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Anchorless forms of prion protein – Impact of truncation on structure destabilization and prion protein conversion

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Prion diseases are a group of fatal neurodegenerative diseases caused by scrapie form of prion protein, PrP^{Sc}. Prion protein (PrP) is bound to the cell via glycophosphatidylinositol (GPI) anchor. The role of GPI anchor in PrP^{Sc} replication and propagation remains unclear. It has been shown that anchorless and truncated PrP accelerate the formation and propagation of prions *in vivo* and further increases the risk for transmission of prion diseases among species. To explain the role of anchorless forms of PrP in the development of prion diseases, we have prepared five C-terminal PrP truncated variants, determined their thermodynamic properties and analyzed the kinetics of conversion into amyloid fibrils. According to our results thermodynamic and kinetic properties are affected both by pH and truncation. We have shown that the shortest variant was the most destabilized and converted faster than other variants in acidic pH. Other variants converted with longer lag time of fibrillization than WT despite comparable or even decreased stability in acidic pH. Our results indicate that even the change in length for 1 amino acid residue can have a profound effect on *in vitro* conversion.

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1. Introduction

Prion diseases are a group of fatal neurodegenerative disease. They occur due to self-propagation and accumulation of pathological form of prion protein, named prion (PrP^{Sc}). PrP^{Sc} is formed by conversion of cellular form of prion protein (PrP^{C}) [1]. PrP^{C} is anchored onto the membrane of most body cells with glycophosphatidylinositol (GPI) anchor. The protein is expressed in most tissues, where its role has not yet been fully explained. The pathological transformation of PrP^{C} into PrP^{Sc} includes the protein's secondary and tertiary structure changes, with no change in its primary sequence. Consequently, the β-sheet rich PrP^{Sc} is formed. PrP^{Sc} molecules are prone to aggregation in brain, which is associated with neuron damage and loss of function.

The role of GPI anchor in PrP^{Sc} replication and propagation remains unclear [2,3], but it has been recently shown on models of transgenic mice, expressing only anchorless prion protein (PrP), that anchorless PrP accelerates the formation and propagation of prions [4–9] and further increases the risk for transmission of prion disease among species [10].

Anchorless PrPs are a group of PrPs truncated at the C-terminus and lack GPI anchor. Truncated forms can be generated from the native form of PrP, using proteolytic degradation by ADAM proteases [11,12], or can occur due to nonsense mutations [13–15]. One such protein, PrP226*, was characterized using monoclonal antibody V5B2, which selectively recognizes the truncated form of PrP ending with amino acids Tyr225 and Tyr226 [16]. A clinical study made by Jansen et al. reported two cases of Gerstmann-Sträussler Scheinker syndrome (GSS) with deposition of C-terminally truncated PrP, ending with Tyr226 and Gln227 [15]. The study serves as confirmation that anchorless truncated PrP can cause the formation of disease *in vivo*.

Deposition of truncated PrP molecules, besides PrP^{Sc} aggregates, was described in the brain of patients with transmissible spongiform encephalopathies (TSEs) [13–15,17–19]. Using V5B2 it has been shown that accumulation of PrP226* in the brain is characteristic for most types of human TSEs, like GSS, sporadic Creutzfeldt-Jakob disease (sCJD), variant Creutzfeldt-Jakob disease

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(vCJD), fatal familial insomnia (FFI) and also animal TSEs, like bovine spongiform encephalopathy (BSE) and scrapie [20,21]. In sCJD, the distribution of PrP226* correlates with the distribution of PrP^{Sc} aggregates, therefore it could be acknowledged as a surrogate marker for PrP^{Sc} [21].

The correlation between PrP226* and amyloid plaques suggests that the truncated protein has an important role in the development of amyloid diseases. According to previous studies, fibrillation can also be propagated by other anchorless PrPs, similar to PrP226* [22,23]. Despite increasing evidence on the importance of truncated PrP variants *in vivo*, these variants have not been characterized *in vitro*. In this study we investigate the effect of stop codon mutations at the C-terminus on PrP stability and fibrillization. We prepared five C-terminal PrP truncated variants. The shortest variant was the most destabilized and converted faster than other variants in acidic pH. Other variants converted with longer lag time of fibrillization than WT despite comparable or even decreased stability in acidic pH. Our results indicate that even the change in length for 1 amino acid residue can have a profound effect on *in vitro* conversion.

2. Materials and methods

2.1. Preparation of PrP mutant constructs

We prepared five mutants of C-terminal truncated PrP. As a template we used the plasmid pProExHTa, containing the HuPrP(90–231, M129) gene [24], that was donated by prof. Giuseppe Legname (SISSA, Trieste, Italy). We prepared mutants Y225X, Y226X, Q227X, R228X and G229X (Fig. 1) using the Quickchange kit II XL Site-directed Mutagenesis (Agilent Technologies). Respectively, we named the expressed proteins PrP224*, PrP225*, PrP226*, PrP227* and PrP228*. The cloned DNA sequences were verified by sequencing.

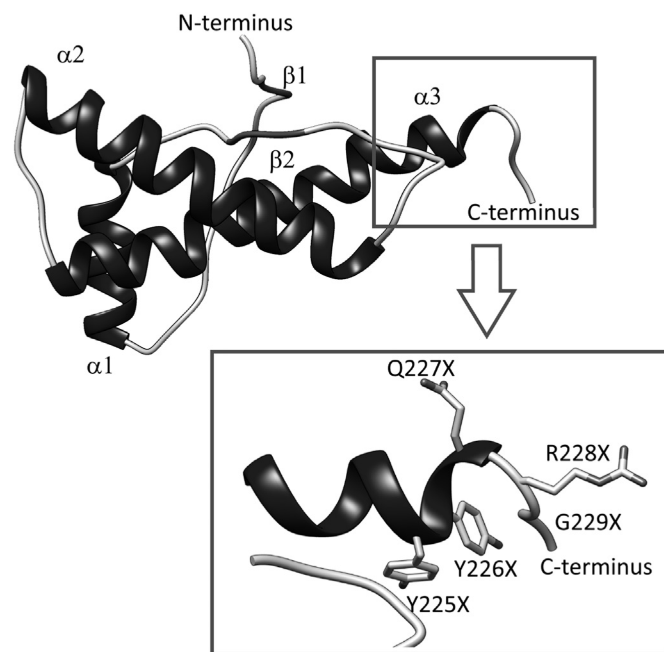

Fig. 1. Schematic representation of the structure of human PrP (PDB ID 2LSB, up) [25] and depicted amino acid residues (down) where we inserted stop mutations to prepare truncated PrP variants.

2.2. Protein expression, purification and refolding

Plasmid pPROEXHTa encoding HuPrP mutants was transformed into competent *E. coli* BL21 (DE3). The proteins were prepared as previously described [24,25]. The isolated inclusion bodies were solubilized in 6 M GndHCl and purified on a 5-mL FF Crude HisTrap column (GE Healthcare). For spectroscopic and thermodynamic studies mutants were refolded by dialysis against refolding buffer (20 mM sodium acetate (pH 4.5) or 5 mM sodium phosphate (pH 7)). For studies of *in vitro* conversion of recombinant prion proteins, mutants were dialyzed against 6 M GndHCl. Purified proteins were analyzed using SDS-PAGE under reducing conditions.

2.3. Circular dichroism spectroscopy and thermal denaturation

Circular dichroism spectra were recorded on a Chirascan spectropolarimeter (Applied Photophysics). Far-UV CD spectra were recorded between 190 nm and 250 nm in a 1 mm path length cuvette at a protein concentration of 0.2 mg/ml. Thermal denaturation of truncated proteins were followed by measuring the CD signal at 222 nm as a function of temperature with a heating rate of 1 °C/min. The reversibility of refolding of denatured proteins to the native state was assessed by recovery of CD signal.

As previously described, CD temperature scans were analyzed by a 2-state transition model with a model equation that accounts for the linear dependence of the pretranslational and posttransitional baselines on temperature [26].

Differences in the stability were also analyzed as previously described [27]. The relation of stability of truncated and wild type (WT PrP) were estimated according to the relationship proposed by Becktel and Schellman [23]: $\Delta\Delta G = \Delta T_m \frac{\Delta H_m^{wt}}{T_m^{wt}}$, where $\Delta\Delta G$ is the difference in stability between truncated proteins and WT protein, ΔT_m is the difference in midpoint temperatures of truncated proteins and WT PrP and ΔH_m^{wt} are reference enthalpy of WT PrP at midpoint temperature T_m^{wt}. In case of the destabilization of WT PrP by acidic pH, the reference values are from WT PrP at pH 7. A negative value of $\Delta\Delta G$ correlates to the destabilization of truncated proteins with respect to WT PrP.

2.4. In vitro conversion of recombinant prion proteins

In vitro conversion was carried out as previously described [27–29]. The denatured proteins were diluted into 1 M GndHCl, 3 M urea and 50 mM PBS (pH 7) or 20 mM sodium acetate with 100 mM NaCl (pH 4.5) to a final protein concentration of 22 μM. Conversion was conducted by shaking at 37 °C, 1000 rpm. Shaking was performed in 96-well microtiter plates with three 3/32 Teflon balls per well. Formation of fibrils was monitored using thioflavin T (ThT). ThT emission (460–535 nm) was tracked by excitation at 442 nm at 5 mM ThT concentration with a Mithras LB 940 microplate reader (Berthold Technologies) [27,29–31]. Kinetic data were fitted to a logistic function [32,33]: $Y = y_i + m_i x + \frac{y_f + m_f x}{1 + e^{\left\{\frac{x_0 - x}{\tau}\right\}}}$, where Y is the fluorescence intensity, x is the time, and x_0 is the time to 50% of maximal fluorescence. Thus, the apparent rate constant, k_{app}, for fibril growth is given by $1/\tau$ and the lag time (t_{LAG}) is given by $x_0 - 2\tau$. Each curve obtained from an individual experiment was fitted separately to extract kinetic parameters.

2.5. Analysis of fibril formation

For analysis of *in vitro* conversion of prion protein we used atomic force microscopy (AFM). Samples were prepared as previously described [31,34] and observed by Agilent Technologies 5500 Scanning Probe Microscope operating in acoustic alternating

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