. Interactions between wild-type and mutant prion proteins modulate neurodegeneration in transgenic mice. *Genes Dev* 1996; 10:1736-50.
11. Hsiao KK, Scott M, Foster D, Groth DF, DeArmond SJ, Prusiner SB. Spontaneous neurodegeneration in transgenic mice with mutant prion protein. *Science* 1990; 250:1587-90.
12. Nator KE, Kuhn F, Seward T, Green M, Zwald D, Purro M, et al. Immunodetection of disease-associated mutant PrP, which accelerates disease in GSS transgenic mice. *EMBO J* 2005; 24:2472-80.
13. Willedeger G, Liemann S, Glockshuber R. Extremely rapid folding of the C-terminal domain of the prion protein without kinetic intermediates. *Nat Struct Biol* 1999; 6:550-3.
14. Asante EA, Gowland I, Grimshaw A, Linehan JM, Smidak M, Houghton R, et al. Absence of spontaneous disease and comparative prion susceptibility of transgenic mice expressing mutant human prion proteins. *J Gen Virol* 2009; 90:546-58.
15. Prusiner SB, Groth DF, Bolton DC, Kent SB, Hood LE. Purification and structural studies of a major scrapie prion protein. *Cell* 1984; 38:127-34.
16. Supattapone S, Bosque P, Muramoto T, Wille H, Aagaard C, Peretz D, et al. Prion protein of 106 residues creates an artificial transmission barrier for prion replication in transgenic mice. *Cell* 1999; 96:869-78.
17. Kitamoto T, Iizuka R, Tateishi J. An amber mutation of prion protein in Gerstmann-Straussler syndrome with mutant PrP plaques. *Biochem Biophys Res Commun* 1993; 192:525-31.
18. Kaneko K, Ball HL, Wille H, Zhang H, Groth D, Torchia M, et al. A synthetic peptide initiates Gerstmann-Straussler-Scheinker (GSS) disease in transgenic mice. *J Mol Biol* 2000; 295:997-1007.
19. Tremblay P, Ball HL, Kaneko K, Groth D, Hegde RS, Cohen FE, et al. Mutant PrP^{Sc} conformers induced by a synthetic peptide and several prion strains. *J Virol* 2004; 78:2088-99.
20. Chiesa R, Drisaldi B, Quaglio E, Migheli A, Piccardo P, Ghetti B, Harris DA. Accumulation of protease-resistant prion protein (PrP) and apoptosis of cerebellar granule cells in transgenic mice expressing a PrP insertional mutation. *Proc Natl Acad Sci USA* 2000; 97:5574-9.
21. Chiesa R, Piccardo P, Ghetti B, Harris DA. Neurological illness in transgenic mice expressing a prion protein with an insertional mutation. *Neuron* 1998; 21:1339-51.
22. Collinge J, Brown J, Hardy J, Mullan M, Rossor MN, Baker H, et al. Inherited prion disease with 144 base pair gene insertion 2. Clinical and pathological features. *Brain* 1992; 115:687-710.
23. Sigurdson CJ, Nilsson KP, Hornemann S, Heikenwalder M, Manco G, Schwarz P, et al. De novo generation of a transmissible spongiform encephalopathy by mouse transgenesis. *Proc Natl Acad Sci USA* 2009; 106:304-9.
24. Gossert AD, Bonjour S, Lysek DA, Fiorito F, Wuthrich K. Prion protein NMR structures of elk and of mouse/elk hybrids. *Proc Natl Acad Sci USA* 2005; 102:646-50.
25. Riek R, Hornemann S, Wider G, Billeter M, Glockshuber R, Wuthrich K. NMR structure of the mouse prion protein domain PrP(121-321). *Nature* 1996; 382:180-2.
26. Zahn R, Liu A, Luhrs T, Riek R, von Schroetter C, Lopez Garcia F, et al. NMR solution structure of the human prion protein. *Proc Natl Acad Sci USA* 2000; 97:145-50.
27. Lopez Garcia F, Zahn R, Riek R, Wuthrich K. NMR structure of the bovine prion protein. *Proc Natl Acad Sci USA* 2000; 97:8334-9.
28. Lysek DA, Schorn C, Nivon LG, Esteve-Moya V, Christen B, Calzolari L, et al. Prion protein NMR structures of cats, dogs, pigs and sheep. *Proc Natl Acad Sci USA* 2005; 102:640-5.
29. Xi YG, Ingrosso L, Ladogana A, Masullo C, Pocchiari M. Amphotericin B treatment dissociates in vivo replication of the scrapie agent from PrP accumulation. *Nature* 1992; 356:598-601.
30. Collinge J, Palmer MS, Sidle KC, Gowland I, Medori R, Ironside J, Lantos P. Transmission of fatal familial insomnia to laboratory animals. *Lancet* 1995; 346:569-70.
31. Lasmezas CI, Deslys JP, Robain O, Jaegly A, Beringue V, Peyrin JM, et al. Transmission of the BSE agent to mice in the absence of detectable abnormal prion protein. *Science* 1997; 275:402-5.
32. Kocisko DA, Come JH, Priola SA, Chesebro B, Raymond GJ, Lansbury PT, Caughey B. Cell-free formation of protease-resistant prion protein. *Nature* 1994; 370:471-4.
33. Scott M, Groth D, Foster D, Torchia M, Yang SL, DeArmond SJ, Prusiner SB. Propagation of prions with artificial properties in transgenic mice expressing chimeric PrP genes. *Cell* 1993; 73:979-88.
34. Legname G, Baskakov IV, Nguyen HO, Riesner D, Cohen FE, DeArmond SJ, Prusiner SB. Synthetic mammalian prions. *Science* 2004; 305:673-6.
35. Legname G, Nguyen HO, Baskakov IV, Cohen FE, Dearmond SJ, Prusiner SB. Strain-specified characteristics of mouse synthetic prions. *Proc Natl Acad Sci USA* 2005; 102:2168-73.
36. Legname G, Nguyen HO, Peretz D, Cohen FE, DeArmond SJ, Prusiner SB. Continuum of prion protein structures enciphers a multitude of prion isolate-specified phenotypes. *Proc Natl Acad Sci USA* 2006; 103:19105-10.
37. Colby DW, Giles K, Legname G, Wille H, Baskakov IV, deArmond SJ, Prusiner SB. Design and construction of diverse mammalian prion strains. *Proc Natl Acad Sci USA* 2009; 106:20417-22.
38. Castilla J, Saa P, Hetz C, Soto C. In vitro generation of infectious scrapie prions. *Cell* 2005; 121:195-206.
39. Saborio GP, Permann B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature* 2001; 411:810-3.
40. Soto C, Saborio GP, Anderes L. Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. *Trends Neurosci* 2002; 25:390-4.
41. Deleault NR, Geoghegan JC, Nishina K, Kascsak R, Williamson RA, Supattapone S. Protease-resistant prion protein amplification reconstituted with partially purified substrates and synthetic polyanions. *J Biol Chem* 2005; 280:26873-9.
42. Deleault NR, Harris BT, Rees JR, Supattapone S. Formation of native prions from minimal components in vitro. *Proc Natl Acad Sci USA* 2007; 104:9741-6.
43. Castilla J, Morales R, Saa P, Barria M, Gambetti P, Soto C. Cell-free propagation of prion strains. *EMBO J* 2008; 27:2557-66.
44. Green KM, Castilla J, Seward TS, Napier DL, Jewell JE, Soto C, Telling GC. Accelerated high fidelity prion amplification within and across prion species barriers. *PLoS Pathog* 2008; 4:1000139.
45. Castilla J, Gonzalez-Romero D, Saa P, Morales R, De Castro J, Soto C. Crossing the species barrier by PrP(Sc) replication in vitro generates unique infectious prions. *Cell* 2008; 134:757-68.
46. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci* 2001; 24:519-50.
47. Parchi P, Giese A, Capellari S, Brown P, Schulz-Schaeffer W, Windl O, et al. Classification of sporadic Creutzfeldt-Jakob disease based on molecular and phenotypic analysis of 300 subjects. *Ann Neurol* 1999; 46:224-33.
48. Barria MA, Mukherjee A, Gonzalez-Romero D, Morales R, Soto C. De novo generation of infectious prions in vitro produces a new disease phenotype. *PLoS Pathog* 2009; 5:1000421.
49. Bush AI. The metallobiology of Alzheimer's disease. *Trends Neurosci* 2003; 26:207-14.
50. Andreoletti O, Levasseur E, Uro-Coste E, Tabouret G, Sarradin P, Delisle MB, et al. Astrocytes accumulate 4-hydroxynonenal adducts in murine scrapie and human Creutzfeldt-Jakob disease. *Neurobiol Dis* 2002; 11:386-93.
51. Kim YK, Lim HH, Song YK, Lee HH, Lim S, Han SM, Kim CJ. Effect of acupuncture on 6-hydroxydopamine-induced nigrostriatal dopaminergic neuronal cell death in rats. *Neurosci Lett* 2005; 384:133-8.
52. Hur K, Kim JI, Choi SI, Choi EK, Carp RI, Kim YS. The pathogenic mechanisms of prion diseases. *Mech Ageing Dev* 2002; 123:1637-47.
53. Kim NH, Park SJ, Jin JK, Kwon MS, Choi EK, Carp RI, Kim YS. Increased ferric iron content and iron-induced oxidative stress in the brains of scrapie-infected mice. *Brain Res* 2000; 884:98-103.
54. Barnham KJ, Bush AI. Metals in Alzheimer's and Parkinson's diseases. *Curr Opin Chem Biol* 2008; 12:222-8.
55. Treiber C, Simons A, Multhaup G. Effect of copper and manganese on the de novo generation of protease-resistant prion protein in yeast cells. *Biochemistry* 2006; 45:6674-80.
56. Basu S, Mohan ML, Luo X, Kundu B, Kong Q, Singh N. Modulation of proteinase K-resistant prion protein in cells and infectious brain homogenate by redox iron: implications for prion replication and disease pathogenesis. *Mol Biol Cell* 2007; 18:3302-12.
57. Li A, Christensen HM, Stewart LR, Roth KA, Chiesa R, Harris DA. Neonatal lethality in transgenic mice expressing prion protein with a deletion of residues 105-125. *EMBO J* 2007; 26:548-58.
58. Baumann F, Tolnay M, Brabeck C, Pahnke J, Klotz U, Niemann HH, et al. Lethal recessive myelin toxicity of prion protein lacking its central domain. *EMBO J* 2007; 26:538-47.
59. Fischer M, Rulicke T, Raeber A, Sailer A, Moser M, Oesch B, et al. Prion protein (PrP) with amino-proximal deletions restoring susceptibility of PrP knockout mice to scrapie. *EMBO J* 1996; 15:1255-64.
60. Flechsig E, Shmerling D, Hegyi I, Raeber AJ, Fischer M, Cozzio A, et al. Prion protein devoid of the octapeptide repeat region restores susceptibility to scrapie in PrP knockout mice. *Neuron* 2000; 27:399-408.
61. Hegde RS, Mastrianni JA, Scott MR, DeFea KA, Tremblay P, Torchia M, et al. A transmembrane form of the prion protein in neurodegenerative disease. *Science* 1998; 279:827-34.
62. Ma J, Wollmann R, Lindquist S. Neurotoxicity and neurodegeneration when PrP accumulates in the cytosol. *Science* 2002; 298:1781-5.
63. Chesebro B, Trifilo M, Race R, Meade-White K, Teng C, LaCasse R, et al. Anchorless prion protein results in infectious amyloid disease without clinical scrapie. *Science* 2005; 308:1435-9.
64. Millhauser GL. Copper and the prion protein: methods, structures, function, and disease. *Annu Rev Phys Chem* 2007; 58:299-320.
65. Jackson GS, Murray I, Hosszu LL, Gibbs N, Waltho JP, Clarke AR, Collinge J. Location and properties of metal-binding sites on the human prion protein. *Proc Natl Acad Sci USA* 2001; 98:8531-5.
66. Jones CE, Abdelrahman SR, Brown DR, Viles JH. Preferential Cu²⁺ coordination by His96 and His111 induces beta-sheet formation in the unstructured amyloidogenic region of the prion protein. *J Biol Chem* 2004; 279:32018-27.

67. Badrick AC, Jones CE. The amyloidogenic region of the human prion protein contains a high affinity (Met)(2)(His)(2) Cu(I) binding site. *J Inorg Biochem* 2009; 103:1169-75.
68. Jobling MF, Huang X, Stewart LR, Barnham KJ, Curtain C, Volitakis I, et al. Copper and zinc binding modulates the aggregation and neurotoxic properties of the prion peptide PrP106-126. *Biochemistry* 2001; 40:8073-84.
69. Belosi B, Gaggelli E, Guerrini R, Kozlowski H, Luczkowski M, Mancini FM, et al. Copper binding to the neurotoxic peptide PrP106-126: thermodynamic and structural studies. *ChemBiochem* 2004; 5:349-59.
70. Crouch PJ, White AR, Bush AI. The modulation of metal bio-availability as a therapeutic strategy for the treatment of Alzheimer's disease. *FEBS J* 2007; 274:3775-83.
71. Bush AI. Metals and neuroscience. *Curr Opin Chem Biol* 2000; 4:184-91.
72. Schlieff ML, Gitlin JD. Copper homeostasis in the CNS: a novel link between the NMDA receptor and copper homeostasis in the hippocampus. *Mol Neurobiol* 2006; 33:81-90.
73. Marijanovic Z, Caputo A, Campana V, Zurzolo C. Identification of an intracellular site of prion conversion. *PLoS Pathog* 2009; 5:1000426.
74. Pauly PC, Harris DA. Copper stimulates endocytosis of the prion protein. *J Biol Chem* 1998; 273:33107-10.
75. Schmitt-Ulms G, Legname G, Baldwin MA, Ball HL, Bradon N, Bosque PJ, et al. Binding of neural cell adhesion molecules (N-CAMs) to the cellular prion protein. *J Mol Biol* 2001; 314:1209-25.
76. Graner E, Mercadante AF, Zanata SM, Forlenza OV, Cabral AL, Veiga SS, et al. Cellular prion protein binds laminin and mediates neuriteogenesis. *Brain Res Mol Brain Res* 2000; 76:85-92.
77. Graner E, Mercadante AF, Zanata SM, Martins VR, Jay DG, Brentani RR. Laminin-induced PC-12 cell differentiation is inhibited following laser inactivation of cellular prion protein. *FEBS Lett* 2000; 482:257-60.
78. Hajj GN, Lopes MH, Mercadante AF, Veiga SS, da Silveira RB, Santos TG, et al. Cellular prion protein interaction with vitronectin supports axonal growth and is compensated by integrins. *J Cell Sci* 2007; 120:1915-26.
79. Hundt C, Peyrin JM, Haik S, Gauczynski S, Leucht C, Rieger R, et al. Identification of interaction domains of the prion protein with its 37-kDa/67-kDa laminin receptor. *EMBO J* 2001; 20:5876-86.
80. Rieger R, Edenhofer F, Lasmez CI, Weiss S. The human 37-kDa laminin receptor precursor interacts with the prion protein in eukaryotic cells. *Nat Med* 1997; 3:1383-8.
81. Vana K, Zuber C, Pflanz H, Kolodziejczak D, Zemora G, Bergmann AK, Weiss S. LRP/LR as an alternative promising target in therapy of prion diseases, Alzheimer's disease and cancer. *Infect Disord Drug Targets* 2009; 9:69-80.
82. Ludewigs H, Zuber C, Vana K, Nikles D, Zerr I, Weiss S. Therapeutic approaches for prion disorders. *Expert Rev Anti Infect Ther* 2007; 5:613-30.
83. Zuber C, Ludewigs H, Weiss S. Therapeutic approaches targeting the prion receptor LRP/LR. *Vet Microbiol* 2007; 123:387-93.
84. Gauczynski S, Peyrin JM, Haik S, Leucht C, Hundt C, Rieger R, et al. The 37-kDa/67-kDa laminin receptor acts as the cell-surface receptor for the cellular prion protein. *EMBO J* 2001; 20:5863-75.
85. Gauczynski S, Nikles D, El-Gogo S, Papy-Garcia D, Rey C, Alban S, et al. The 37-kDa/67-kDa laminin receptor acts as a receptor for infectious prions and is inhibited by polysulfated glycanes. *J Infect Dis* 2006; 194:702-9.
86. Horonchik L, Tzaban S, Ben-Zaken O, Yedidia Y, Rouvinski A, Papy-Garcia D, et al. Heparan sulfate is a cellular receptor for purified infectious prions. *J Biol Chem* 2005; 280:17062-7.
87. Warner RG, Hundt C, Weiss S, Turnbull JE. Identification of the heparan sulfate binding sites in the cellular prion protein. *J Biol Chem* 2002; 277:18421-30.
88. Hijazi N, Kariv-Inbal Z, Gasser M, Gabizon R. PrP^{Sc} incorporation to cells requires endogenous glycosaminoglycan expression. *J Biol Chem* 2005; 280:17057-61.
89. Aguzzi A, Baumann F, Bremer J. The prion's elusive reason for being. *Annu Rev Neurosci* 2008; 31:439-77.
90. Spielhauer C, Schatzl HM. PrP^C directly interacts with proteins involved in signaling pathways. *J Biol Chem* 2001; 276:44604-12.
91. Santuccione A, Sytnyk V, Leshchynska I, Schachner M. Prion protein recruits its neuronal receptor NCAM to lipid rafts to activate p59fyn and to enhance neurite outgrowth. *J Cell Biol* 2005; 169:341-54.
92. Mouillet-Richard S, Ermonval M, Chebassier C, Laplanche JL, Lehmann S, Launay JM, Kellermann O. Signal transduction through prion protein. *Science* 2000; 289:1925-8.
93. Coitinho AS, Lopes MH, Hajj GN, Rossato JI, Freitas AR, Castro CC, et al. Short-term memory formation and long-term memory consolidation are enhanced by cellular prion association to stress-inducible protein 1. *Neurobiol Dis* 2007; 26:282-90.
94. Zanata SM, Lopes MH, Mercadante AF, Hajj GN, Chiarini LB, Nomizo R, et al. Stress-inducible protein 1 is a cell surface ligand for cellular prion that triggers neuroprotection. *EMBO J* 2002; 21:3307-16.
95. Lee HP, Jun YC, Choi JK, Kim JI, Carp RI, Kim YS. Activation of mitogen-activated protein kinases in hamster brains infected with 263K scrapie agent. *J Neurochem* 2005; 95:584-93.
96. Anfinsen CB. Principles that govern the folding of protein chains. *Science* 1973; 181:223-30.
97. Sandal M, Valle F, Tessari I, Mammi S, Bergantino E, Musiani F, et al. Conformational equilibria in monomeric alpha-synuclein at the single-molecule level. *PLoS Biol* 2008; 6:6.
98. Cacace MG, Landau EM, Ramsden JJ. The Hofmeister series: salt and solvent effects on interfacial phenomena. *Q Rev Biophys* 1997; 30:241-77.
99. Polano M, Bek A, Benetti F, Lazzarino M, Legname G. Structural Insights into Alternate Aggregated Prion Protein Forms. *J Mol Biol* 2009; 393:1033-42.