

New insights into structural determinants of prion protein folding and stability

Federico Benetti^{1,2} and Giuseppe Legname^{1,3,*}

¹Laboratory of Prion Biology; Department of Neuroscience; Scuola Internazionale Superiore di Studi Avanzati (SISSA); Trieste, Italy;

²Current affiliation: ECSIN-European Center for the Sustainable Impact of Nanotechnology; Veneto Nanotech S.C.p.A.; Rovigo, Italy;

³ELETTRA - Sincrotrone Trieste S.C.p.A; AREA Science Park; Basovizza, Trieste, Italy

ABSTRACT. Prions are the etiological agent of fatal neurodegenerative diseases called prion diseases or transmissible spongiform encephalopathies. These maladies can be sporadic, genetic or infectious disorders. Prions are due to post-translational modifications of the cellular prion protein leading to the formation of a β -sheet enriched conformer with altered biochemical properties. The molecular events causing prion formation in sporadic prion diseases are still elusive. Recently, we published a research elucidating the contribution of major structural determinants and environmental factors in prion protein folding and stability. Our study highlighted the crucial role of octarepeats in stabilizing prion protein; the presence of a highly enthalpically stable intermediate state in prion-susceptible species; and the role of disulfide bridge in preserving native fold thus avoiding the misfolding to a β -sheet enriched isoform. Taking advantage from these findings, in this work we present new insights into structural determinants of prion protein folding and stability.

KEYWORDS. prion protein, folding, stability, N-terminal domain, octarepeat, globular domain, intermediate state, disulfide bridge

ABBREVIATIONS. TSE, transmissible spongiform encephalopathies; CJD, Creutzfeldt-Jakob disease; GSS, Gerstmann-Sträussler-Scheinker syndrome; FFI, fatal familial insomnia; PrP^C, cellular prion protein; PrP^{Sc}, prion; GPI, glycosylphosphatidylinositol; OR, octarepeats; NMDA receptor, N-methyl-D-aspartate receptor; ADAM family, A Disintegrin And Metalloproteinase family

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*Correspondence to: Giuseppe Legname; Email: legname@sissa.it

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INTRODUCTION

Prion diseases or transmissible spongiform encephalopathies (TSE) are a group of fatal neurodegenerative diseases such as Creutzfeldt–Jakob disease (CJD), Gerstmann–Sträussler–Scheinker (GSS) syndrome, fatal familial insomnia (FFI), and kuru in humans, bovine spongiform encephalopathy in cattle, scrapie in sheep and goats, and chronic wasting disease in elk, deer, and moose.¹ These maladies can be sporadic, genetic or infectious disorders.² Prions, the etiological agents of these disorders, are composed of a conformational isoform of cellular prion protein (PrP^C) known as PrP^{Sc}.³ Compared to PrP^C, prions present altered biochemical properties, such as resistance to limited proteolysis and insolubility in non-denaturant detergents, and secondary and tertiary structures. While PrP^C contains 40% α -helix and 3% β -sheet, PrP^{Sc} is highly enriched in β -sheets. Fourier-transform infrared spectroscopy analysis showed higher β -sheet (43%) and lower α -helix (30%) content in PrP^{Sc} than PrP^C.⁴ The reduction of α -helical content in PrP^{Sc} has been also confirmed by hydrogen-deuterium exchange experiments showing no native α -helices in prions.⁵ Although the insoluble nature of prions hampers the use of high-resolution analytical techniques such as nuclear magnetic resonance or X-ray crystallography, a number of studies using low resolution approaches such as limited proteolysis, Fourier transform infrared spectroscopy, antibody-labeling, electron microscopy, fiber X-ray diffraction and small angle X-ray scattering provided important information on both secondary and tertiary PrP^{Sc} structure.^{6,7}

According to the ‘protein-only hypothesis’, the transmission of the disease is due to the ability of a prion to convert PrP^C into the pathological form, acting as a template.⁸ During the course of prion diseases, PrP^C is converted into the abnormal form by a conversion process whereby most α -helix motives are replaced by β -sheet secondary structures.⁷ Point mutations and seeds trigger PrP^C to overcome the energy barriers, causing genetic and infectious TSE.⁹ Information on molecular events leading to sporadic CJD is still lacking, though epidemiological studies indicate being a majority of all

prion diseases.¹⁰ Understanding the molecular mechanisms by which PrP^C is converted into PrP^{Sc} is a major challenge in prion research.

PRION PROTEIN STRUCTURE AND FUNCTION

The PrP^C is a sialoglycoprotein tethered to the outer leaflet of the plasma membrane via a C-terminal glycosylphosphatidylinositol (GPI) anchor. Following the cleavage of N-terminal signal peptide, PrP^C is exported to the cell surface as a N-glycosylated protein. Its tridimensional structure is highly conserved among mammals,^{11,12} and it is composed of a flexible unfolded N-terminal domain and an α -helical enriched globular domain (**Fig. 1**).¹³ The unfolded N-terminal domain consists of unusual glycine-rich repeats. Residues 59–90 (*mouse numbering*) form 4 octarepeats (OR), PHGG[GS]WGQ, while residues 51–58 form an octapeptide lacking of the histidine residue (PQGGT~~W~~GQ). The OR segment binds copper and other divalent cations such as zinc, nickel, iron and manganese.^{14–18} Although PrP^C and metals share many common physiological functions, such as neuroprotection against apoptosis and oxidative stress, neurite outgrowth, maintenance of myelinated axons, copper homeostasis and synapse formation and functioning, the role of PrP^C–metal complexes is still elusive.¹⁹ Recently, the role of PrP^C–copper complex in protecting neurons by modulating NMDA receptor through S-nitrosylation has been shown.²⁰

The C-terminal domain presents 3 α -helices (spanning residues 143–152, 171–192 and 199–222), a short 2-stranded antiparallel β -sheet, and a short C-terminal tail.^{13,21} The V-shaped arrangement of the 2 longest helices, the second and the third, forms the scaffold onto which the β -sheet and the first α -helix are anchored. This domain also contains a disulfide bridge linking α_2 – α_3 helices, and the 2 N-linked carbohydrates.

Like many other cell surface proteins, PrP^C is exposed to low dielectric constant and different pH values because of its proximity to membranes and the constitutive cycling between

FIGURE 1. Schematic representation of PrP^C structural determinants. Highlighted: N-terminal domain (red line), globular domain (blue line), octarepeat region (OR) with octapeptide sequences (see main text), α -helices (H1, H2 and H3), β -strands (S1, S2), N-linked glycosylation sites (CHO), disulfide bridges (S-S) and glycosylphosphatidylinositol (GPI) anchor.



plasma membrane and endocytic compartments. As a general mechanism for modulating its activity,²² PrP^C can be differentially cleaved *in vivo* in the central region generating a soluble N-terminal domain and an anchored C-terminal globular domain (truncated form).^{22–24}

PRION PROTEIN FOLDING AND STABILITY

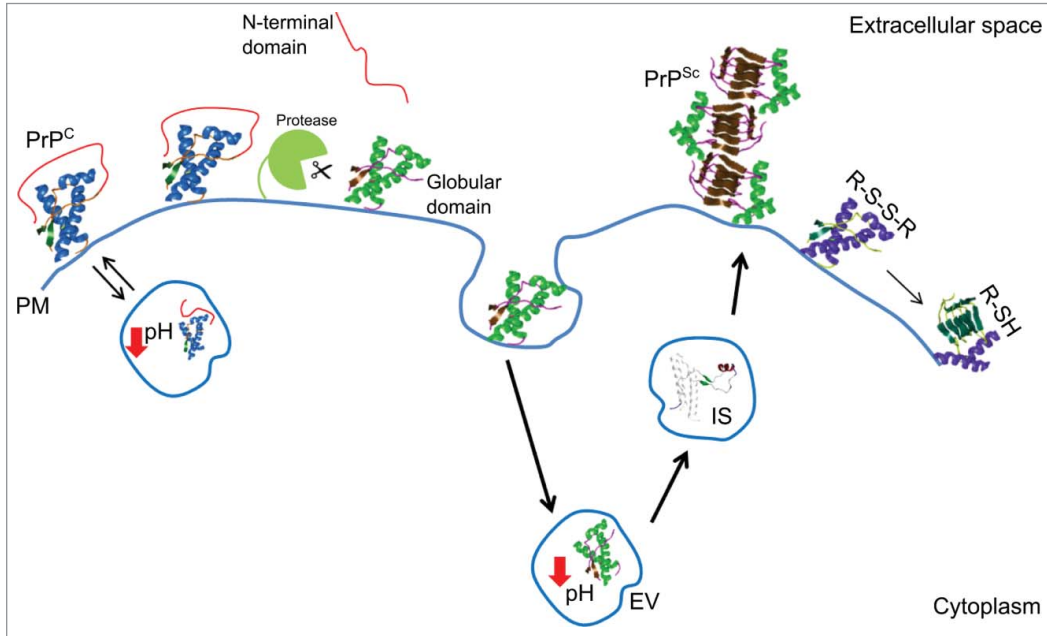
PrP^C is formed by several structural determinants such as unfolded N-terminal domain, octapeptide region, globular domain, and disulfide bridge. In physiological conditions PrP^C mostly folds in α -helical conformation, whereas in prion pathogenesis it is converted into the β -sheet enriched isoform PrP^{Sc}. To disclose molecular mechanisms inducing the pathological conversion in sporadic forms of prion diseases, a detailed study on the major structural determinants and environmental key factors affecting prion protein folding and stability is required. Recently, we published a study elucidating the contribution of major structural determinants and environmental factors in prion protein folding and stability.²⁵

Comparing folding of full-length and truncated forms, we observed an α -helical conformation for both proteins at neutral and acidic pH, with the full-length more stable than the truncated form. Calorimetric measurements on full-length protein showed $\Delta G_{25^\circ\text{C}}$ values 4–5 Kcal/mol higher than those obtained with spectroscopic analysis. Since calorimetry measures the amount of released or absorbed heat during chemical reactions and physical changes, taking into account all interactions occurring in protein folding and unfolding, the higher $\Delta G_{25^\circ\text{C}}$ values obtained by differential

scanning calorimetry are due to the interaction of N-terminal tail with C-terminal domain thus suggesting a role for the unfolded N-terminal domain in stabilizing the folded globular part. The flexible N-terminal domain interacts with the folded C-terminal part stabilizing it and favoring its folding, as confirmed by the reversible folding/unfolding process of full-length and the fully irreversible thermal unfolding transition of the truncated form. Therefore the flexible tail of the protein affects folding and stability of globular domain. The contribution of N-terminal domain in stabilizing the protein is pH-dependent because of histidine residues in the OR part acting as pH sensor. At acidic conditions similar to those present in endocytic compartments, histidine residues are mostly positively charged and stability of full-length is reduced. Also in these conditions, full-length is more stable than the truncated form, as indicated by $[\text{Urea}]_{1/2}$ value of 5.7 M and 5.2 M respectively.

As describe above, OR segment represents the major structural determinant in N-terminal domain. In addition to metal binding, OR region is involved in stabilizing full-length protein, preserving its native folding. The lack of only one octarepeat causes the decrease of α -helical content and determines the loss of reversibility, similarly to the truncated form. To conclude, the N-terminal domain modulates the stability of C-terminal domain through the OR segment. Aberrant interactions of N-terminal domain with surrounding proteins or molecules, such as divalent cations, may alter the stabilizing effect on the globular domain permitting its misfolding. Metal dys-homeostasis may therefore induce the formation of non-physiological PrP^C-metal complexes, aberrant

FIGURE 2. Cartoon of the major structural determinants and environmental key factors in prion protein folding and stability. Abbreviations correspond to: cellular prion protein (PrP^{C}); prion (PrP^{Sc}); highly enthalpically stable intermediate state (IS); plasma membrane (PM); endosomal vesicles (EV); disulfide bridge (R-S-S-R); free thiol (R-SH).



N-terminal/C-terminal interactions and then globular domain destabilization.

The cleavage of PrP^{C} by zinc metalloprotease ADAM family induces the release of N-terminal domain and the formation of the truncated form. This globular domain is still tethered to membrane and undergoes to the constitutive cycling between plasma membrane and endosomes, with subsequent exposure to different environmental pH values. In the presence of acidic conditions, globular domain unfolds with a 3-state transition mechanism. The transition state is favored at low temperatures, as confirmed by free-energy and $[\text{Urea}]_{1/2}$ values, and it is enthalpically stabilized with respect to the native state by approximately 20 kcal/mol. Interestingly, the highly enthalpically stable intermediate state corresponds to a peculiar metastable folding of PrP only in species susceptible to scrapie such as human, mouse, cattle, and some sheep variants. Scrapie-resistant species, as the sheep variant ARR, do not show the highly enthalpically stable intermediate state. Therefore, this metastable state may

represent a common path in the pathological conversion of PrP^{C} to PrP^{Sc} . This finding is in agreement with previous observations showing the endosomal recycling compartment as the intracellular site of the conversion.²⁶ The highly enthalpically stable intermediate state is characterized by a repositioning of α_1 -helix, a new β -sheet between residues 124–128 and residues 151–155, and a larger number of intramolecular salt-bridges and intermolecular hydrogen bonds with water not accessible from the native conformation. The structural rearrangements leading to the formation of the metastable state are compatible with results obtained with low resolution techniques on N-terminally truncated $\text{PrP}27\text{--}30$,^{6,7} and the left-handed β -helix model proposed by Govaerts et al.²⁷ In this model, PrP residues from 90 to 170 are converted into β -strands and subsequently in β -helices, while the globular domain retains its α -helical structure with the disulfide bridge and the glycan moieties located outside the oligomeric core.

TSE, as other neurodegenerative diseases, are characterized by oxidative damage.^{28–30} The impact of oxidative stress on PrP^C disulfide bridge and then on prion conversion is unsolved. For this reason, we investigated the role of disulfide bridge in preserving native folding and protein stability. Previously published *in vitro* studies have shown that disulfide bridge reduction under denaturing conditions causes the disruption of native tertiary interactions and protein unfolding. In our study, we reduced disulfide bridge in quasi-native conditions, preserving protein folding. While the reduced form of full-length shows a decrease in α -helical content, the reduced globular domain is characterized by larger β -sheet content and hydrophobic surface exposed to the solvent. The increased hydrophobicity on protein surface leads to misfolding and aggregation.

CONCLUSIONS

During prion pathogenesis, PrP^C undergoes to structural rearrangements leading to the formation of prions. Prions propagate acting as a template, and thus converting new PrP^C molecules into PrP^{Sc}. In sporadic forms of prion diseases, the early events causing PrP^C misfolding and conversion are still unclear. Understanding the major structural determinants in PrP^C folding and stability is crucial to disclose the early events leading to prion conversion in sporadic TSE. In our study, we identified the structural elements and environmental key factors in prion protein folding and stability (**Fig. 2**). These findings allow us to conclude that aberrant N-terminal domain/C-terminal domain interactions or PrP^C cleavage may cause protein misfolding in endosomal vesicles, therefore favoring prion conversion.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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