Summary of the Ph.D. thesis

Dimerization of the prion protein studied by site specific mutagenesis to *p*-benzoyl-L-phenylalanine

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INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of rare, infectious and fatal neurodegenerative disorders that can affect both humans and animals. They include, among others, Creutzfeldt–Jakob disease (CJD), Gerstmann Straussler-Scheinker syndrome (GSS), fatal familial insomnia (FFI) and kuru in humans, and bovine spongiform encephalopathy (BSE) in cattle, scrapie in sheep and chronic wasting disease (CWD) in deer and elk. TSEs are known to occur as a result of the conformational conversion of the α-helical, non-infectious and protease sensitive form of the normal cellular prion protein, PrP^{C}, into a ß-sheet-rich, infectious and protease resistant form, PrP^{Sc}. The hallmark histopathological characteristics of these diseases include widespread spongiform encephalopathy, neuronal loss, gliosis, and deposits of prion protein aggregates of various sizes, ranging from small, soluble oligomers to long, thin, unbranched fibrils [1]. The mechanism of developing these diseases is not yet known as well as a cure to TSEs has not been found to date.

Two models have been proposed for the mechanism of scrapie-prion replication: the template-assisted model and nucleation (seed) dependent polymerization model [2]. In the template-assisted model, pre-existing PrP^{Sc} provides the template for conversion of PrP^{C} when that transiently interacts with PrP^{Sc}, i.e., $PrP^{C} + PrP^{Sc} \rightarrow PrP^{Sc} * PrP^{C} \rightarrow 2PrP^{Sc}$, while in the nucleation dependent polymerization model, PrP^{Sc} is formed by accumulated assimilation of PrP^{C} monomers into a propagating structure. However, the exact mechanism and early steps of PrP^{C} oligomerization are not yet clear.

The cellular prion protein and its structure:

PrP^{C} is a sialoglycoprotein anchored to the outer surface of the plasma membrane via glycosylphosphatidylinositol (GPI) anchor. PrP^{C} is predominantly expressed in the central nervous system (CNS), but it is also found in multiple other organs, including, the heart, liver and kidneys. PrP^{C} possesses a large [amino acids (aa.) 23-124, human numbering] intrinsically disordered N-terminal domain, which is about half the protein and which contains an octarepeat region (OR) comprised of five consensus sequences of PHGGGWGQ, region that is unique among all proteins. The histidine residues of the OR confer affinity towards various divalent cations such as, copper, zinc, nickel, iron and manganese, to the protein. The other half (aa. 125-231) of the protein presents a structured, globular, C-terminal domain with three α-helices and two short antiparallel β-sheet strands. Acting also as a transition region

between the N- and C-terminal domains, there is a hydrophobic domain (HD, aa. 111-134) that has been reported to be involved in dimerization and interaction with various protein partners. PrP^{C} possesses also N-linked glycans, at residues 181 and 197, as well as one disulfide bond between residues 179 and 214. The exact physiological function of PrP^{C} is not yet known, however PrP^{C} binds to a variety of protein partners and it has been reported to participate in several biological processes, including stress protection, cellular differentiation, neuronal excitability, myelin maintenance, circadian rhythm, metal ion homeostasis, mitochondrial homeostasis, regulating levels of amyloid-ß and tau, and modulation of the immune system.

Two paralogs of the prion protein had been uncovered: the doppel (Dpl), and the Shadoo (Sho) proteins, which together with PrP^{C} constitute the prion family proteins. Dpl and Sho possess C-terminal GPI anchors as PrP^{C}, but have differing structures. Dpl has a short, unstructured N-terminal tail and a globular C-terminal domain composed of three α-helices, two disulphide bonds, and two N-glycosylation sites. Dpl is expressed primarily in the testis of adult mammals and its ectopic expression can result neurodegeneration in the CNS. Contrary, Shadoo is an unstructured protein and resembles the flexible N-terminal tail of PrP^{C}, and it is expressed in the CNS. Sho had been reported to exert a number of similar protective activities as PrP^{C} and had been proposed that it may act on similar signaling pathways. However, the functional relations of these two paralogs with PrP^{C}, or their involvement if any information of PrP^{Sc}, are not fully understood at present.

PrP dimerization:

In normal cellular conditions, a percentage of PrP^{C} is known to be in homodimeric form at the cell surface [3] and it has been demonstrated that dimerization plays an important role in prion biology, regulating PrP^{C}-mediated signaling processes, which result inneuroprotective or neurotrophic activities [4]. On the contrary, however, formation of unwanted PrP dimers can lead to propagation of PrP^{Sc}, which is associated to neuronal toxicity [5]. Despite numerous investigations, the mechanism of transition of PrP^{C} from monomeric to oligomeric forms and/or to infectious, PrP^{Sc} form is still elusive. The exact role played by PrP^{C} dimers in PrP^{Sc} formation is not yet known, however, the dimeric state of the cellular PrP^{C} could lie as an important intermediate form on the pathway of formation of PrP^{Sc} from PrP^{C}.

The G127V variant of PrP confers resistance to kuru:

The prion disease kuru, developed in the Fore tribe from Papua New Guinea as a result of a ritual cannibalism and it became the prime cause of death in the region by 1957. Later it was found that a group of people among the tribe presented resistance to kuru. Genetic analysis conducted in 2009 uncovered that these individuals possessed a novel protective mutant variant of the prion protein, containing a valine substitution at position 127 in heterozygous state and on a M129 allele [6]. Transgenic mice studies performed in 2015 validated the protective effect of this mutation, moreover, in mice the G126V (equivalent to G127V in human sequence) mutation in homozygous state confers complete resistance to all kinds of prion diseases [7]. The molecular mechanism underlying this protection and how this point mutation leads to resistance to propagating the pathogenic isoform of prion *in vivo* it is not known. Molecular dynamics (MD) simulations along with nuclear magnetic resonance (NMR) spectroscopy studies revealed that the G127V variant of human PrP (HuPrP) prevents the formation of dimers by ß-sheet and fibril formation [8,9]. However, whether this is also the case in more physiological conditions or not, it remains to be elucidated, as well as, whether or not it plays a role in the mechanism of rescue effect of the G127V mutation.

Expansion of the genetic code and incorporation of genetically encoded non-canonical amino acids in prokaryotes to facilitate protein interaction and structural studies:

Biological processes usually rely on a complex network of protein-protein interactions (PPIs) taking place between different proteins and also between same type of proteins (self-interacting proteins or SIPs). Alterations within these interactions can disrupt the physiological processes and may lead to pathophysiological events. Regarding the prion protein, the proposed vast number of protein interacting partners and its self-interaction mechanisms remain poorly validated. Understanding the interactions at a residue level could provide valuable insights into both normal and pathological events that PrP^{C} participates to. Several methods can be used to map protein interactions, however, one of the most promising methods, which could prove to become a powerful strategy in this respect, is the use of site-specific, genetic incorporation of photocrosslinkable nonnatural amino acids to protein sequences, for precise identification of protein interactions and interaction sites at the same time. Genetic code expansion in this way, has enabled the genetic incorporation of noncanonical amino acids (ncAAs) site specifically into proteins using *in vivo* systems.

To date, more than 230 different ncAAs, including aryl azide, diazirine and p-benzoyl-L-phenylalanine (*p*Bpa) had been incorporated into proteins by *in vitro* or *in vivo* methods.

The *in vivo* method is based on an orthogonal t-RNA and an amino acyl-tRNA synthetase (tRNA/aaRS) pair able to recognize an amber stop codon, TAG and incorporate the ncAA site specifically into the growing polypeptide chain during protein synthesis, and was successfully used in various hosts [*Escherichia coli (E. coli)*, *Saccharomyces cerevisiae*, *Pichia pastoris* and mammalian cells] [10]. Among the ncAAs, *p*Bpa possesses favorable characteristics such as, low reactivity to water and light, favorable photokinetics, high site- specificity and ease of incorporation into proteins during bacterial synthesis. Upon UV-irradiation at 350 to 365 nm, the -C=O group of *p*Bpa can react and form covalent bond with -C-H group of adjacent protein molecule situated within a 3.1 A° distance, permitting this way covalent bonding of the interacting partners. Introducing this method to study prion biology could provide valuable information on the homo- and heterodimerization interactions of PrP.

MAIN OBJECTIVES OF THE THESIS

The main goal of the thesis is to address current questions related to the dimerization of the prion protein, such as: "Which residues participate in forming of the dimerization interface?" and "What is the effect of a novel disease-protective mutation on dimerization?", by employing an *in vitro* approach that allows studying the process at single amino acid level resolution along the sequence of the prion protein. In line with this goal, the specific aims set are as follows.

1. To interrogate the dimerization interface of full length recombinant mPrP by site specific incorporation of the crosslinkable amino acid *p*Bpa.

1.1. To develop an *in vitro* model system consisting of a set of purified, recombinant full length (aa. 23–230) mouse prion proteins (mPrPs) that bear genetically coded single-substitutions of selected residues to the photocrosslinkable amino acid, *p*Bpa, placed strategically at various positions along the sequence in order to allow testing of the sites involved in the formation of a dimer.

1.2. To test whether site specific insertion of *p*Bpa into the mPrP sequence occurred correctly and whether *p*Bpa insertion has any disruptive effect on the structure or conformational stabilities of the mPrP variants.

1.3. To use the system to study the sites involved in the homodimerization of mPrP, by evaluating the efficiency of homodimer formation of each *p*Bpa-mutant variant, using photocrosslinking, and identification of covalently crosslinked dimers by sodium dodecyl

sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and densitometry analysis of protein bands.

2. To investigate the effects of the novel disease-protective mutation, G127V, on the dimerization of the prion protein.

2.1. We aimed to develop further mutants, including G126V mutants and site-specific *p*Bpa mutants of full length recombinant mPrP, untagged and mCherry tagged variants, to have an appropriate set of proteins by which the effect of G126V mutation upon the dimerization of the mPrP can be studied.

2.2. Using the produced set of mPrP mutant variants, to test the efficiency of dimerization of mPrP in the presence and absence of the disease-protective G126V mutation, by using protocrosslinking and densitometry analysis after SDS-PAGE and by assessing both hetero- and homodimerization.

2.3. To compare the results obtained by our *in vitro* approach to the results of experiments conducted in parallel in an *in cellulo* experimental setup using HeLa cell cultures transfected by various mutant mPrP constructs designed to test the dimerization efficiency of mPrP in the presence and absence of the disease-protective mutation G126V.

EXPERIMENTAL METHODS

DNA-plasmids construction:

In order to produce recombinant proteins in *E. coli*, all DNA-plasmids encoding the desired proteins were constructed in pRSET-B bacterial protein expression vectors. For the prion protein, in all of the studies presented here, the gene coding for the full length mPrP (aa. 23-230) was used. Two sets of constructs were prepared for PrP: one set where the encoded PrP was present as untagged, and another set, where the protein coding DNA sequence (CDS) of an mCherry was used downstream of the CDS of mPrP to result a fusion protein with mCherry for the PrP at its C-terminal. The two sets comprised each a plasmid coding for the corresponding wild type (WT) prion protein (untagged or mCherry-tagged) and several other plasmids coding for various *p*Bpa-mutants of the mPrP (untagged or mCherry tagged).

1. To generate *p*Bpa-mutant mPrPs in *E. coli*, an amber stop codon (TAG) was used, which was placed at specific positions into the CDS of mPrP. In this manner, a set of various (over 40 different) DNA plasmid constructs were created for including both the untagged and the

mCherry-tagged set of PrPs, to encode mPrP variants with one *p*Bpa mutation present in the mPrP sequence at varying positions:

1.1. For mapping the dimerization interface of mPrP the amber stop-codons (one for each construct) were placed such that the positions of *p*Bpa-point mutations cover key regions, by replacing residues as follows: W80, G89, Q90 within the highly flexible segment of N-terminal region; N107, K109, V111, G113, A116, A117, G118, A119, V120, V121, L124, G125 within the central region (CR, aa. 105 to 125) and also, together with G126, Y127, M128, L129, M133 within the hydrophobic domain (HD, aa. 111 to 134), as well as, R135, E206 and E210, belonging to the C-terminal domain. Amino acids 206 and 210 are buried within the protein fold and are not expected to be taking part in the dimerization surface, therefore, could serve as negative controls. Using this strategy, we created 24 constructs with individual positions for *p*Bpa insertion in the mCherry tagged mPrPs, and 14 constructs for untagged mPrPs, along with two constructs corresponding to the wild type mPrP, coding for untagged and mCherry tagged mPrP.

1.2. For the studies on the effect of the G126V disease-protective mutation on dimerization of the protein, additional DNA plasmid constructs, to the above mentioned ones, were also generated. These encode for: an untagged and an mCherry tagged G126V mutant mPrP; and three untagged mPrPs possessing both a G126V mutation and also one *p*Bpa mutation at positions 127, 128 and 131.

Protein expression:

Plasmids that were constructed for expression of proteins with no *p*Bpa insertion, were expressed in *E. coli* BL21(DE3) cells, whereas, all plasmids encoding for a *p*Bpa-insertion into a protein were transfected into pre-transformed *E. coli* BL21(DE3) cells, which already harbored a plasmid encoding for an orthogonal t-RNA and an amino acyl-tRNA synthetase (tRNA/aaRS) pair in order to produce mPrP with *p*Bpa mutant variants. Single colonies of transformed bacteria were used to inoculate starter cultures in Luria–Bertani (LB) media containing the corresponding antibiotics and were grown, in general, for ~16 h at 37 °C. 1% inoculum was added to 800 ml LB media and cultures were induced, usually, at an $O.D_{600}$ within 0.5–0.6 range, by adding either 1 mM isopropyl ß-D-1-thiogalactopyranoside alone (for protein expressions without a *p*Bpa insertion) or together with 0.02% arabinose and 1 mM *p*Bpa, for the expression of *p*Bpa-mutant PrPs. The cultures were further grown, usually for at least 4 h at 37 °C, before harvesting by centrifugation.

Protein purification:

Recombinant mPrPs and variants were purified from bacterial inclusion bodies, briefly as follows. Bacterial pellets were disrupted by sonication at 4 °C in presence of protease inhibitors. The inclusion bodies were solubilized in denaturation buffer containing 6 M guanidine hydrochloride. Extracted prion proteins were captured using nickel-nitrilotriacetic acid (Ni-NTA) beads and were allowed to refold overnight in a redox buffer, in the presence of glutathione oxidase and glutathione reductase, on ice. Refolded proteins were eluted from the beads by increasing concentration of imidazole and were dialyzed in storage buffer, 20 mM sodium acetate, pH 5.5. Dialyzed proteins were tested by SDS-PAGE for proper molecular weight and purity. The concentration of the proteins was measured using the Bradford method. Aliquots of 1 ml were flash-frozen in liquid nitrogen and stored at -80 °C for later use.

Mass spectrometry:

MALDI-TOF mass spectrometry analysis was performed to confirm the correct site specific insertion of *p*Bpa into the mPrPs.

Urea gradient assay:

Conformational stabilities of wild type and mutant PrP-s were compared using an urea gradient assay to test if mutations had any altering effect on the stability. Briefly, protein samples were treated with increasing concentrations of urea in presence of reducing agents. The non-reduced and reduced populations of proteins in the samples of increasing urea content were visualized on 10% polyacrylamide (PA) sodium dodecyl sulphate (SDS) gels. Midpoints of transitions from non-reduced to reduced states were compared for wild type and *p*Bpa-mutant mPrP variants.

Photocrosslinking:

Proteins were photocrosslinked at 365 nm UV light for 2 h, on ice. In parallel, similar aliquots of samples were kept at dark to serve as negative controls. Crosslinking of each protein sample was performed in two conditions in parallel: in presence of 0.06% and 2% SDS, which favor dimer and monomer formation, respectively. Wild type mPrP (untagged or mCherry tagged) without containing *p*Bpa, were also used in both the UV-irradiated and non-irradiated sets of samples, to serve as controls.

Evaluation of crosslinking efficiencies:

Crosslinked samples were run on 10% PA SDS gels and the percentages of the crosslinked products were evaluated by gel-densitometry analysis and using the ImageJ program. The percentage of dimers was calculated as the dimer area divided by the sum of the

monomer area and the dimer area on the densitogram of one lane on the image of gel, containing the sample. Specificity of dimer formation was tested at monomer forming condition (2% SDS), and values obtained were subtracted from those of dimer-condition (0.06% SDS). Values obtained in a similar way for the non-irradiated control samples were also subtracted to correct for background crosslinking. The resulting positive values significantly different from control protein, were considered as specific crosslinking events.

Circular dichroism spectroscopy:

Far-UV circular dichroism (CD) spectra of wild type- and *p*Bpa-mutant PrPs were compared to test whether *p*Bpa insertion had any altering effect on the proper folding of PrP. CD spectra of photocrosslinked samples were also recorded and analyzed to detect any secondary structural changes upon crosslinking and/or dimerization of proteins.

Identification of hetero- and homodimers of G126V mPrP variants:

1. For testing heterodimerization by crosslinking, the purified proteins were selected in pairs: one mPrP possessing mCherry tag, while the other being an untagged mPrP that usually contained the *p*Bpa insertion as well. By such pairing the heterodimers formed upon photocrosslinking could be easily identified on SDS-PA gels.
2. Single protein variants of untagged mPrP containing *p*Bpa at position 127, 128 or 131, with and without the presence of a G126V mutation, were selected to study homodimer formation efficiency.
3. For determining the percentages of the crosslinked heterodimer PrPs, the area corresponding to the heterodimer band on the intensity plot was divided by the total area corresponding to the sum of areas of all protein bands in the lane.

RESULTS

I. The study of dimerization interface of mPrP:

1. A set of over 40 DNA plasmid constructs encoding the full length mPrP, suitable for protein expression in *E. coli*, was constructed in order to systematically map the dimerization site of the prion protein. Among these DNA constructs are a subset encoding for untagged- and a subset for mCherry-tagged mPrPs, each set containing a plasmid for the corresponding wild type mPrP and the rest for mPrPs with an amber stop-codon (TAG) mutation, placed at various specific position in the mPrP-CDS sequences in order to initiate the insertion of one *p*Bpa residue, site specifically into each of the protein constructs when expressed in *E. coli*.

2. Using the set of plasmids constructed, WT- and a series of *p*Bpa containing recombinant mPrP protein variants, both untagged and mCherry tagged, were successfully expressed and purified using *E. coli* expression system and Ni-NTA chromatography.

3. The correct site specific insertion of *p*Bpa into the mPrP sequence was successfully validated by MALDI-TOF mass spectrometry analysis using the individual protein samples mPrP(S131Y)-mCherry (control) and mPrP(S131*p*Bpa)-mCherry, grown in presence and absence of *p*Bpa, and of three mixtures of different ratios of these two protein variants (75:25, 88:12 and 94:6%). The analysis confirmed that *p*Bpa was correctly incorporated at position 131 of mPrP, in at least of ~90% of protein molecules expressed.

4. By comparing the conformational stabilities of the WT and *p*Bpa-mutant PrPs employing an urea gradient assay in presence of reducing agents, similar unfolding patterns could be detected for WT- and *p*Bpa mutant PrPs on SDS-PA gels, revealing that *p*Bpa did not significantly disturb the conformation of PrPs.

5. An efficient photocrosslinking method was set up, by which mPrP dimers could be covalently UV-crosslinked in case of both untagged and mCherry-tagged mPrP variants possessing *p*Bpa.

6. SDS-PAGE followed by densitometry analysis of the photocrosslinked samples revealed that:

6.1. Among 24 positional *p*Bpa variants of mCherry tagged mPrPs, the most efficient dimer formations are present in the case of those with *p*Bpa at positions: 107, 127, 128 and 131 and, although less efficient, but also for 80-, 111-, 117-, 118- and 121*p*Bpa variants. Altogether, nine out of the 24 positions yielded crosslinked protein amounts significantly higher than the amounts produced by non-specific association of the proteins in 2% SDS.

6.2. In case of untagged mPrP variants, most efficient dimer formations were found for those containing *p*Bpa at positions 107, 127, 128 and 131, and also, but somewhat less efficient, for those with *p*Bpa at positions 80, 90, 111,113 and 116. Nine out of the 14 mutants displayed significant amount of crosslinked products compared to the controls (monomeric, 2% SDS, condition).

These findings suggest that the same positional *p*Bpa mutants provide efficient dimer-crosslinking in both tagged and untagged forms, and that the *p*Bpa mutants have generally similar rankings regardless of mPrP being tagged by an mCherry or untagged.

7. The far-UV CD spectra of the monomeric and crosslinked dimeric forms of untagged wild type- and *p*Bpa-mutant mPrP variants confirmed that only slight variations in the secondary

structural elements of the proteins occur upon crosslinking and that the helical structure of mPrP is preserved during dimerization.

II. <u>The study of the disease-protective G126V mutation on the dimerization of mPrP:</u>

1. We created various DNA constructs encoding the full length mPrP, with the disease protective mutation G126V, either alone or in combination also with a *p*Bpa encoding mutation, both in untagged form of mPrP or tagged form in fusion with an mCherry coding sequence as C-terminal tag, in order to investigate the effect of the G126V disease-protective mutation on the formation of mPrP dimers. We successfully expressed and purified these proteins, as earlier, using *E. coli* expression system and Ni-NTA chromatography.

2. Applying the crosslinking method established for these set of proteins, the crosslinking efficiency data revealed that the highest percentage of heterodimers are crosslinked when both mPrP protein partners are possessing their original wild type sequence, i.e. without the presence on G126V mutation, and when crosslinking is performed with *p*Bpa-mutation placed at 127 or 131 aa. positions of the untagged PrP. When the valine mutation is present at 126 position of PrP, the heterodimer formation significantly diminished in all of the protein-combinations selected.

3. Homodimer formation studied with untagged mPrP-s containing *p*Bpa insertions at positions 127, 128 and 131, revealed that the efficiency of homodimerization is also highest when the proteins possess their original amino acid, G126, and when *p*Bpa is at position 127 or 131 of mPrP, whereas, homodimerization is significantly diminished by V126 mutation in the protein sequences.

CONCLUSIONS

In this work, we created a set of 44 variants of recombinant full length mouse prion protein, mPrP, in order to permit site-specific covalent photocrosslinking and the study of the dimerization interface of mPrP and the homo- and heterodimerization of the disease-protective G126V variant of mPrP. These proteins included 41 *p*Bpa-mutant variants (each possessing one site specifically inserted photocrosslinkable amino acid, *p*Bpa, and included 24 mCherry tagged and 14 untagged mPrP variants, and additional three untagged mPrP *p*Bpa-variants with a G126V mutation); as well as one mPrP(G126V) mutant tagged with

mCherry, and two wild type, untagged and mCherry tagged, mPrPs. We proved the usefulness of the approach and of the set of proteins created as a tool, in two studies. First, we used this system to interrogate the dimerization interface of the full length mPrP. Second, we used this approach to test the effect of the prion disease-refractive mutation G126V (equivalent to V127V in HuPrP) on the homo- and heterodimerization of mPrP. Our major findings include the following.

1. Insertion of the photocrosslinkable nonnatural amino acid *p*Bpa as a point mutation into the sequence of the mPrP using *E. coli* system was efficient. The presence of the *p*Bpa at the chosen positions did not disturb the stability of the protein, and provided efficient crosslinking in order to study the dimerization of mPrP by SDS-PAGE and densitometry analysis. Proving also that the developed system can be used as a site-specific tool to interrogate the interaction surfaces of PrP.
2. Using this system, we found that the N-terminal part of the prion protein, specifically the regions around position 127 and 107, is integral part of the dimerization interface of the full length mPrP, and that the formed dimers are alpha helical.
3. By using selected proteins from the set of *p*Bpa-mutant variants, and by creating targeted additional mutants, we studied the effect of the prion disease-refractive mutant G126V of mPrP on the homo- and heterodimerization of the protein, and found that this mutation has a diminishing effect on dimerization of the protein *in vitro*. These results are in agreement with previous NMR and MD simulation studies. However, our results obtained in a collaborative work in corresponding experiments using cell culture systems showed no major effects of this mutation on dimerization *in cellulo*, indicating a more complex nature for the mechanism of effect of G126V in protecting against disease at cellular level.

Taken together, the system we developed together with the *p*Bpa-variants of mPrP that we created here, are useful in the study of interactions and interaction surfaces of the PrP. Thus, it may also prove as a useful tool in studies of other interactions of the prion protein, such as, it may facilitate to gain further structural insights into its oligomeric and fibrillar species, including the pathological variants of prion, and/or it may also aid in PrP's protein binding-partner studies.

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