The Structural Basis of Prion Disease Susceptibility and the Transmission Barrier

by

Braden Sweeting

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Medical Biophysics
University of Toronto

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2013

Abstract

When prions are transmitted between species, there can be a delay in pathogenesis due to a phenomenon referred to as the transmission barrier. Some species also show very low susceptibility to prion disease. In this study I hypothesized that the susceptibility of species to prion disease is proportional to the tendency of their endogenous prion protein, PrP, to adopt the β-state, an oligomeric form of misfolded recombinant PrP that is rich in β sheet.

Using a novel method of two-wavelength CD analysis, it could be shown that recombinant PrP from prion-susceptible species have a higher propensity to refold to the β-state than resistant species. The crystal structure of rabbit PrP$^{C121-230}$ revealed a helix-cap motif at the N-terminus of helix-2 that contributes to the reduced β-state propensity of rabbit PrP.

Single amino acid changes in the sequence of PrP can lead to a transmission barrier and/or resistance in species. Mutating single residues in rabbit PrP$^{C}$ to those found in...
corresponding positions in hamster PrP\textsuperscript{C}, ablated the helix-cap observed in the wild-type and caused an increase in the β-state propensity of rabbit PrP. Conversely, a decrease in β-state propensity was observed when rabbit mutations were introduced into PrP of hamster, a susceptible species.

A dimeric association is hypothesized to be involved in the function of PrP and/or the conversion mechanism to infectious prion. In the structures of the wild-type and mutant rabbit PrP\textsuperscript{C}s a dimeric arrangement was observed in the asymmetric unit of the crystals. Using 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), a dimer of rabbit and hamster PrP\textsuperscript{C} was crosslinked in solution. The dimer crosslink was specific and dependent on the tertiary structure of PrP\textsuperscript{C}. Crosslinking of the β-state octamer with EDC showed that similar contacts may be present in this oligomeric form.

Together these data provide strong evidence that species susceptibility is linked to β-state propensity.
Acknowledgments

Many people contributed to my research and help me in my work and home life throughout my studies. First and foremost my supervisor, Dr. Pai, who provided an excellent environment to excel, mentorship when needed and space to explore interests, no matter how far fetched. Also, my co-supervisor, Dr. Chakrabartty, for an alternate perspective, speculative rap sessions and good company at conferences. I would also like to acknowledge committee members Drs Julie Forman-Kay and John Glover for their invaluable input during committee meetings.

A special thanks to all the members of both the Pai and Chakrabartty labs, both past and present. Former Pai lab members Drs. Shekeb Khan and Jian Payandeh for their guidance and feedback and Terence To for his help and assistance, Kim Hed and Eric Brown for their diligent work as research students. Thanks to current lab members Ondrej Halgas, Natasha Kruglyak, Angela Lee and Pedram Mehrabi for all their help, support and gummi bears while I was writing up. Also, thanks to Qasim Khan and Sylvia Ho from the Chakrabartty labs for the great collaborations.

Also, a big thank you to my entire family, especially my parents Dave and Sheila for the all of the love, encouragement and support from the early years catching snakes to later years of helping pay the tuition bills. Also my parents-in-law Craig and Sue for weekends away and baby sitting on demand.

Lastly and most importantly, I couldn’t have done any of this without my wife, Nicole. Nothing I can say here can every thank you enough. Our adventure in graduate school has been one that we went through together which made it all the more fun and challenging. In the end, aside from our doctorates, we ended up with the biggest price of all, our daughter, Libby. I can’t wait to see where the next adventure takes us.
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<tr>
<td>ANS/bisANS</td>
<td>4-4’-Bis(1-anilinonaphthalene 8-sulfonate)</td>
</tr>
<tr>
<td>BSE</td>
<td>Bovine Spongiform Encephalopathy</td>
</tr>
<tr>
<td>CD</td>
<td>Circular Dichroism</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob Disease</td>
</tr>
<tr>
<td>CWD</td>
<td>Chronic Wasting Disease</td>
</tr>
<tr>
<td>Δ/CR</td>
<td>PrP central region (residues 100-125)</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>DRM</td>
<td>Detergent Resistant Microdomain</td>
</tr>
<tr>
<td>EDC</td>
<td>1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>ESI - MS</td>
<td>Electron Spray Ionization Mass Spectrometry</td>
</tr>
<tr>
<td>FFI</td>
<td>Fatal Familial Insomnia</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infrared Raman spectroscopy</td>
</tr>
<tr>
<td>GPI Anchor</td>
<td>Glycosyl Phosphate Inositol anchor</td>
</tr>
<tr>
<td>GSS</td>
<td>Gerstmann-Sträussler-Scheinker Syndrome</td>
</tr>
<tr>
<td>ML</td>
<td>Maximum Likelihood</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RiboNucleic Acid</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular Weight Cut Off</td>
</tr>
<tr>
<td>NCAM</td>
<td>Neural Cell Adhesion Molecule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NCS</td>
<td>Non-Crystallographic Symmetry</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-Aspartate</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>PK</td>
<td>Proteinase K</td>
</tr>
<tr>
<td>PrP</td>
<td>Prion Protein (or protease resistant protein)</td>
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<tr>
<td>PrP&lt;sup&gt;C&lt;/sup&gt;</td>
<td>Cellular form of prion protein</td>
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<td>Proposed toxic form of PrP</td>
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<td>Transmissible Spongiform Encephalopathy</td>
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<td>United Kingdom</td>
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<tr>
<td>vCJD</td>
<td>Variant CJD</td>
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Chapter 1

Introduction

The content of this chapter has been adapted from:


"Structural factors underlying the species barrier and susceptibility to infection in prion disease."

Sweeting, B.\textsuperscript{1,3}, Khan, M.Q.\textsuperscript{1,2}, Chakrabartty, A.\textsuperscript{1,2,3}, Pai, E.F.\textsuperscript{1,2,3,4}

\textsuperscript{1}Campbell Family Institute for Cancer Research, Ontario Cancer Institute/University Health Network. \textsuperscript{2}Department of Biochemistry, University of Toronto. \textsuperscript{3}Department of Medical Biophysics, University of Toronto. \textsuperscript{4}Department of Molecular Genetics, University of Toronto.

Statement of contribution: B.S. conceptualized and wrote the manuscript. MQK, AC and EFP aided in manuscript writing and editing.
1.1 Introduction

Prion diseases, also known as the transmissible spongiform encephalopathies (TSEs) are a group of fatal, neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD), Gerstmann-Sträussler-Scheinker Syndrome (GSS), Fatal Familial Insomnia (FFI) and Kuru in humans, Scrapie in goats and sheep as well as Chronic Wasting Disease (CWD) in deer and elk, among others. In humans, the majority of TSEs are sporadic (~85%) or inherited as the result of genetic defects (~10%) (Collins et al., 2004).

The remainder of cases are acquired through exposure to infectious material. Some of this infectious material is from human sources through iatrogenic means via blood products, grafts or contaminated equipment that has been obtained from or been in contact with a person infected with a TSE (inherited or otherwise). Prions are very persistent in the environment and extremely difficult to remove (Georgsson et al., 2006).

The final and most terrifying source of prion disease infection is from tainted meat, obtained from infected animals. Due to an effect referred to as the species or transmission barrier, infectious prions from one species of animal generally have some difficulty infecting a new host species. However, when this does occur the results can be disastrous as evidenced by the outbreak of variant-CJD (vCJD) in the UK caused by bovine spongiform encephalopathy (BSE) contaminated beef (Bruce et al., 1997; Hill et al., 1997).

Since the BSE crisis, regulations have been introduced to protect food from exposure to possibly infectious material. Although it currently appears as though the BSE crisis has been contained, prion disease infection threats remain, such as the spread of CWD through the North American Midwest (Williams, 2005) and the possibility of unknown new sources of infection (Yokoyama and Mohri, 2008). Recent research efforts have revealed several aspects of prion disease and especially of the transmission barrier, but a detailed mechanism of how prion disease spreads from one species to another remains elusive. A more in depth knowledge of the source of prion disease, the infectious unit and the transmission barrier are needed in order to insure that future outbreaks can be prevented.
One avenue of gaining understanding is to study species that have an intrinsically low susceptibility or resistance to prion infection. Years of research and analysis of accidental exposures during the BSE outbreak have revealed that several species have comparably low susceptibility to prion disease (Fernández-Borges et al., 2012). Insight into the mechanism of how these species are able to avoid prion infection may allow further discovery into how prion infection, its exchange between species, and perhaps prion disease in general can be prevented.

1.2 The discovery of prions

Creutzfeldt-Jakob disease (CJD) was the first human prion disease to be discovered; it was described by Hans Gerhard Creutzfeldt and Alfons Maria Jakob in 1920-21 (Creutzfeldt, 1920; Jakob, 1921), but its transmissibility remained unknown for several more decades. Scrapie was the most intensely studied of the TSEs in the early 20th century, due to its impact on wool and textiles at the height of the industrial revolution. The first breakthrough in TSE research came with the experimental transmission of scrapie from sheep to goats in 1939 (Cuille and Chelle, 1939) establishing it as the first identified transmissible spongiform encephalopathy.

In humans, CJD was not identified as a TSE until after Carlton Gajdusek successfully performed the first experimental transmission into chimpanzees of Kuru (Gajdusek et al., 1966), a neurodegenerative disease affecting tribes in Papua New Guinea that practiced ritual cannibalism (Gajdusek and Zigas, 1957). The first experimental transmission of CJD to chimpanzees followed shortly after (Gibbs, C.J. et al., 1968).

For many years, the search for an infectious particle of scrapie centered on a hypothesized "slow virus". The gene Sinc was identified as affecting the incubation period of scrapie in mice (Dickinson et al., 1968a). In 1967, J.S. Griffith proposed that rather than a virus, an infectious unit only composed of protein was responsible for scrapie transmission (Griffith, 1967). In his Nobel Prize winning work, Stanley Prusiner provided experimental evidence in strong support of this proposal; he identified a protease-resistant, hydrophobic protein particle that was enriched in scrapie-infected hamster brains (Prusiner et al., 1981). He determined that the host protein PrP was the principal component of these infectious particles and that infectivity could not be attenuated using methods that would kill a contagion containing genetic material (i.e. DNA or
RNA). He then proposed that these were the hypothesized protein-only infectious particles and coined the term “prion” to describe them (McKinley et al., 1983; Prusiner, 1982).

Subsequent study identified that Prnp was the gene encoding PrP and that it was identical to the Sinc gene involved in scrapie incubation times (Chesebro et al., 1985; Oesch et al., 1985). Also it was found that the infectious particle (PrP\textsuperscript{Sc}) and cellular PrP (PrP\textsuperscript{C}) are encoded by the same gene (Basler et al., 1986). The identification of the Prnp gene allowed the production of Prnp\textsuperscript{0/0} mice. It was found that these PrP-lacking mice, when inoculated with infectious material, were resistant to scrapie infection (Büeler et al., 1993; Sailer et al., 1994). This experiment confirmed a crucial tenant of the prion hypothesis; demonstrating that infectious PrP\textsuperscript{Sc} requires endogenous host PrP in order to infect the host.

### 1.3 PrP

Since the discovery of its role in prion disease, a great deal of research has been focused on PrP, its nominal function and structure and the mechanism of prion infection and pathogenesis.

#### 1.3.1 Prnp and PrP expression

The prion protein, or protease-resistant protein (PrP) is found in many different species and is highly conserved among mammals, with close to 90% identity (Wopfner et al., 1999). It is ubiquitously expressed, but is found at its highest levels in the nervous system (Ferrer et al., 2000; Ford et al., 2002a) and at lower levels in the immune system and muscle (Ford et al., 2002b). The human Prnp gene contains two exons, but only one codes for PrP\textsuperscript{C} (Choi et al., 2006) and it is believed that its transcription is controlled via the chromatin structure (Cabral et al., 2002). The PrP transcript mRNA also contains a pseudoknot structure that may contribute to regulation at the translational level (Barrette et al., 2001).

#### 1.3.2 The structure of PrP\textsuperscript{C}

Human PrP is a 209-residue protein, normally tethered to the extracellular membrane via a C-terminal GPI-anchor. After removal of a 22-residue leader sequence, the PrP\textsuperscript{C} fold consists of two domains: a largely disordered N-terminal domain from residue 23 to residue 125 and an ordered domain from residues 126-231 (Fig. 1.1).
Figure 1.1 – The structure of PrP$^C$. Residues 1-22 compose a signal peptide that is removed during processing and directs export to the cell membrane. Immediately following the signal peptide is a small polybasic region from residues 23-27 (orange). Residues 55-95 contain a series of histidine containing octapeptide repeats (black) that are known to bind copper and other divalent cations. There is a positively charged region spanning residues 95-111 (purple) and a hydrophobic domain from residues 111-143 (blue). The N-terminal half of the protein (residues 23-121) is disordered, whereas the C-terminal half (residues 121-230) is ordered and composed of a small, two-stranded $\beta$-sheet and three $\alpha$-helices. Whenever structural features of PrP are mentioned or discussed in this thesis it will refer to the ordered C-terminal half of the molecule, if not specifically stated otherwise.
The structure of the C-terminal ordered domain was first determined from mouse PrP using NMR in 1995 (Riek et al., 1996) and structures from many other species have since been added. All mammalian PrP show a similar structure, consisting of three α-helices and a small two-stranded anti-parallel β-sheet. A disulfide links the second and third α-helices and there are two N-glycosylation sites at N181 and N197.

The N-terminal domain contains a small poly-basic domain from residues 23-27 (Solomon et al., 2011), several octapeptide repeats containing histidine (residues 50-91), which have been shown to bind copper (Brown et al., 1997a), as well as a stretch of highly conserved hydrophobic amino acids from residue 112 to residue 130 that has been implicated as a possible transmembrane domain (Hegde et al., 1998). Interestingly, if this domain is deleted it generates a lethal phenotype (Li et al., 2007).

Expressed PrP<sub>C</sub> is directed to the endoplasmic reticulum by its signal peptide where the N-linked oligosaccharides and GPI-anchor are added. After modifications of the oligosaccharides in the Golgi, the PrP<sub>C</sub> is exported to the cell surface where it primarily resides in detergent resistant micro domains (DRM, also known as lipid-rafts or caveolae) (Peters et al., 2003). PrP<sub>C</sub> has a half-life of approximately 5 hours (Yedidia et al., 2001) and cycles between surface and early endocytic pathway via caveolae-containing endocytic structures or clathrin-coated pits depending on the cell type (Peters et al., 2003; Shyng et al., 1995).

1.3.3 The function of PrP<sub>C</sub>

Although the role of PrP in prion disease has been well characterized, its putative physiological function still remains a topic of intense research. The ubiquitous expression and conservation of the gene sequence between species suggests an important function, but attempts to clarify its role have identified several properties of the protein in the cell but no "cut and dry" essential function.

In efforts to determine the function of PrP, several PrP knockout mice have been produced but initial observations showed that the absence of PrP had little to no effect on mice aside from rendering them immune to prion disease infection (Büeler et al., 1993; Manson et al., 1994). However, closer study has led to further clues into the function of PrP. Although knock-outs did not display any gross developmental or morphological abnormalities, some more subtle electrophysiological changes have been discovered suggesting that PrP is necessary for normal
synaptic function (Collinge et al., 1994; Maglio et al., 2004; Mallucci et al., 2002) as well as myelin maintenance (Bremer et al., 2010).

Early research efforts showed that PrP<sub>C</sub> binds copper ions via the octapeptide repeats in the N-terminal region (Brown et al., 1997a); it also has lower affinity for other metal ions such as Ca<sup>2+</sup> and Zn<sup>2+</sup> (Brown et al., 2000; Jackson et al., 2001). Binding of metal cations enables PrP to play a protective role against oxidative stress (Brown et al., 1997a, 1997b) and it is believed that PrP may prevent the formation of reactive oxygen species by binding copper that, if free in the cytosol, is known to generate free radicals of oxygen. Also, PrP may be involved in transport of copper into the endosomal pathway as the affinity of the octapeptide repeats for copper is lower under the conditions present in endosomal vesicles (Miura et al., 1999; Pauly and Harris, 1998).

Changes in the expression levels of PrP<sub>C</sub>, ligand binding by PrP<sub>C</sub> and crosslinking of PrP<sub>C</sub> by antibodies have all been shown to alter signal transduction pathways within the cell [for review see (Linden et al., 2008)]. Transmembrane signal transduction by PrP appears to be dependent on its interaction with caveolin (Mouillet-Richard et al., 2000) possible with NCAM as an intermediary (Santuccione et al., 2005). PrP engagement via the above mechanisms has been shown to activate the MAPK (Chiarini et al., 2002) and cAMP/PKA pathways (Martins et al., 1997) as well as non-receptor kinase including Fyn and Lck (Mouillet-Richard et al., 2000) resulting in changes in cell proliferation, differentiation and survival responses. These results show that PrP plays an important role in cell signaling, but further study is needed to identify the specific effects of PrP<sub>C</sub>-mediated-signaling.

Some experiments have also shown that PrP may protect against excitotoxic stress in neurons. Removing a 20 amino acid stretch from the N-terminal disordered domain of PrP (105 - 125, ΔCR PrP) causes mice to develop degeneration of cerebellar granule neurons, a typical symptom of excitotoxic stress. Cells expressing this construct or point mutations in the deleted region display spontaneous ionic currents suggesting that PrP may be involved in the formation of membrane channels (Solomon et al., 2010, 2011). The effects of ΔCR PrP can be silenced by co-expression of WT PrP, and over-expression of PrP has been shown to protect cell lines from apoptosis suggesting that the function of normal PrP may be to serve a neuroprotective role (Kim et al., 2004; Shyu et al., 2005).
This neuroprotective role may coincide with a role in neuronal development and interactions with neurotransmitters as PrP<sup>C</sup> also co-localizes with synaptic sites (Moya et al., 2000). PrP<sup>0/0</sup> mice show subtle abnormalities in cerebellar and hippocampal neurons, suggesting that PrP<sup>C</sup> may be necessary for normal neuronal development (Kanaani et al., 2005). This effect may be due to irregular activation of the NMDA receptor, which has enhanced activity in PrP<sup>0/0</sup> mice (Khosravani et al., 2008). Excitotoxicity occurs in PrP<sup>0/0</sup> mice as aberrant activity of the NMDA receptor allows entry of Ca<sup>2+</sup> ions into the cell causing nerve cell damage and death. Co-expression of WT-PrP<sup>C</sup> reverses this effect, allowing PrP<sup>C</sup> to interact with the NR2D subunit of the NMDA receptor and suppresses excitotoxicity (Khosravani et al., 2008).

PrP localizes on the cell surface in lipid rafts, suggesting a possible role in cell adhesion (Schmitt-Ulms et al., 2001). The protein also interacts with surface proteins such as the neural cell adhesion molecule (NCAM)(Schmitt-Ulms et al., 2001) laminin and the laminin receptor precursor (Graner et al., 2000; Rieger et al., 1997). Recent work in zebrafish has provided further evidence that PrP plays an important role in neuronal growth and differentiation (Málaga-Trillo et al., 2009).

### 1.4 Pathogenesis of prion disease.

The primary event in prion disease pathogenesis is the conversion of normal host PrP<sup>C</sup>, which is monomeric and primarily α-helical in structure, to an aggregated form rich in β-sheet secondary structure. PrP<sup>res</sup> is used as a generic term for the infectious unit of any prion disease, whereas specific forms are annotated with their associated disease i.e. PrP<sup>CJD</sup> for CJD and PrP<sup>Sc</sup> for scrapie. PrP<sup>res</sup> often accumulates in amyloid plaques and is accompanied by spongiform changes (vacuolation) in the brain, neurodegeneration and death (Collins et al., 2004). Although there are some similarities, the transmissibility of prion disease is what sets it apart from other neurodegenerative diseases that involve the accumulation of aggregated host proteins (Alzheimer's, Parkinson's, etc.). However, some recent evidence has suggested some others may also be infectious (Morales et al., 2012; Stöhr et al., 2012).
1.4.1 Conversion of $\PrP^C$ to $\PrP^{res}$

It is hypothesized that $\PrP^{res}$ transmits prion disease by interacting with the host $\PrP^C$ and directing its conversion to $\PrP^{res}$. The amino acid sequences of $\PrP^C$ and $\PrP^{res}$ are exactly the same and expression levels of PrP do not change in diseased brain (Oesch et al., 1985). Instead, the disease state involves conformational changes in PrP rather than covalent modification.

There are currently two competing hypotheses for the mechanism of conversion from $\PrP^C$ to $\PrP^{res}$. The “template-directed misfolding” hypothesis proposes that a large free energy barrier separates the conformations of $\PrP^C$ and $\PrP^{res}$. It is then proposed that the infectious form can associate with $\PrP^C$ to catalyze its conversion to $\PrP^{res}$, thus propagating the infectious form. The other hypothesis, termed “seeded nucleation”, proposes that the conformations $\PrP^C$ and $\PrP^{res}$ are in equilibrium with each other, with the equilibrium constant greatly favouring the normal cellular form. In this case, the infectious unit is composed of a stable nucleus of $\PrP^{res}$, which is capable of recruiting and converting additional $\PrP^C$; the nucleus upon growth can fragment to form new nuclei, which, in turn, will propagate the disease further. The major difference between these two hypotheses is that in “seeded nucleation”, the infectious conformation is ubiquitous and the formation of a stable nucleus happens only occasionally whereas in “template-directed misfolding” the conformational change itself is a rare event. To date, there is some experimental evidence to support both of these hypotheses, but it is insufficient to unequivocally support one hypothesis over the other.

Under normal conditions $\PrP^C$ is degraded by the proteasome with a half-life of 5 hours and misfolded PrP conformers are not detected. Once converted, $\PrP^{res}$ becomes relatively insoluble and protease-resistant (Meyer et al., 1986). Thus, $\PrP^{res}$ is able to sustain and propagate itself by converting additional $\PrP^C$. The cellular location of conversion of $\PrP^C$ to $\PrP^{res}$ is suspected to be on the cell surface in detergent resistant microdomains (DRMs), or in the endosomal pathway (Caughey et al., 1991a). However, some inherited prion disease mutations such as familial CJD cause PrP to accumulate in the endoplasmic reticulum (ER) and cytoplasm in aggregates with properties similar to $\PrP^{res}$ when the proteasome is inhibited (Ivanova et al., 2001).
1.4.2 The structure of PrP\textsuperscript{res}

Although the fold of the ordered domain of PrP\textsuperscript{C} has been well characterized, there is a lack of detailed structural knowledge of PrP\textsuperscript{res}. The aggregated and heterogeneous nature of PrP\textsuperscript{res} from natural sources, together with the inability to produce uniform infectious prions \textit{in vitro} makes it difficult to obtain samples suitable for high-resolution structural studies. PrP\textsuperscript{res} appears to have a structured core domain that is protease resistant and composed of the C-terminal region of PrP from residues 80-90 to the C-terminus (Bolton et al., 1982; Prusiner et al., 1984). However, it must be noted that the degree of protease resistance varies and protease-sensitive PrP\textsuperscript{Sc} has been isolated from scrapie-infected brains (Pastrana et al., 2006), implying that protease resistance may not be a necessary property for infectivity.

Low-resolution optical methods have shown that PrP\textsuperscript{res} has an increased proportion of β-sheet secondary structure but with some remaining α-helical structure as shown by infrared Fourier transform spectroscopy and circular dichroism (Caughey et al., 1991b; Pan et al., 1993). X-ray diffraction studies of two-dimensional crystals formed from purified mouse PrP\textsuperscript{Sc} show the characteristic diffraction pattern of an amyloid cross-β core structure where β-sheet strands run perpendicular to the axis of the fibril (Sunde et al., 1997) similar to the cross-β structures found in yeast prions (King and Diaz-Avalos, 2004; Tanaka et al., 2004).

Fibrils of PrP\textsuperscript{res} can be quite large, but there is compelling evidence that the minimal infectious unit may be smaller oligomers of PrP\textsuperscript{res}. Infectivity is preserved when PrP\textsuperscript{res} is partially disaggregated (Riesner et al., 1996) and the highest relative infectivity resides in particles composed of 14-28 PrP protomers (Silveira et al., 2005).

In the absence of high-resolution data, several structural models have been produced based on available biochemical data, molecular dynamics simulations and low-resolution methods, such as amyloid fiber diffraction, electron microscopy reconstructions (DeMarco and Daggett, 2004; Govaerts et al., 2004). The β-helical model (Fig. 1.2 A) is based on the results of x-ray diffraction experiments on two-dimensional crystals of partially protease digested and purified mouse PrP\textsuperscript{Sc} (Govaerts et al., 2004; Wille et al., 2002). It proposes that the N-terminal portion of the PrP\textsuperscript{res} protease resistant domain (residues 89-175) is in a β-sheet conformation forming a central cross-β helix, while the secondary structure of helices 2 and 3 are largely preserved on the outside of the cross-β helix allowing for glycosylation. The cross-β helix portion was
modeled by threading the proposed β-sheet portion of PrP\textsuperscript{res} onto the β-helical portion of the structure of streptococcal urydiltransferase and modeled into a trimer to conform to the three-fold symmetry observed from the x-ray diffraction data of the two-dimensional mouse PrP\textsuperscript{Sc} crystals. The resulting model bears a resemblance to the structures of fungal prions such as HET-s (Wasmer et al., 2008).

Another model, the β-spiral model (Fig 1.2 B), is derived from molecular dynamics simulations with monomeric PrP\textsuperscript{C} as a starting point combined with structural constraints from biochemical observations of PrP\textsuperscript{res} (DeMarco and Daggett, 2004). In general, the model retains a large portion of the native PrP\textsuperscript{C} ordered domain structure, but the molecular dynamics simulations suggested that two short β-strands of PrP\textsuperscript{C} elongate into a larger β-sheet with a new strand formed from the loop connecting β-strand-1 and helix-1. The filament protomers interact along these new β-strands fitting into a trimeric arrangement. The trimers then associate forming a spiral around the filament axis with the β-strands at a 45º angle relative to the fibril axis and spiraling along the axis. The orientation of the β-strands relative to the filament axis does not agree with x-ray diffraction data and with studies on recombinant PrP fibrils that both indicate that the β-strands of PrP\textsuperscript{res} run perpendicular to the filament axis.

As an alternative to PrP\textsuperscript{res} isolated from natural sources, bacterially recombinant PrP can be induced to form amyloid fibrils with properties similar to PrP\textsuperscript{res} (Apetri and Surewicz, 2002; Baskakov et al., 2002, 2004; Bocharova et al., 2005). Experiments with recombinant PrP fibrils using hydrogen deuterium exchange and site-directed spin labeling have shown that the region of residues 160-170 to 220 forms a parallel, in-register cross-β structural core (Fig. 1.2 C) (Cobb et al., 2007; Lu et al., 2007). Solid state NMR measurements of recombinant PrP amyloid fibrils further support such an arrangement (Tycko et al., 2010). Additionally, crystallographic studies of short peptide segments from amyloidogenic proteins such as PrP among others, show that they form parallel and anti-parallel cross-β structures with self-complimentary interfaces that exclude water, called steric zippers (Sawaya et al., 2007). In the absence of data from infectious prions of natural sources, the properties of recombinant PrP can tell us much about the structural characteristics and behavior of ordered PrP aggregates.
Figure 1.2: Proposed models of PrP<sup>res</sup>. (A) The β-helical model (Govaerts et al., 2004). Protomers were modelled by threading residues 89-174 of PrP<sup>C</sup> onto the structure of the beta-helical portion of uridintransferase (GlmU) from Streptococcus pneumonia. Protomers were then modelled into a trimer based on symmetry observed from electron micrographs of two-dimensional crystals of mouse PrP<sup>Sc</sup>. (B) The β-spiral model (DeMarco and Daggett, 2004). Molecular dynamics simulations incorporating biochemical restraints were used to model the two short strands of the PrP<sup>C</sup> β-sheet associating with a third strand formed from the random coil between β-strand 1 and helix-1. The protomers then interact along these new β-strands into a trimeric arrangement along the filament axis. (C) Parallel, in-register β-sheet model (Cobb et al., 2007). Site-directed spin labeling and EPR were used to probe the molecular architecture of amyloid formed from recombinant PrP. Data showed that a core of residues ~160-220 made of single molecule layers with parallel, in-register alignment of β-strands makes up PrP fibrils.
1.4.3 The β-oligomer

Although infectivity in prion disease has been traditionally associated with large aggregates and amyloid formed from PrP, recent evidence suggests that smaller aggregates (non-fibrillar, 20-50 monomers) and/or oligomers (< 20 monomers) may also play the more important role. Toxic soluble oligomers have been implicated in other misfolding diseases including Alzheimer’s Disease (Haass and Selkoe, 2007) and it has been hypothesized that a misfolded oligomeric form or forms may be crucial for the conversion of PrP$^C$ to PrP$^{res}$ and/or the mechanism of pathogenesis (Prusiner, 1998). By varying solution conditions, recombinant PrP can be induced to form different oligomeric states, all with enriched β-sheet content (Hornemann and Glockshuber, 1998; Swietnicki et al., 1997). Although the solution conditions that produce these in vitro misfolded forms are not fully representative of native PrP in its in vivo environment, they still provide valuable insight into the folding behaviour of PrP, e.g. through the elucidation of folding conformations available to the protein.

PrP$^C$ can be made amyloidogenic by simply reducing the disulfide bond that links its helices 2 and 3 (Jackson et al., 1999). Although of general interest, this disulfide reduction is unlikely to be involved in the conversion mechanism as it has been shown that the PrP disulfide bridge most likely remains intact (Welker et al., 2002). With the disulfide intact, PrP can also refold into a β-sheet rich, oligomeric form (β-oligomer, β$^O$) with properties (ANS binding, partial protease resistance) similar to those of PrP$^{res}$ (Baskakov et al., 2002). The conditions conducive to such a transformation are low pH (<4.5), mildly denaturing conditions (3 M - 5 M urea) and moderate ionic strength of the buffer (Morillas et al., 2001; Swietnicki et al., 2000), conditions supposedly found in the late endosome or lysosome, both sites proposed as locations where the conversion of PrP$^C$ to PrP$^{Sc}$ could be triggered (Hornemann and Glockshuber, 1998).

According to ESI MS, the β-oligomer has a molecular mass of approximately 130 kDa, suggesting an octameric arrangement (Baskakov et al., 2002) whereas other techniques indicate a 10-mer arrangement (Tahiri-Alaoui et al., 2006). It appears to be formed by an ordered core of the PrP residues 126-228 leaving the N- and C-termini of the subunits disordered as determined by NMR experiments (Gerber et al., 2007, 2008a). Kinetic analysis of oligomer formation aligned assembly of the oligomer with the conformational change towards increased β-sheet
content. The process should be under kinetic control given that the \(\alpha\)-helical and \(\beta\)-oligomer forms are only separated by a free energy barrier which can be overcome simply by mildly denaturing conditions (Baskakov et al., 2001; Swietnicki et al., 2000). Recombinant mouse PrP 89-231 can also form amyloid-like fibrils that appear to be off pathway from the formation of \(\beta\)-oligomers (Baskakov et al., 2002). Interplay between these two pathways may be important in the pathogenesis of prion disease (Baskakov et al., 2002). It still remains controversial whether the \(\beta\)-oligomer or fibrils produced from recombinant PrP are completely valid representations of PrP\text{res}. Oligomers and amyloid produced at low pH and moderately denaturing conditions are both toxic (Novitskaya et al., 2006) and, in at least one controversial case, have led to a report of synthetic prions with low infectivity (Baskakov and Breydo, 2007; Legname et al., 2004).

Another point in supporting the relevance of recombinant \(\beta\)-oligomer and amyloid for drawing conclusions about the \textit{in vivo} systems is that their formation responds to polymorphisms and species differences in PrP sequences. The M/V polymorphism at amino acid 129 in human PrP, which is a determinant in prion disease susceptibility, does not affect the structure or stability of PrP\text{C} but does appear to influence its ability to self-associate. When the rates of \(\beta\)-oligomer formation were tested for the M129 and V129 variants of recombinant human PrP 90-231 it was found that the M129 variant forms oligomers faster and matures more quickly into \(\beta\)-sheet rich oligomers (Tahiri-Alaoui et al., 2004); the V129 variant is faster in forming the off-pathway amyloid-like fibrils (Baskakov et al., 2005). Atomic force microscopy revealed that \(\beta\)-oligomers can build stacks of two or three oligomers, the heights of which are affected by the polymorphism at position 129. The M129 PrP variant formed the highest proportion of these stacked oligomers (Gerber et al., 2008b). When one considers that methionine at position 129 of PrP promotes the formation and stacking of \(\beta\)-octamers and that human M129/M129 PrP homozygotes are the ones most susceptible to prion disease (Palmer et al., 1991) it is tempting to suggest that formation of the octamer is related to susceptibility to prion disease.

1.4.4 \textit{The neurodegenerative mechanism}

Although a detailed knowledge of the structure of the infectious form of PrP\text{res} would give valuable insight into how prion disease is transmitted, there is a lot of recent evidence that suggests that PrP\text{res} may not be directly responsible for neurodegeneration in prion disease.
Interestingly, although PrP\textsuperscript{res} is postulated as the infectious unit of prion disease, \textit{in vitro} toxicity does not correlate with appearance of PrP\textsuperscript{res} (Ma et al., 2002). A great deal of insight has been gained into the pathological mechanism from studying mutations of PrP that are known to lead to disease states. Some inherited forms of prion disease are not transmissible and do not accumulate protease-resistant aggregates (Brown et al., 1994; Piccardo et al., 2001). There are also cases that develop abundant PrP\textsuperscript{res}, but with no neuropathological changes (Hill et al., 2000; Race et al., 2001). This suggests that infectivity and neurodegeneration have separate mechanisms and that PrP\textsuperscript{res} may be involved with transmission, but not with pathogenesis.

The discovery that \textit{Prnp}\textsuperscript{0/0} mice are immune to prion disease indicated that endogenous PrP\textsuperscript{C} acts as a mediator for toxic effects of prion disease (Büeler et al., 1993). Also, the neurotoxic effects of prion disease require the expression of GPI-anchored PrP. Mice expressing anchorless PrP can propagate infectivity but do not develop neurodegenerative symptoms (Chesebro et al., 2005). It has been hypothesized that the neurodegenerative mechanism may involve a loss function of PrP (Biasini et al., 2012). Although the exact function of PrP is not known, PrP null mice do not display any prion disease phenotypes so it is unlikely that pathogenesis is due to a loss of function (Büeler et al., 1993).

Some N-terminal mutations of PrP have been shown to cause incorporation of PrP into the plasma membrane \textit{via} a central hydrophobic stretch of amino acids (Hegde et al., 1998). Transmembrane PrP causes neurotoxic effects and it has been proposed that the neurodegenerative mechanism of PrP may involve transmembrane PrP. However, there has been no observed increase in the levels of transmembrane PrP in prion disease states, as one would expect if it was responsible for neurotoxic effects (Stewart and Harris, 2003).

The hydrophobic portion (CR) of the N-terminus of PrP has shown some interesting properties that suggest that it is involved in the neurodegenerative mechanism of PrP. It was demonstrated 15 years ago that expression of truncated PrP\textsuperscript{C} 121-231, lacking the disordered N-terminal portion, would lead to ataxia and degeneration of cerebellar granule neurons (Shmerling et al., 1998). More recently, further investigation has shown that deletion of a highly conserved central portion of PrP, residues 105-125 (PrP\textsuperscript{C} ΔCR), causes similar neurotoxic effects that can be rescued by co-expression with wild-type PrP\textsuperscript{C} (Li et al., 2007). These observations suggest that the CR region of PrP interacts with an as yet undiscovered molecular target, which when bound...
to ΔCR PrP causes a neurotoxic signal. Binding of the CR to this target in wild-type PrP \(^C\) suppresses this neurotoxic signal. The neurotoxic mechanism of PrP \(^{res}\) may act by subverting this neurotoxic signal function of PrP. Analysis of PrP \(^{res}\) has shown that it is altered in the CR region (Peretz et al., 1997) and may only be able to interact with the target via the C-terminal domain of PrP, eliciting the toxic response. Interestingly, none of the ΔCR deletion mutations lead to the formation of infectious PrP \(^{res}\) but neuropathological symptoms similar to infectious prion diseases, a further suggestion that infectivity and pathogenesis may be separate processes in prion disease (Biasini et al., 2012).

If PrP \(^{res}\) were to invoke the neurotoxic capabilities of PrP \(^C\), it would be expected that appearance of PrP \(^{res}\) itself would be enough to trigger pathogenesis and clinical symptoms. Furthermore, there are reported cases in which prion disease pathogenesis occurs with low levels of PrP \(^{res}\) (Collinge et al., 1995; Hsiao et al., 1990). However, PrP \(^{res}\) can also accumulate without pathogenesis in cases of subclinical infection. For example, heterozygous Prnp \(^{+/-}\) mice expressing 50% of the wild-type level of PrP have a delayed pathogenesis but develop a comparable amount of PrP \(^{res}\) (Collinge and Clarke, 2007). Conversely, tga20 mice that express 10-fold more PrP than normal mice succumb to the disease relatively quickly compared to wild-type (Fischer et al., 1996).

To satisfy these observations, an intermediate state in the conversion of PrP \(^C\) to PrP \(^{res}\). PrP \(^L\) has been proposed to be the toxic species in prion disease (Fig. 1.3) (Collinge and Clarke, 2007). In this model, rather than cause toxicity itself, PrP \(^{res}\) acts as a “catalytic surface” for the formation of PrP \(^L\) and the resulting toxicity. PrP \(^L\) subsequently “matures” into infectious, albeit non-toxic, PrP \(^{res}\). In this model, toxicity is governed by the rate of accumulation of PrP \(^L\) by conversion of PrP \(^C\) after interaction with PrP \(^{res}\). This rate of accumulation (\(k_1\)) is affected by the efficiency of conversion of host PrP \(^C\) by PrP \(^{res}\) and the rate of proteolytic clearance by the cell. The extent of PrP \(^{res}\) accumulation is determined by the rate of maturation of PrP \(^L\) into PrP \(^{res}\). In cases of subclincal infection, a slow rate of PrP \(^L\) accumulation (\(k_1\)) and fast rate of maturation (\(k_2\)) would lead to delayed toxicity from a low level of PrP \(^L\), but a normal accumulation of PrP \(^{res}\). Instances of rapid pathogenesis would be the result of a fast rate of PrP \(^L\) accumulation (\(k_1\)) due to any combination of efficient PrP \(^C\) conversion, high concentration PrP \(^C\) or slow clearance of PrP \(^L\).
Although PrP\textsuperscript{L} is proposed as the toxic entity in prion disease, it is unknown how such a toxic species causes cell death in prion disease. Toxicity may occur due to sustained translational repression caused by the accumulation of misfolded PrP. Rising levels of misfolded proteins cause the transient shut down of protein translation via the phosphorylation of eukaryotic translation initiation factor 2 (eIF2). It has been shown that during prion replication in neuronal cells, eIF2 becomes phosphorylated prior to cell death and can be rescued by dephosphorylation of eIF2 by GADD34 a specific eIF2 phosphatase (Moreno et al., 2012).

Alternatively, cell death may be the result of glutamate-induced excitotoxicity. Monitoring with whole cell-patch clamping has shown that expression ofΔCR-PrP induces large, spontaneous inward ionic currents (Solomon et al., 2010). These ionic currents sensitize neurons to glutamate-induced excitotoxicity via calcium mediated death. A similar mechanism may be induced by β-oligomers (Biasini et al., 2013).

1.5 The species barrier and strains

For decades since their discovery, prion diseases had been diseases of animals and livestock, except for some notable exceptions. However, in the United Kingdom in 1986, a new TSE was recognized in cattle which previously had not shown to be susceptible to prion diseases (Wells et al., 1987). Fears quickly arose that this disease could spread to humans through tainted beef, and these fears were confirmed in 1995 when Stephen Churchill became the first victim of a new TSE, variant-CJD, which could transmit zoonotically from cows to humans (Will et al., 1996). Besides humans, several other species contracted vCJD through contaminated feed including several species of felines, whereas other species that were exposed appeared immune, such as canines (Aldhous, 1990a; Polymenidou et al., 2008) in what was an accidental demonstration of the phenomenon referred to as the prion disease species barrier.

1.5.1 The species barrier

The prion disease species barrier occurs when prions from a donor species are transmitted to a different recipient species and an uncharacteristically long incubation period before the onset of symptoms is observed in the recipient species. If prions are then re-isolated from the recipient
host and re-transmitted to another host of the recipient species, the incubation period is greatly reduced and the prions are said to have “adapted”. The first experimental transmission of a TSE between species was performed with scrapie between sheep and goats in 1939 (Cuille and Chelle, 1939). However, the first description of a species barrier was reported by Gajdusek with the experimental transmission of Kuru from humans to chimpanzees. Kuru was a disease that affected cannibalistic Fore tribes of Papua and after its first description was suspected to be a TSE but several attempts at experimental transmission failed. Gajdusek hypothesized that a longer incubation time was necessary and he was successful in transmitting Kuru to chimpanzees (Gibbs, C.J. et al., 1968). Shortly thereafter, CJD was also transmitted to chimpanzees in a similar manner demonstrating that it also was a TSE (Gibbs, C.J. et al., 1968).

Shortly after the discovery of the species barrier, further experiments were performed with a myriad of other species to test their susceptible to various TSEs. These transmission experiments revealed that some animals such as rabbits and guinea pigs display intrinsically very low susceptibility to prion infection (Gibbs and Gajdusek, 1973) in strong contrast to other species such as hamsters and bank voles, which are unusually susceptible (Nonno et al., 2006; Prusiner et al., 1990).

After the discovery by Prusiner and colleagues that the principal component of infectious prions is PrP, PRNP was identified as the gene that encodes PrP. Research into the source of the species barrier revealed that single base pair changes in the sequence of PRNP, resulting in single amino acid differences in PrP, are sufficient to create a barrier between donor and receptor species (Priola et al., 1994; Prusiner et al., 1990). Subsequently, Vorberg et al. showed in mouse neuroblastoma cells that resistance to PrPSc conversion could be conferred when a single residue in mouse PrP (a susceptible species) was changed to the amino acid found at the corresponding position in the sequence of PrP from rabbits (a resistant species) (Vorberg et al., 2003). Thus, single amino acid changes exert similar effects on the species barrier and the susceptibility to prion disease suggesting that the two aspects may share similar mechanisms.
Figure 1.3 – The kinetic selection model of PrP conversion. Rather than acting itself as the toxic unit, PrP\text{res} acts as a catalytic surface where PrP\text{C} is converted into the (perhaps oligomeric) toxic form, PrP\text{L}. PrP\text{C} first interacts with the surface of PrP\text{res} and is then converted to PrP\text{L} in a reaction with the rate constant k1. PrP\text{L} then matures and is further converted to produce additional PrP\text{res} with the rate constant of k2. Kinetic selection of a pathogenic prion occurs when the rate constants k1 and k2 are such that they allow the accumulation of the toxic species, PrP\text{L}. If the conversion rate is too slow or the maturation rate is too fast, then the concentration of PrP\text{L} never reaches its toxic threshold and sub-clinical infection may occur.
1.5.2 Prion strains

Further confounding the observations of the species barrier, identical animals infected with prions from different sources can develop varying pathologies, which upon passage to new hosts of the same species will maintain their biochemical properties and clinical outcomes. Infectious prions that present and maintain different pathologies when passaged to a new host are referred to as prion "strains" (for review see (Aguzzi et al., 2007)). Prion strains were first described by Pattison and Millson in 1961, who observed that transmission of infectious prions into two goats resulted in two different pathologies, termed "scratching" and "drowsy" (Pattison and Millson, 1961).

Normally, strains of disease refer to those of different genetic make-up but prions do not contain any genetic material. Rather experiments with FTIR, atomic force microscopy and conformational immunoassay have shown that prion strains are the result of alternative stable conformations of PrP\textsuperscript{Res} that can be faithfully propagated in new hosts (Bartz et al., 2000; Peretz et al., 2001). The differences in these conformations result in differences in resistance to proteinase-K digestion and electrophoretic mobility after PK digestion (Bessen and Marsh, 1992a; Collinge et al., 1995), varying glycosylation patterns (Parchi et al., 1996), sedimentation velocities and resistance to urea and guanidinium hydrochloride denaturation (Safar et al., 1998).

The combination of different hosts and prion strains can also result in different pathologies and clinical symptoms. The most common variation between strains is in the time between inoculation and symptom onset, referred to as the incubation period, which is often reproducible within hosts of the same species (Bessen and Marsh, 1992b; Bruce, 1993). Differences in the location and scale of vacuolation damage in the brain are also common and can be consistently scored using a lesion profile (Fraser and Dickinson, 1973). Strains can also result in differing clinical symptoms such as motor-incoordination, ataxia, rough coat and hunch (Bruce et al., 1991; Dickinson et al., 1968b) as well as dementia, depression and insomnia in humans but these symptoms are often variable and inconsistent (Dell’Omo et al., 2002).

These variations in pathologies and biochemical properties between prion strains can and have been used to effectively classify and track prion strains through intra- and inter-species
transmission (Bessen and Marsh, 1992a; Bruce, 1993) using classification systems such as that by Hill (Hill et al., 2003) or Gambetti (Gambetti et al., 2011). For example, after the first cases of vCJD in humans, comparisons of the glycosylation and PK digestion patterns as well as lesion profiles led to the conclusion that these cases were not the result of sporadic CJD but were indeed a new form of CJD resulting from inter-species transmission of BSE (Bruce et al., 1997).

1.5.3 The transmission barrier and conformational selection hypothesis

As previously stated, prion transmission between species occurs most easily when the PrP\textsuperscript{res} of the donor and the PrP\textsuperscript{C} of the target host have the same PrP sequence; even single amino acid differences between donor and host can lead to a species barrier (Collinge, 2001; Collinge et al., 1991; Palmer et al., 1991). This relationship between PrP\textsuperscript{res} and the primary, hence detailed tertiary structure of PrP\textsuperscript{C} implies that the source of the species barrier is a structural incompatibility of interaction between donor and acceptor. However, observations of prion strains and the species barrier indicate that they are symptoms of the same phenomenon. Different strains of prions can be propagated in the identical hosts, producing different incubation periods and pathologies (Fraser and Dickinson, 1973) and prion strains that are propagated in identical hosts can have completely different barriers for transfer to other species. Also, transmission of strains between species can have a marked difference in tissue dependent attack rates; e.g. lymphoid tissues are markedly more permissive than nervous tissues (Béringue et al., 2012) Thus, the “species” is not solely responsible for the observations of interspecies transmission of prions and the term “transmission” barrier is more appropriate (Collinge, 1999).

To unify the observations of the species barrier and prion strains, Collinge and Clarke proposed the conformational and kinetic selection model (Fig. 1.3)(Collinge and Clarke, 2007). The model proposes that a given strain of PrP represents a thermodynamically stable conformation of PrP\textsuperscript{res} that can replicate itself in a host faster than the rate of clearance \textit{in vivo}. Each of these stable and persistent conformations represents a strain of PrP\textsuperscript{res} and the ensemble of these conformations represents the “portfolio” of all possible strains.

This portfolio of strains includes all primary sequences of PrP. However, the PrP sequence from a given species can only accommodate a particular subset of these possible strain conformations.
Figure 1.4 – The conformational selection model of the prion disease transmission barrier. The ensemble of all possible conformations of PrP\textsuperscript{res}, each representing a possible prion strain, is (sparingly) represented by the six different colours/shapes. The sequence of PrP from a species A can adopt PrP\textsuperscript{res} conformation 1-3, whereas species B can adopt conformations 2-4. Prions would transmit readily between these two species because they share the subset of prions 2 and 3 whereas a transmission barrier would occur with species C as it can only adopt PrP\textsuperscript{res} conformations 5 and 6 and does not share a subset with species A and B.
If a species is exposed to a PrP\textsuperscript{res} strain within its strain subset, then they would be infected relatively easily, whereas another species which lacks that particular conformation in its subset would be resistant to infection.

Subsequently when prions are transferred between species, for which the PrP\textsuperscript{res} conformational subsets of two species overlap, then transmission of that strain can occur quite easily. However, if the susceptible subsets of the two species do not overlap, a transmission barrier will result. This model provides a basis for the varying structures observed in prion strains and explains how they can give rise to a species barrier.

By extension, a species with a promiscuous primary sequence of PrP and a subsequently large subset of possible prion strain conformations would be highly susceptible to infection from many strains. Alternatively, a species with an unfavorable sequence of PrP would have fewer possible PrP\textsuperscript{res} conformations and would be relatively resistant. Therefore, the ultimate source of these conformational selections is the amino acid variation in the sequence of PrP itself.

1.6 Variations of PrP between and within species

The ability of a single amino acid difference to affect the conformational subset of PrP proteins leads to the question of what are the structural consequences that these differences will cause.

1.6.1 Structural variations in PrP\textsuperscript{C} between species

By comparing the structure and biophysical properties of PrP from species of differing susceptibilities, we may be able to extract information regarding the source of the postulated conformational differences. The sequence of PrP is highly conserved among mammals (between 85-95% identity (Wopfner et al., 1999), and it has been proposed that the amino acid changes in PrP that give rise to a species barrier cause structural changes in PrP that make the protein itself more resistant to conversion. Determination of the structures of PrP\textsuperscript{C} from first mouse and then hamster, bovine and human sources followed by a large number of other PrP\textsuperscript{C} structures, have made it apparent that the overall folds of mammalian PrPs are very similar. However, differences in structural dynamics have been observed when prions from a variety of species were compared. In particular, one feature of recent interest has been the stretch of residues 165-
175, which connects the second β-strand to α-helix 2 (the β2-α2 loop). This loop has been proposed as a possible interaction site for the hypothetical protein X, a postulated co-factor in the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} (Kaneko et al., 1997). In addition, a peptide of the same sequence is a potent amyloid former adopting a steric zipper conformation (Sawaya et al., 2007). When, in the loop of the mouse protein, native Asn173 is changed to serine the mutant protein now resists conversion to PrP\textsuperscript{Sc} (Vorberg et al., 2003).

In the first NMR structure of a prion protein, that of mouse PrP\textsuperscript{C} 121-231, no resonances could be assigned for the residues of the β2-α2 loop, most probably due to conformational heterogeneity of this part of the protein chain (Riek et al., 1996). In contrast, in the following NMR structure of PrP\textsuperscript{C} from hamster, the resonances for the loop residues could be clearly identified; residues 166-168 folded into a short 3\textsubscript{10}-helix and amino acids 171-174 formed the first turn of α-helix 2 (Donne et al., 1997). In subsequent structures, e.g. in PrP from humans, cows, sheep, cats, dogs and pigs, the appearance of these NMR resonances covered a broad range from unquestionable assignment to being completely absent (López Garcia et al., 2000; Lysek et al., 2005; Zahn et al., 2000). In the structure of elk PrP\textsuperscript{C}, the β2-α2 loop was clearly defined adopting a conformation similar to that of hamster PrP. Elk and deer are species susceptible to CWD; they are also quite unique in incorporating a threonine residue at position 174, which in most known sequences is an asparagine or, less commonly, serine. Mutating two residues in the mouse β2-α2 loop to mimic that of elk PrP resulted in a clearly defined “rigid” loop as observed in the actual elk protein. The great susceptibility of elk to CWD combined with the rigidity of the loop in its prion protein led to the hypothesis that structural homogeneity in the β2-α2 loop results in increased susceptibility to prion disease (Gossert et al., 2005). This hypothesis received support from an \textit{in vivo} model in which the rigid loop mutant of mouse PrP was expressed in transgenic mice, which subsequently became highly susceptible to prion infection (Sigurdson et al., 2009).

Giving further weight to this hypothesis, the structure of PrP\textsuperscript{C} from bank voles, a species extremely susceptible to prion diseases, also showed the β2-α2 loop in a clearly defined conformation (Christen et al., 2008). The same is true for Tamar wallaby PrP\textsuperscript{C}, which also revealed that long-range contacts to the C-terminus of helix-3 may help to stabilize the β2-α2 loop (Christen et al., 2009). Despite growing evidence that rigidity in the β2-α2 loop generally correlates well with susceptibility to prion disease there are also some exceptions. PrP\textsuperscript{C} from pigs
and rabbits, which are quite resistant to infection, also have clearly defined β2-α2 loops (Lysek et al., 2005; Wen et al., 2010). This finding, however, can still be accommodated in the framework of the conformational selection model when one replaces simple rigidity of the β2-α2 loop with the conformational subset available to it as the determinant of resistance and susceptibility. Supporting this hypothesis, it has been found that amino acid identity at position 170 and its resulting conformational effects are determinant in inter-species transmission (Sigurdson et al., 2010).

1.6.2 PrP polymorphisms within species

Aside from differences in PrP sequence affecting susceptibility and resistance between species, there are also several cases of known polymorphisms in the primary structure of PrP within a species. This can have profound effects and one of the best documented cases of intra-species variability are varying genotypes in sheep livestock where amino acid polymorphisms at residues 136, 154 and 171 dictate the level of susceptibility and resistance to Scrapie. Animals expressing the V136R154Q171 and A136R154Q171 variants are susceptible to the disease, whereas those expressing the A136R154R171 and A136H154Q171 variants are more resistant (Goldmann et al., 1990, 1994), although rare instances of infection have been reported (Groschup et al., 2007).

Comparison of the biophysical effects of these polymorphisms on the structure of sheep PrP revealed that the susceptible variants VRQ and ARQ have higher global thermal stability than the resistant variants ARR and AHQ (Rezaei et al., 2002). This contradicts the intuitive assumption that the susceptible variants simply represent a less stable PrPC structure. During unfolding, all four variants also formed an intermediate state rich in β-sheet structure. The activation energy for its formation was significantly higher in the resistant variants, ARR and AHQ, and accompanied by a lower β-sheet propensity when compared to the susceptible variants, VRQ and ARQ (Rezaei et al., 2002). The crystal structures of the ARR and VRQ variants complexed to Fab fragments of anti-PrP antibodies showed variations in surface hydrogen bonding patterns that were interpreted as lowering the stability of the ARR variant (Eghiaian et al., 2004). According to molecular dynamics simulations, at low pH values the mobility within the β2-α2 loop correlates well with the respective resistance of the variants, ARR displaying the largest movements and VRQ the smallest (Bujdoso et al., 2005). The same calculations also linked variant susceptibility to the ease of unwinding α-helix 2, suggesting that β2-α2 loop mobility and helix-2 stability could be connected (Fitzmaurice et al., 2008).
Another well-documented case of PrP variations within a species effecting susceptibility to infection is the methionine/valine polymorphism at position 129 in the amino acid sequence of human PrP. During the Kuru outbreak in Papua New Guinea, the age of onset for patients homozygous for methionine or valine at position 129 was 19 years compared to over 30 years for heterozygotes (Cervenáková et al., 1998). Also during the BSE outbreak in the United Kingdom, all documented human cases of vCJD have been in individuals homozygous for methionine at residue 129 (Collinge et al., 1996). This amino acid sits at the centre of the first strand of the protein’s β-sheet, close to the N-terminus of the ordered domain; adjacent to Y128, which forms a hydrogen bond with D178, a residue that is mutated to asparagine in cases of inherited TSE Fatal Familial Insomnia (FFI). This association may be significant as the amino acid at position 129 determines the development of either FFI (methionine) or familial CJD (valine) (Goldfarb et al., 1992). The thermodynamic stability of PrP\textsubscript{C} is not affected by polymorphism at position 129 (Hosszu et al., 2004; Liemann and Glockshuber, 1999) but, similar to the variants in sheep mutations, does effect the propensity of recombinant human PrP to self-associate (Apetri et al., 2005; Lewis et al., 2006; Tahiri-Alaoui and James, 2005).

1.7 Experimental summary and hypothesis

Since the discovery of TSEs and prions, the proteinaceous infectious agent responsible for them, a great deal has been learned about what causes prion diseases. Although the structure of PrP\textsubscript{C} has been determined, a detailed mechanism of infectivity and pathogenesis of prion disease remains elusive. Recent evidence suggests that infectivity and pathogenesis in prion disease involve separate mechanisms. The conformational and kinetic selection model proposes that infectivity resides in PrP\textsubscript{res} and pathogenesis is caused by a hypothesized toxic intermediate state, PrP\textsubscript{L}. Although PrP\textsubscript{L} has not been positively identified \textit{in vivo}, the recombinant PrP β-octamer has been found to have properties similar to what has been proposed for PrP\textsubscript{L}.

Several species including pigs and rabbits have shown widespread resistance to infection by prions whereas others such as hamsters and bank voles appear to be susceptible to prions from a wide range of sources. These variations in prion susceptibility may be related to the rate of formation of PrP\textsubscript{L}. If the rate and extent of conversion of PrP\textsubscript{C} to PrP\textsubscript{L} is low, then pathogenesis is delayed and the host may never develop symptoms and thus appear resistant. On the other
hand, if conversion is fast then a species will be more susceptible. Given these assumptions, I proposed the following hypothesis:

“The susceptibility of a species to prion disease is proportional to the tendency of their endogenous PrP to adopt a toxic, misfolded conformation, the β-state.”

To test my hypothesis I performed two studies:

To determine the β-state propensity, colleagues and I developed a novel method of two-wavelength CD analysis. Using this method, we showed that recombinant PrP from prion susceptible species have a higher propensity to refold to the β-state relative to PrP from less susceptible species. The crystal structure of rabbit PrP<sub>C</sub> 121-230, a prion disease resistant species, revealed a helix-cap motif at the N-terminus of helix-2 that contributes to the reduced β-state propensity of rabbit PrP. Additionally, further characterization of the β-state revealed that it is toxic and consists of β-state monomers and octamers in equilibrium. These findings support the hypothesis that the propensity of PrP is related to the susceptibility of a species to prion disease.

In my second study, I determined that single amino acid changes in the helix-cap at the N-terminus of helix-2 of hamster and rabbit PrP, modulate β-state propensity. Structures of a double and two single mutants of rabbit PrP<sub>C</sub>, analogous to the sequence of hamster PrP<sub>C</sub>, showed that the mutations ablated the helix-cap. Folding studies using the two-wavelength CD method on the rabbit PrP<sub>C</sub> mutants as well as hamster PrP<sub>C</sub> mutants analogous to rabbit PrP<sub>C</sub>. These experiments showed that ablation of the rabbit helix-cap increased β-state propensity, whereas introducing the helix-cap into hamster PrP<sub>C</sub> decreased propensity. This is an excellent demonstration of how single amino acid changes in PrP can affect its structure and its conversion to PrP<sub>L</sub> or PrP<sub>res</sub>, which in turn could contribute to the transmission barrier and/or species susceptibility and resistance.

Together these data provided strong evidence that species susceptibility is linked to β-state propensity.

Appendix A describes an additional study that was performed to explore the implications of a dimeric arrangement that was observed in the asymmetric unit of the structures of the wild-type
and mutant rabbit PrP$_C$s. A dimeric association is hypothesized to be involved in the function of PrP and/or the conversion mechanism to infectious prion. Using the chemical crosslinker 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC), and disulfide crosslinking of an N197C mutant of rabbit PrP$_C$, dimers of rabbit and hamster PrP$_C$ were crosslinked in solution. Controls showed that the dimer crosslink was specific and dependent on the tertiary structure of PrP$_C$ and may alter the folding behaviour of PrP upon association. EDC crosslinking of the β-state octamer with EDC showed that similar contacts may also be present in the β-state octamer.
Chapter 2


“Prion disease susceptibility is affected by β-structure folding propensity and local side-chain interactions in PrP.”

Khan, M.Q. *,1,2, Sweeting, B. *,1,3, Mulligan V.K.1,2, Arslan, P.E.1,2, Cashman, N.R.4, Pai, E.F.1,2,3,4, Chakrabartty, A.1,2,3

* - M.Q.K and B.S contributed equally to this work. 1Campbell Family Institute for Cancer Research, Ontario Cancer Institute/University Health Network. 2Department of Biochemistry, University of Toronto. 3Department of Medical Biophysics, University of Toronto. 4Brain Research Centre, Division of Neurology, Department of Medicine, University of British Columbia and Vancouver Coastal Health, University of British Columbia Hospital. 5Department of Molecular Genetics, University of Toronto.

Statement of contribution:  M.Q.K, developed and validated two-wavelength CD method and monomer-octamer equilibrium analysis.  B.S. developed PrP expression and purification protocol, determined rabbit PrP C crystal structure and contributed to validation of two-wavelength CD method and monomer-octamer equilibrium analysis.  V.K.M. contributed to development of two-wavelength CD method.  P.E.A. performed toxicity studies.  All authors contributed to experimental design and writing of the manuscript.
2.1 Introduction

According to the “protein-only” hypothesis, prion diseases are caused by the misfolding of the prion protein (PrP) into a conformation that is pathogenic and infectious (Griffith, 1967; McKinley et al., 1983). PrP exists normally in a monomeric, mostly α-helical state (PrP\textsuperscript{C}) and upon prion infection it refolds into an aggregation-prone, mostly β-structured state that is infectious (PrP\textsuperscript{res}).

However, prions are not universally infective. When a strain of prions is transferred between hosts that are genetically different or of a different species, a delay between infection and onset of symptoms can occur in a phenomenon that is referred to as the transmission barrier.

The transmission barrier is the result of the PrP sequence of the host and the prion strain itself (Collinge and Clarke, 2007). The structure of PrP\textsuperscript{res} is not homogenous between prion strains, and each strain is the result of a different conformation of PrP\textsuperscript{res}. Not every sequence of PrP is compatible with every strain conformation of PrP\textsuperscript{res}. If the host PrP sequence and the PrP\textsuperscript{res} conformation are compatible, then transmission will occur easily. However, if the host PrP sequence is incompatible with the PrP\textsuperscript{res} conformation, then a transmission barrier will result.

Experiment and observation of the transmission of prions between species has suggested that not all species are equally susceptible to prion disease (Gibbs and Gajdusek, 1973). Although it is difficult to rank the susceptibility of different species to contract prion disease with the limited data available, certain trends are clear.

Previous studies of prion transmission in hamsters indicated that this species is highly susceptible to prion diseases, for hamsters develop prion disease when inoculated with various prion isolates from different donors, including humans, cows, sheep, mice, mink, and other hamsters (Bessen and Marsh, 1992b; Gibbs and Gajdusek, 1973; Kimberlin and Walker, 1977; Thomzig et al., 2006). Mice show comparable prion disease susceptibility (Bessen and Marsh, 1992b; Chandler, 1961; Gibbs and Gajdusek, 1973; Hill et al., 2000; Lasmézas et al., 1997; Thomzig et al., 2006), but rabbits have a low susceptibility to prion disease despite having been inoculated with human, sheep, and mouse prions; they can resist prion infection for at least 3 years (Barlow and Rennie, 1976; Gibbs and Gajdusek, 1973).
Furthermore, reports from the BSE crisis in the United Kingdom indicate clear differences in BSE-susceptibility among larger mammals. Humans and many feline species, including cheetahs, pumas, and domestic cats, were susceptible to the BSE agent, while no cases of prion disease were reported in canine or equine species (Aldhous, 1990b; Kirkwood and Cunningham, 1994). However, due to a lack of experimental data, it is unclear how dogs and horses would fare when challenged with a variety of prion isolates.

Although understanding the determinants of prion susceptibility would yield insights into the mechanism of pathogenesis in prion diseases, the varying susceptibilities of mammalian species has yet to be explained mechanistically.

It has been known for a number of years that PrP can form a β-structured state (β-state) under slightly destabilizing conditions (Hornemann and Glockshuber, 1998; Swietnicki et al., 2000; Zhang et al., 1997). β-structured octameric species of the mouse prion protein have been observed before using electrospray ionization mass spectrometry, and of the hamster, by dynamic and static light scattering (Baskakov et al., 2001, 2002). β-structured monomers have been observed as well, and have been proposed to be on the pathway to oligomerization (Sokolowski et al., 2003).

The proposal that β-state PrP plays a role in the mechanism underlying prion diseases (Gerber et al., 2008a) raises the possibility that the propensity to form the β-state is a major determinant of prion disease susceptibility. To date, a quantitative measure of the propensity of prion proteins to populate β-structured monomers and octamers has not been available.

We have developed a novel, two-wavelength method of circular dichroism (CD) analysis to quantify the propensity of PrP to transform into the β-state, and have applied this, along with analytical ultracentrifugation experiments, to compare hamster, mouse, rabbit, horse and dog PrP. Using urea and acid denaturation to induce structural transitions, we find that the PrP proteins from these five species can each populate four distinct conformational states: native monomers, unfolded monomers, and a β-structured state (β-state) that can be monomeric or octameric. We find that the β-state propensity of PrP from these species varies with their prion disease susceptibility. Using this two-wavelength CD method in conjunction with X-ray crystallography, we have been able to identify a key helix-capping motif that controls the formation of the β-state, which may govern the susceptibility to prion disease.
2.2 Materials and methods

2.2.1 Protein expression

DNA constructs of golden Syrian hamster (hamster), mouse, and rabbit PrP 23-231 were generous gifts from M. Coulthart (Health Canada, Winnipeg, MA, Canada), D. Westaway and J. Watts (University of Alberta, Edmonton, AB, Canada), and S. Priola (Rocky Mountain Laboratories, Hamilton, MT, USA), respectively. Horse and dog PrP 23-231 encoding DNA constructs were generous gifts from J. Castilla (The Scripps Research Institute, Jupiter, FL, USA). The DNA sequences coding for hamster, mouse, rabbit, horse and dog PrP 90-231 were cloned and expressed using the pProEX-Htb plasmid (Invitrogen, Burlington, ON, Canada) with an N-terminal 6xHis-tag and a Tobacco Etch Virus (TEV) protease cleavage site. Rabbit PrP 121-231 was cloned and expressed using pET28a (Novagen, Gibbstown, NJ, USA) with an N-terminal 6xHis-tag and a thrombin protease cleavage site.

2.2.2 Protein purification

All PrP proteins were expressed as inclusion bodies in E. coli BL21 AI cells (Invitrogen) and refolded and purified according to Zahn et al. (Zahn et al., 1997), with slight modifications. Frozen cell pellets of previously expressed PrP were thawed and resuspended in 50 mL of 20 mM Tris, pH 8.0. Suspended cells were then lysed by sonication using a Branson Sonifier 450 (Denby, CT, USA). Inclusion bodies were separated from the cell lysate by centrifugation at 15,000 g for 30 minutes. Pelleted inclusion bodies were then resuspended in 50 mL of buffer G (6 M guanidinium hydrochloride, 20 mM Tris, pH 8.0, 100 mM Na₂HPO₄, and 10 mM reduced glutathione) overnight on an inverting shaker, at 4°C. Insoluble debris was separated from solubilized inclusion bodies by centrifugation at 15,000g for 30 minutes. The supernatant was then added in drops to a suspension of 25 mL of Ni-NTA resin (Qiagen, Mississauga, ON, Canada) in 50 mL of buffer G and incubated at room temperature for 30 minutes with stirring. The protein and resin mixture was then loaded onto a 100 mL gravity-driven Ni-NTA column and washed, refolded and eluted with a cycle of: 75 mL of buffer G wash, 200 mL linear gradient
of buffer G to buffer B (20 mM Tris, 100 mM Na₂HPO₄, pH 8.0), eluted with 75 mL of buffer B + 500 mM imidazole and a final wash of 75 mL of buffer B. The cycle was repeated four times and all eluents were combined and dialyzed overnight against 4 L of 20 mM Tris pH 8.0.

The dialysate was then loaded onto a 5 mL Ni-NTA column, washed with 15 mL of 20 mM Tris, pH 8.0 and eluted with 15 mL of Tris, pH 8.0, 500 mM imidazole. For all variations of PrP 90-231, the His-tag was then cleaved with His-tagged TEV protease (Invitrogen) and both the His-tag and TEV were removed with Ni-NTA. For PrP 121-231, 10 U of thrombin was then added to the eluent and the PrP/thrombin mixture was dialysed against 2 L of 20 mM Tris, pH 8.0, overnight at 4°C. The dialysate was then loaded onto a HiTrap Q-sepharose 5/5 anion exchange column on an ÄKTA FPLC system (GE Bioscience, Mississauga, ON, Canada) washed with 20 mL of 10 mM Tris, pH 8.0 and eluted with a 100 mL gradient of 0-250 mM NaCl in 10 mM Tris, pH 8.0.

2.2.3 Unfolding and refolding of PrP 90-231 monitored by CD

Stocks (95 μM) of folded PrP 90-231 were dialyzed into the following buffers that were of identical ionic strength, each with 5 mM EDTA; pH 7.0 (50 mM sodium phosphate), pH 5.0 (50 mM sodium acetate, 67 mM NaCl), pH 4.5 (50 mM sodium acetate, 70 mM NaCl), pH 4.0 (50 mM sodium acetate, 74 mM NaCl). Equilibrium samples were prepared by diluting the PrP stock 10-fold to 9.5 μM into buffered solutions containing urea at concentrations ranging from 0 M - 9 M and incubated for 5 days at room temperature before making CD measurements.

Unfolded PrP stocks (95 μM) were prepared by denaturing folded PrP in 9.9 M urea in pH 7.0 and pH 4.0 buffers using 3,500 MWCO dialysis cups (Pierce, Rockford, Illinois). Urea-refolding samples were prepared by diluting the PrP stock 10-fold (to a final concentration of 9.5 μM) into pH-buffered solutions containing urea to final concentrations ranging from 1 M to 9 M urea. Samples were incubated for 5 days at room temperature to reach equilibrium before CD measurements were taken.

2.2.4 CD spectroscopy

CD spectroscopy was performed on an Aviv circular dichroism spectrometer model 62DS. All CD measurements were taken using a 1 mm quartz cuvette and a spectral bandwidth of 1 nm at
25 °C. Far-UV CD spectra were averaged from three wavelength scans from 250 nm to 190 nm with a 5 second averaging time, and blanked with respective buffers.

2.2.5  **Urea unfolding of PrP 90-231 monitored by CD at 220 nm and 229 nm.**

CD measurements were obtained at wavelengths of both 220 nm and 229 nm; 100 measurements at a rate of 1 measurement/s were averaged. For each pH, equilibrium curves were normalized according to Santoro & Bolen (Santoro and Bolen, 1988). The fraction β-state PrP at each urea concentration was determined applying Eq. 6 (see section 2.3.3). The z parameters in Eq. 6 represent the normalized CD signal of β-state PrP at wavelengths 220 nm and 229 nm; the values of these critical points were determined graphically (Barrick and Baldwin, 1993).

2.2.6  **Analytical ultracentrifugation**

Equilibrium sedimentation experiments were performed on hamster, mouse and rabbit PrP 90-231 at pH 4.0 in urea concentrations ranging from 2.5 M to 5.0 M, and horse and dog PrP 90-231 at pH 3.5 in urea concentrations ranging from 1.33 M to 3.67 M, at protein concentrations of 9.5 μM. Samples were loaded into six-sectored cells with quartz windows using rotor speeds of 3,000, 10,000, 15,000, 20,000, 25,000, 30,000, 35,000 and 40,000 rpm at 25 °C. For each speed, 5 replicates of data were collected every 10 μm at a wavelength of 280 nm after 18 to 34 hours. Molecular weight determinations involved global analysis of data acquired at 5 different speeds using Beckman XL-I software, where absorbance versus radial position data were fitted to the sedimentation equilibrium equation using non-linear least squares techniques. The partial specific volume and density of the sample were calculated using the program SEDNTERP (Hayes et al., 1995) from the amino acid sequence and buffer composition, respectively.

2.2.7  **Determination of the fraction octamer**

Sedimentation data collected from hamster, mouse and rabbit PrP 90-231 at pH 4.0 in urea concentrations ranging from 2.5 to 5.0 M were fit to a double-exponential model, where the absorbance of the PrP sample equals the summation of the absorbance of the monomer and the absorbance of the octamer, as a function of radius:

\[
\text{Absorbance (PrP sample)} = \text{Absorbance (Monomer)} + \text{Absorbance (Octamer)} \quad [1]
\]

\[
\text{Absorbance (PrP sample)} = A*\exp(k*\text{radius}^2) + B*\exp(8*k*\text{radius}^2), \quad [2]
\]
where A, B represent proportionality constants. \( k \) equals the term \( (\omega^2/2RT) \times (M*(1- \nu \rho)) \), where \( \omega \) represents the angular velocity of the rotor (in radians per second), \( R \) represents the gas constant \( (8.314 \times 10^7 \text{ erg/mol*K}) \), \( T \) represents the temperature in Kelvin, \( M \) represents the gram molecular weight of the protein, \( \nu \) represents the partial specific volume of the protein and \( \rho \) represents the density of the solvent. For each sample, the fraction octamer was determined at the radius value where the sedimentation absorbance readings at speeds ranging from 10,000 to 15,000 rpm intersect with the absorbance readings at 3,000 rpm. Since the absorbance readings at 3,000 rpm represent the absorbance of the sample before protein sedimentation has occurred, the total PrP concentration at this radius equals the concentration prior to any sedimentation. The fraction octamer was calculated using 3-6 separate data sets.

2.2.8 Size exclusion chromatography.

Hamster PrP 90-231 at 9.5 \( \mu \)M in 3.6 M Urea, 50 mM sodium acetate pH 4.0, 80 mM NaCl was incubated at room temperature for a minimum of 72 hours. An aliquot of 300 \( \mu \)L was then injected onto a Superdex 200 10/30 column (GE Bioscience) equilibrated in the same buffer as above. The flow rate was 0.25 mL/min and the column elution was monitored by absorbance at 280 nm using an ÄKTA FPLC system at room temperature.

2.2.9 Sulforhodamine B cytotoxicity assay.

Pheochromocytoma (PC-12) cells were plated at 1,000 cells per well in a 96-well plate and incubated in 60 ng/mL NGF diluted in N2 supplement/Dulbecco's modified eagle medium (Gibco BRL). Cells were differentiated for four days. Samples (0 to 9 mM) of \( \beta \)-state mouse PrP 90-231, native state mouse PrP90-231, and the bee venom mellitin were prepared by diluting concentrated stock solutions into cell culture medium and then dialyzing the samples against large volumes of cell culture media in a 3,500 MWCO dialysis cup to remove urea from the samples. After differentiation, the medium was exchanged with the PrP/mellitin samples and the cells were incubated for 4 days at 37°C. Toxicity was assayed using the sulforhodamine B (SRB) assay. Briefly, cells were fixed with 8.5% trichloroacetic acid for 30 minutes. The plates were washed with water and air-dried. Protein was stained with 0.4% SRB (Molecular Probes Inc.) in 1% acetic acid for 30 minutes. Plates were washed with 1% acetic acid and air-dried. The dye was extracted in 10 mM Tris, pH 9.0, and absorbance was assayed at 550 nm on a plate reader.
2.2.10 Crystallization of rabbit PrP 121-231 and diffraction data collection

Initial screens using Crystal Screens I & II (Hampton Research, Aliso Viejo, CA, USA) yielded showers of crystalline plates in 100 mM Hepes, pH 7.5, 4.3 M NaCl. Further optimization and microseeding gave single, plate-like crystals (300 μm x 300 μm x 30 μm) that grew overnight in 100 mM Tris, pH 7.0, 2.5 M NaCl and diffracted to a resolution of 1.6 Å. Diffraction data were collected from a single crystal flash-frozen at 100 K in 100 mM Tris, pH 6.5, 2.5 M NaCl, and 30 % v/v glycerol as cryoprotectant at a wavelength $\lambda = 0.97934$ Å using a Marmosaic CCD300 detector at the CMCF-I beamline at the Canadian Light Source Synchrotron (Saskatoon, SK, Canada).

2.2.11 Data processing, refinement and model building

Data were processed with the program package XDS (Kabsch, 2010). Statistics for data collection and unit cell parameters are summarized in Table 1. The structure was solved applying molecular replacement techniques contained in the program PHASER (McCoy et al., 2007) with sheep PrP (PDB ID: 1UW3) as a search model. The model was refined without NCS with the maximum likelihood-method using REFMAC (Murshudov et al., 2011) and TLS refinement (Winn et al., 2003) assisted by TLSMD (Painter and Merritt, 2006). Model building was done using Coot (Emsley and Cowtan, 2004). Summary of refinement statistics are given in Table 1. Atomic coordinates have been deposited in the Brookhaven Protein Data Bank (PDB ID: 3O79).

2.3 Results

2.3.1 Urea denaturation of hamster PrP 90-231 produces α-helical, β-structured, and unfolded states

Purified, recombinant, hamster PrP (9.5 μM) encompassing residues 90 to 231 (hamster PrP 90-231) was incubated in urea (0 M - 9 M, pH 4, 25 °C, 5 days) and CD measurements were made at 220 nm. When plotted as a function of urea concentration, the mean residue ellipticity of hamster PrP 90-231 shows a biphasic transition (Fig. 2.1a). The CD spectrum of hamster PrP 90-231 measured at 0, 2.5, 7.5 M urea (pH 4), respectively, displays a typical α-helical structure, a
β-sheet-rich structure, and an unfolded structure (Fig. 2.1b). By relating the three unique CD spectra of hamster PrP 90-231 in Figure 2.1b to the biphasic unfolding curve in Figure 2.1a, it is apparent that urea does not cause unfolding of the native state to a partially folded intermediate state, which then completely unfolds. Instead, moderate amounts of urea at pH 4 cause the native α-helical state of hamster PrP 90-231 to convert to a very different, non-native, β-sheet-rich conformation and addition of higher amounts of urea results in complete unfolding of this unique β-sheet-rich state. We denote this β-sheet-rich conformation as β-state PrP.

2.3.2 A monomer-octamer equilibrium best describes the oligomerization state of hamster PrP 90-231 in the β-state

In usual practice, denaturation curves that display a characteristic biphasic shape are fit to a three-state transition model to determine the thermodynamic stabilities of the three stably populated species. However, these models usually assume that the protein remains monomeric at all denaturant concentrations. Therefore, before fitting our CD data we determined the oligomerization states of native, β-state and unfolded PrP.

Sedimentation data indicated that native (no urea, pH 4.0) and unfolded PrP (7.5 M urea, pH 4.0) were monomeric with a molecular weight of 16 kDa. However, sedimentation analysis indicated that β-state PrP self-associates in a monomer-octamer equilibrium (Fig. 2.2a), which was confirmed by analysis of the residuals of the fits to various monomer-oligomer models (Fig. 2.2b). Size exclusion chromatography (SEC) (Fig. 2.2c) supports the results from sedimentation equilibrium ultracentrifugation. Thus, β-state PrP, unlike native or unfolded PrP, exists as an equilibrium mixture of two species: monomers and octamers.

2.3.3 A novel two-wavelength method of circular dichroism analysis allows measurement of fractional concentrations of β-state PrP

The finding that β-state PrP exists as a mixture of monomers and octamers indicates that a simple three-state model is inadequate to fit the biphasic, urea unfolding curves of PrP. Analysis of the data using a four-state equilibrium model is also not straightforward because various sequential
Figure 2.1: Hamster PrP 90-231 adopts three distinct secondary structural states as a function of urea concentration and pH value. (A) Urea-unfolding curve of hamster PrP 90-231 at pH 4 monitored by CD at 220 nm. (B) CD spectra of hamster PrP 90-231 in the native (○), β-state (●), and unfolded (□) conformations at pH 4.
Figure 2.2: The β-state of hamster PrP 90-231 exists as an equilibrium between monomers and octamers (a) Goodness-of-fit (variance) of sedimentation equilibrium data of hamster PrP 90-231 analyzed by various self-association models in 2.5 M (▲) and 3.2 M (○) urea at pH 4. (b) Sedimentation equilibrium data of hamster PrP 90-231 in 3.2 M, pH 4.0 at 15,000 rpm, fit to a monomer-octamer equilibrium model (lower panel). Residuals to the fit of the data to a monomer-octamer model (upper panel) (c) Size exclusion chromatography of hamster PrP 90-231 in 3.6 M urea at pH 4.0.
or branched models are equally probable and cannot be discerned from the urea unfolding curves. Furthermore, the greater complexity of the four-state model, with its higher number of parameters, increases the difficulty in differentiating local goodness-of-fit minima in the parameter landscape from the global minimum. For these reasons, we decided to develop an approach that does not make any presumptions about oligomerization state. Our method takes advantage of the fact that β-state PrP possesses a β-sheet CD spectrum that is distinct from both the α-helical native and random coil-like unfolded PrP spectra (Fig. 2.1b). Because the CD spectrum is a sum of the CD contributions from all protein molecules present in their particular conformations, our observed CD signal \( \theta_{220, \text{obs}} \) measured from a sample can be expressed as follows:

\[
\theta_{220, \text{obs}} = [\theta]_{220, \text{Native}}[\text{Native}] + [\theta]_{220, \beta\text{-state}}[\beta\text{-state}] + [\theta]_{220, \text{Unfolded}}[\text{Unfolded}]
\]

In the above, \([\theta]_{220, \text{Native}}, [\theta]_{220, \beta\text{-state}}, \text{ and } [\theta]_{220, \text{Unfolded}}\) represent the molar ellipticity of native, β-state, and unfolded state PrP at 220 nm, respectively. The above expression can be simplified by normalizing the CD data to fraction apparent values where the native state and unfolded state have values of 1 and 0, respectively:

\[
F_{\text{app} 220} = 1(F_{\text{Native}}) + z_{220}(F_{\beta\text{-state}})
\]

Here, \(F_{\text{app} 220}\) is the observed normalized CD signal, \(z_{220}\) is the normalized CD signal for β-state PrP at 220 nm, and \(F_{\text{Native}}\) and \(F_{\beta\text{-state}}\) are fractional concentrations of the native and β-state PrP, respectively.

To solve for \(F_{\beta\text{-state}}\), a system of two equations can be generated by monitoring the unfolding of PrP at a second wavelength. In order to solve for \(F_{\beta\text{-state}}\), the normalized CD signal \(z_{\lambda}\) for the β-state at the second wavelength would need to be different from \(z_{220}\), and also distinct from the native and the unfolded CD signals. Based on the spectra in Figure 2.1b, a wavelength of 229 nm fulfills these criteria, yielding the following second equation:

\[
F_{\text{app} 229} = (F_{\text{Native}}) + z_{229}(F_{\beta\text{-state}})
\]

Combining Eqs. 2 and 3 and solving for \(F_{\beta\text{-state}}\) yields:

\[
F_{\beta\text{-state}} = (F_{\text{app} 220} - F_{\text{app} 229}) / (z_{220} - z_{229})
\]
Thus, by monitoring urea unfolding of PrP at both 220 nm and 229 nm, the relative fraction of β-state PrP can be calculated at any given urea concentration and pH value. Note that this two-wavelength method treats the CD spectrum of the native, β-state, and unfolded PrP as a unique signature of that particular state. Procedures that deconvolve CD spectra into percent helix, percent β-sheet, and percent random coil were not used.

2.3.4 Urea treatment of PrP causes conversion of PrP to the β-state at low pH

Figure 2.3 shows the normalized urea unfolding curves of hamster PrP 90-231 at pH values of 7.0, 5.0, 4.5 and 4.0. The CD signals were monitored at wavelengths of 220 nm and 229 nm and the data were normalized to F\textsubscript{app}. A relationship is observed between the shape of the urea unfolding curves and the difference between the normalized CD signals at the two wavelengths. At pH 7.0, the urea unfolding curves are monophasic in shape and the curves at the two different wavelengths are superimposable (Fig. 2.3a). At pH 5.0, the biphasic shape and the differences between the curves monitored at two wavelengths are both diminished (Fig. 2.3b). At pH values of 4.0 and 4.5, the urea unfolding monitored by normalized CD at the two wavelengths display strikingly different biphasic curves (Fig. 2.3c and 2d). Since, unfolding curves measured at the two wavelengths do not overlay at intermediate urea concentrations, at low pH, this gives a qualitative indication that β-state PrP must be significantly populated. Quantification of the amount of β-state PrP is possible with our two-wavelength method.

2.3.5 The fractional concentration of β-state PrP from hamster, mouse, rabbit, horse and dog PrP 90-231 correlates with prion susceptibility

Mouse, rabbit, horse, and dog PrP 90-231 formed monomer-octamer equilibrium mixtures at pH 3.5-4, and 2-6 M urea that were similar to hamster PrP 90-231 (Fig. 2.4). Urea-induced unfolding of PrP 90-231 constructs of each of these species at pH values of 7.0, 5.0, 4.5 and 4.0 was monitored by CD measurements at 220 nm and 229 nm (Fig. 2.5 and 2.6). A clear gradation is observed between the five species. Differences between the two wavelength curves are greatest with hamster followed by mouse, and then by rabbit, horse, and dog, thus providing an index of β-state concentrations in the five species. Importantly, refolding experiments of these PrP proteins demonstrate that at pH values of 7.0 and 4.0, unfolding and refolding of the proteins are completely reversible, indicating that equilibrium has been achieved (Fig. 2.7).
Figure 2.3: Urea-unfolding curves of hamster PrP 90-231 monitored by CD at 220 (▲) and 229 nm (○) at pH (A) 7.0, (B) 5.0, (C) 4.5, and (D) 4.0. The lines are intended as a guide to the eye.
Figure 2.4: (a) Goodness-of-fit (variance) of equilibrium sedimentation data of mouse (▲) and rabbit (○) PrP 90-231 to various self-association models in 5.0 M and 4.0 M urea, respectively, at pH 4. Sedimentation data of mouse (b) and rabbit (c) PrP 90-231 at 15,000 rpm fitted to a monomer-octamer equilibrium model. (d) Goodness-of-fit (variance) of equilibrium sedimentation data of horse (▲) and dog (○) PrP 90-231 to various self-association models in 2.33 M urea at pH 3.5. Sedimentation data of horse (e) and dog (f) PrP 90-231 at 15,000 rpm fitted to a monomer-octamer equilibrium model.
Figure 2.5: Urea-unfolding curves monitored by circular dichroism at 220 nm (▲), and 229 nm (○) at pH 7.0, 5.0, 4.5, and 4.0 of mouse PrP 90-231 (top panels) and rabbit PrP 90-231 (bottom panels). The lines are intended as a guide to the eye.

Figure 2.6: Urea-unfolding curves monitored by circular dichroism at 220 nm (▲), and 229 nm (○) at pH 7.0, 5.0, 4.5, and 4.0 of horse PrP 90-231 (top panels) and dog PrP 90-231 (bottom panels). The lines are intended as a guide to the eye.
Figure 2.7: Urea-unfolding (○) and refolding (●) at pH 7.0, and urea-unfolding (□) and refolding (■) at pH 4.0 for (a) hamster, (b) mouse and (c) rabbit PrP 90-231. Circular dichroism data were collected at 220 nm.
Figure 2.8: The propensity to populate β-state PrP correlates with species susceptibility to prion disease. Comparison of the β-state PrP fraction between hamster (red line), mouse (green line), rabbit (blue line), horse (dark yellow line), and dog PrP 90-231 (purple line) as a function of urea concentration at pH (A) 7.0, (B) 5.0, (C) 4.5, and (D) 4.0. The lines are intended as a guide to the eye.
The value of $z_{220} - z_{229}$ was calculated at pH values that rendered a biphasic curve for the PrP constructs; this value was $0.144 \pm 0.020$, and the same, within error, for all PrP proteins examined. Using a value of 0.144 for $z_{220} - z_{229}$, the fractional concentrations of β-state PrP were calculated for each of the five species at various urea concentrations and pH values (Fig. 2.8). At pH 7.0, none of the five PrP proteins populates the β-state (Fig. 2.8a). At pH 4.0, all five species populate the β-state fraction to some degree (Fig. 2.8d). At pH 5 (Fig. 2.8b) and 4.5 (Fig. 2.8c), they show varying fractional concentrations of the β-state. The propensity to form the β-state is greatest for hamster PrP followed by mouse PrP, and then by rabbit, horse, and dog PrP. This correlates with prion disease susceptibility in these mammals.

2.3.6 Comparison of the fraction octamer with the β-state fraction: detection of a monomeric, β-state PrP species

The fractional concentrations of octamer in the various samples from hamster, mouse and rabbit PrP 90-231 can be determined from sedimentation equilibrium data by fitting the data to a double-exponential equation (Fig. 2.9). The fraction octamer was calculated at urea concentrations of 2.5, 3.2, 4.0 and 4.5 M for hamster PrP, at 3.7, 4.3 and 5.0 M urea for mouse PrP and at 3.0, 3.7 and 4.4 M urea for rabbit PrP 90-231, all at pH 4.0 (Fig. 2.10). It is clear that the fractional concentration of β-state PrP is significantly higher than the fractional concentration of the octamer. The maximum fraction octamer reaches a value of 70% for hamster PrP (Fig. 2.10a), 60% for mouse PrP (Fig. 2.10b), and 50% for rabbit PrP (Fig. 2.10c). Under these conditions the fractional concentration of β-state PrP is 100% for each of these species. Since sedimentation equilibrium and SEC analysis indicated that only monomers and octamers are present, we conclude that the β-state PrP fraction is a mixture of β-structure-containing monomers and octamers for hamster, mouse and rabbit PrP 90-231.

To further demonstrate the existence of β-structured monomers, hamster PrP 90-231 was diluted into 3.6 M urea, pH 4.0 and secondary structure and oligomerization status were examined by CD and size exclusion chromatography, respectively. The data show that the reduction in the levels of monomers and the appearance of octamers occur over a period of hours (Fig. 2.11); however, formation of β-sheet structure is complete at the start of the measurements (Fig. 2.11b, 2.11c). Thus the monomers that are well populated at early time points are β-structured.
Figure 2.9: Determining octamer fraction: (a) Sedimentation data of hamster PrP 90-231 (2.5 M urea, pH 4.0) collected at 10,000 rpm (○) fitted to a monomer octamer equilibrium model (solid curve). The dashed line represents the radius at which the data collected at 3,000 rpm (■) intersect with the fitted data at 10,000 rpm. (b) The monomer (□) and octamer (▲) absorbance fractions can be plotted as a function of radius from the fitted data in (a). The dashed line represents the radius at which the octamer fraction is calculated. At this radius the total PrP concentration equals the initial concentration in the cell before sedimentation.
Figure 2.10: The β-state PrP fraction is a mixture of β-structure-containing monomers and octamers. Comparison of the β-state PrP fraction (●) with the octamer fraction (black bars) as a function of urea concentration at pH 4.0 for (a) hamster, (b) mouse and (c) rabbit PrP 90-231. The lines are intended as a guide to the eye. The error bars represent the standard error based on 3 to 6 measurements of the fraction octamer.
Figure 2.11: Direct evidence of a β-structured monomer. (a) The percentages of the area of both monomer (○) and octamer (■) peaks were plotted as a function of time after diluting folded hamster PrP 90-231 into 3.6 M urea, pH 4.0 buffer. The lines are intended as a guide to the eye. (b) The CD signal at a wavelength of 218 nm was used to monitor the CD changes over the course of 5 hours. (c) CD spectra of hamster PrP 90-231 diluted in 3.6 M urea, pH 4.0 at time zero (■) and after 48 hours (○). (d) Elution profile of hamster PrP 90-231 showing octamer and monomer peaks at equilibrium in 4.1 M urea, pH 4.5 buffer. (e) Size exclusion chromatography of the monomer after collecting and re-applying the monomer peak in (d) to the size exclusion column. (f) CD spectra of monomeric hamster PrP 90-231 (○) from the same protein sample that was applied to the column in (e), and CD spectra of the monomer-octamer mixture (■) from the same protein sample that was applied to the column in (d).
2.3.7 β-state PrP from mouse PrP 90-231 is cytotoxic

We performed cytotoxicity studies to confirm that β-state PrP has similar toxicity to oligomeric β-structured PrP examined previously (Novitskaya et al., 2006). We have observed that once formed, β-state PrP remains stable in solution for at least one week, when the conditions are changed from 4.0 M urea, pH 4.0 to phosphate buffered saline, pH 7.4. This stability of β-state PrP allowed investigation of its cytotoxic properties using differentiated PC12 cells, a common cell culture model of neurons. Native-state and β-state mouse PrP 90-231 were added to differentiated PC12 cells and their effect on cell survival was monitored (Fig. 2.12). Bee venom mellitin, a known cytotoxin, was used as a positive control. While native state PrP did not display any toxic properties, β-state PrP was as cytotoxic as bee venom mellitin on a molar basis. These data indicate that these β-state PrP preparations possess potent cytotoxic activity under physiological conditions.

2.3.8 X-ray crystal structure of rabbit PrP 121-230

To find out whether the structure of natively-folded rabbit PrP contains any unique structural elements that might contribute to its resistance to conversion into the β-state, we solved the X-ray crystal structure of rabbit PrP 121-230 to 1.6 Å resolution (see Table 2.1 for data collection and refinement statistics)(Fig. 2.13). While numerous structures of PrP from various species have been solved by nuclear magnetic resonance spectroscopy, to date X-ray crystal structures are available only for sheep PrP, human PrP (as a domain-swapped dimer), and human PrP bound to an antibody (Haire et al., 2004; Knaus et al., 2001; Simoneau et al., 2007). These have all been refined at resolutions of 2.0 Å or greater.

The asymmetric unit of the rabbit PrP 121-231 crystal structure contains two molecules, which associate closely along a non-crystallographic 2-fold symmetry axis that is almost parallel to helix-2 (Fig. 2.14). The root-mean squared deviation (RMSD) between all backbone atoms in the two molecules is 0.38 Å, indicating that the folds of the two molecules are practically identical. They are also very similar to those of other structurally known PrP proteins; for example, the
Figure 2.12: β-state PrP from mouse PrP 90-231 is cytotoxic. Toxicity of β-state PrP when exposed to neuronal-differentiated PC-12 cells. Absorbance readings were taken at 550 nm after performing the sulforhodamine B (SRB) cytotoxicity assay to measure cell viability of cells exposed to no protein (*), β-state PrP of mouse PrP 90-231 (▲), native PrP (●), and melittin (○). The SRB assay was performed after 4 d cultivation of cells at 37 °C.
Table 2.1: Summary of crystallographic data collection and model refinement statistics.

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<td>Unique reflections</td>
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<tr>
<td>Completeness (%)</td>
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<tr>
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</tr>
<tr>
<td>&lt;I&gt;/σI</td>
<td>18.35 (7.71)</td>
</tr>
</tbody>
</table>

| Refinement Statistics                     |       |
| Final R Cryst (%)                         | 16.1  |
| R-free (%)                                | 21.8  |
| Solvent (%)                               | 44.7  |
| No. of molecules                          | 2     |
| No. of all atoms                          | 1902  |
| No. of water molecules                    | 192   |
| No. of sodium ions                        | 4     |
| No. of Chloride ions                      | 2     |
| Average B-factor (Å²)                     | 19.60 |
| Ramachandran plot                         |       |
| Favored                                   | 99.5% |
| Allowed                                   | 0.5%  |
| R.M.S from ideal values                   |       |
| Lengths                                   | 0.022 |
| Angles                                    | 1.790 |
Figure 2.13: The crystal structure of rabbit PrP\textsuperscript{C} 126-230. The observed monomeric fold is similar to previously observed structures of the ordered domain of PrP\textsuperscript{C}. The $\beta_2$-$\alpha_2$ loop is highlighted in the Inset box.
Figure 2.14: Observed dimeric arrangement in the asymmetric unit of wild-type rabbit PrP$^C$ 121-231 crystal lattice. The two molecules are not domain-swapped and closely associate between the helix 2–loop–helix 3 region of the C-terminal half of the ordered domain.
Figure 2.15: Omit $F_0-F_C$ electron density (at 2.0 $\sigma$) for residues V166-N168 (left) and Y169-S174 (right) of the $\beta_2$-$\alpha_2$ loop. Residues 166-168 are well ordered forming a $3_{10}$ helical loop and show good electron density for the main and side chains. Residues 169-174 also show good electron density except for S170 for which no side chain electron density was observed. Electron density for S174 shows that the side-chain hydroxyl points inward toward the backbone, making a hydrogen bond with the backbone amide.
Figure 2.16: Long range contacts between the β2-α2 loop and helix-3. V166 and F175 make hydrophobic contacts with Y218. Polar contacts are also formed between the backbone of helix 3 and N172.
RMSD between the backbone atoms of rabbit PrP 121-230 and sheep PrP C is 0.80 Å (Haire et al., 2004).

The loop between β-strand-2 and helix-2 (residues 166-174), sometimes referred to as the β2-α2 loop or rigid-loop (Gossert et al., 2005), has been implicated as a possible amyloidogenic motif that forms a steric zipper (Sawaya et al., 2007). In the rabbit PrP C 121-230 structure, electron density for residues 165-175 is well defined (Fig. 2.15) indicating that the region is clearly ordered. The main-chain carbonyls of P165 and V166 form hydrogen bonds with the amides of Q168 and Y169, respectively, generating a short 3_10 helix, as seen in previous structures (Christen et al., 2008, 2009; Gossert et al., 2005; Liu et al., 1999). The side chains of D167 and Q168 are surface exposed but that of V166 forms hydrophobic interactions with Y218 of helix 3 possibly contributing to the stability of this loop (Fig. 2.16). The 3_10-helix preceding the N-terminus of helix 2 produces a small kink in the loop before the 171NQNS174 sequence.

2.3.9 Side chain interactions between residues 171 and 174 form a helix-capping motif that affects β-state propensity of rabbit PrP

The amino acid at position 174 (serine in rabbit PrP and asparagine in mouse PrP) is of particular interest because it is known to affect prion susceptibility (Vorberg et al., 2003). In rabbit PrP 121-231, the side chain carbonyl of N171 forms a hydrogen bond with the backbone amide of S174 and, in turn, the side chain hydroxyl of S174 binds to the backbone carbonyl of N171 (Fig. 2.17). These reciprocal side-chain to backbone hydrogen bonds, together with the flanking hydrophobic residues Y169 and F175, form a helix-capping motif similar to the so-called “hydrophobic staple” (Aurora and Rose, 1998; Seale et al., 1994) thereby bestowing additional stability upon the N-terminus of helix-2. The S174N mutation in this helix-capping motif causes rabbit PrP 121-231 to populate the β-state fraction in 4 M urea, pH 4.5, while the wild type rabbit PrP 121-231 does not populate the β-state under these conditions (Fig. 2.18). Disruption of this helix-capping motif increases the β-state propensity of rabbit PrP to match that of PrP from mouse, a more susceptible species to prion disease.
Figure 2.17: The helix-capping motif in rabbit PrP$^C$. Comparison of residues 170–174 of the rigid loop from rabbit PrP$^C$ structures and the lowest energy structures from the hamster and mouse PrP$^C$ NMR structure ensembles.
Figure 2.18: Comparison of the β-state PrP fraction between rabbit PrP 121-231 (○) and the S174N mutant (▲) as a function of urea concentration at pH (a) 5.5 and (b) 4.0. The lines are intended as a guide to the eye.
2.4 Discussion

Previous studies have reported that PrP may populate β-structured octameric and monomeric states (Baskakov et al., 2001, 2002; Swietnicki et al., 2000). We have investigated whether the propensity to form these states could underlie susceptibility to prion disease. The work presented here provides a novel method to quantify the populations of β-state equilibrium species under a broad range of conditions, and to assess the propensity for PrP from a given animal species to populate the β-state, allowing interspecies comparisons. By using a two-wavelength CD method, combined with analytical ultracentrifugation, we were able to measure the fractional concentrations of all conformational states present at equilibrium for several animal PrP proteins. We found that there was a gradation of propensities to populate the monomeric and octameric β-states, with a descending rank order of hamster, mouse, rabbit, horse, and dog PrP. Because PrP from susceptible species (hamsters and mice) had significantly higher β-state propensity than PrP from apparently resistant species (rabbits, horses, and dogs), this shows that β-state propensity varies with prion disease susceptibility.

The fact that PrP can populate a β-structured, non-native state has been known for a number of years (Hornemann and Glockshuber, 1998; Swietnicki et al., 2000; Zhang et al., 1997), and some have suggested that β-structured, oligomeric forms of PrP represent the more toxic and/or infectious form underlying prion diseases (Gerber et al., 2008a; Silveira et al., 2005). Much has been done to map the pathway of conversion, but historically, it has been difficult to quantify the relative abundances of potentially disease-relevant folding intermediates. It has been shown by stopped-flow techniques that prion disease-causing variants of human PrP have larger populations of partially folded, kinetic intermediates than the wild-type protein (Apetri et al., 2004). To my knowledge, the present study is the first to quantify relative abundances of octameric and monomeric β-structured species at equilibrium. Our finding that the β-state propensity tracks with prion disease susceptibility across several animal species lends support to the notion that β-state monomers and/or octamers play a prominent role in the mechanism of prion disease.
2.4.1 Contributions of sequence versus structure to prion disease susceptibility

Early experimental findings on prion susceptibility suggested that primary sequence similarity between the prions from the donor animal and the PrP from the recipient animal plays an important role in determining the outcome of prion transmission (Prusiner et al., 1990). For example, wild-type mice show low susceptibility to hamster prions, yet transgenic mice expressing hamster PrP are much more susceptible (Scott et al., 1989). However, there have been cases where primary sequence similarity does not determine a mammal’s susceptibility to prion strains. Bank voles are more susceptible to human prions than to hamster or mouse prions, despite the fact that there is greater sequence similarity between bank vole PrP and hamster or mouse PrP than between bank vole PrP and human PrP (Nonno et al., 2006). Also, variant CJD isolates from humans infect wild-type mice more readily than transgenic mice expressing human PrP (Hill et al., 1997). Furthermore, the fact that many prion strains exist as a single sequence of PrP, and still exhibit varying biochemical, histopathological, and neuropathological characteristics when introduced in the same species of mammal, suggests that prion susceptibility involves more than just primary sequence similarity between donor prions and host PrP (Béringue et al., 2008).

Our finding that PrP proteins from hamsters, mice, rabbits, horses, and dogs display a gradation of resistance to adopting the β-state, which correlates with prion susceptibility in these mammals, suggests that the conformational malleability of PrP, which is encoded in the primary sequence, is a determinant of prion susceptibility.

2.4.2 Structural features of native rabbit PrP that may impede conformational transition into the β-state

Studies have shown that by exposing scrapie-infected mouse neuroblastoma cells to molecules that stabilize PrP\(^\text{C}\), such as antibodies (Antonyuk et al., 2009) or chemical chaperones like trimethylamine N-oxide and dimethylsulfoxide (Tatzelt et al., 1996), one hinders the conformational transition into the PrP\(^\text{Sc}\) form, enabling improved survival when cultured cells are exposed to infectious scrapie material. Thus, stabilizing certain aspects of the native structure of PrP\(^\text{C}\) hinders the conformational transition into the PrP\(^\text{Sc}\) form. From our studies it appears that the difference in the β-state propensity of the five species studied resides within the covalent
structure of PrP. Therefore, certain atomic interactions in the natively-folded rabbit PrP structure may stabilize segments of secondary structure that reduce conversion to the β-state.

Structural studies on PrP from a variety of mammalian species indicate that single amino acid changes do not have drastic structural effects on the overall fold. Rather, variation in local interactions in the PrP monomer may affect the mechanism of conversion to the infectious form. For example, while mouse neuroblastoma cells expressing wild-type PrP are susceptible to scrapie infection, an N174S mutant of mouse PrP (the analogous residue in rabbit PrP) is not (Vorberg et al., 2003). In our high-resolution crystal structure of rabbit PrP 121-231, N171 and S174 are part of a hydrophobic staple-like helix-capping interaction at the N-terminus of α-helix 2 (Fig. 2.17). Such an arrangement is the first one described for any PrP structure. Our demonstration that disruption of this motif through the S174N mutation increases the β-state propensity of rabbit PrP (Fig. 2.18) to match that of mouse PrP implicates this motif as a determinant of prion infectivity.

2.4.3 Concluding remarks

Our current findings demonstrate that the propensity to form β-state PrP is a valid marker of prion disease susceptibility in hamsters, mice, rabbits, horses, and dogs; this relationship is likely to apply to other species. β-state propensity measurements were used in conjunction with high-resolution structural techniques to identify key amino acid side chain interactions that affect the conformational transition between helical and β-structured states of PrP - namely the hydrophobic staple-capping motif. The methods employed here have the potential to be used to test the efficacy of compounds that may reduce the effective β-state propensity of PrP, and which could therefore be of therapeutic benefit in prion disease.
Chapter 3

Public Library of Science ONE 8(5): e63047 (2013)

“N-Terminal Helix-Cap in α-Helix 2 Modulates β-State Misfolding in Rabbit and Hamster Prion Proteins.”

Sweeting, B., 1,3 Brown, E., 1,3 Khan, M.Q., 1,2 Chakrabartty, A., 1,2,3 Pai, E.F. 1,2,3,4

1Campbell Family Institute for Cancer Research, Ontario Cancer Institute/University Health Network. 2Department of Biochemistry, University of Toronto. 3Department of Medical Biophysics, University of Toronto. 4Department of Molecular Genetics, University of Toronto.

Statement of contribution: B.S. designed and performed experiments. E.B. contributed to protein production and crystallization. M.Q.K. contributed to experimental design and β-state propensity experiments. B.S., E.F.P. and A.C. contributed to writing of manuscript.
3.1 Introduction

Pathogenesis in prion disease involves conversion of the host protein, PrP, from the monomeric, primarily α-helical cellular form (PrP<sup>C</sup>) to a β-sheet enriched, aggregated infectious form (PrP<sup>res</sup>) (Aguzzi and Polymenidou, 2004). Susceptibility to prion disease varies depending on the donor species and strain of infectious prion as well as the species and genotype of the recipient. However, some species appear to be susceptible to prions from multiple sources, e.g. bank voles and hamsters (Bessen and Marsh, 1994; Kimberlin and Walker, 1977; Nonno et al., 2006; Piening et al., 2006), whereas others show a lower susceptibility to prion disease, e.g. rabbits, dogs, and horses (Gibbs and Gajdusek, 1973; Loftus and Rogers, 1997) or are even completely unaffected like birds (Moore et al., 2011). The determining factor in interspecies prion transmission appears to be intrinsic to the amino acid sequence of PrP (Prusiner et al., 1990). Even single amino acid differences between donor and recipient can give rise to a species barrier (Priola et al., 1994) or confer resistance to conversion of the recipient PrP<sup>C</sup> to the infectious form PrP<sup>Sc</sup> (Vorberg et al., 2003).

The sequence of PrP is highly conserved among mammals. Mutations in PrP could affect susceptibility and transmission of prion disease by causing changes in the structure of PrP<sup>C</sup> and/or the mechanism of its conversion to PrP<sup>res</sup>. The structure of PrP<sup>C</sup> from many different species has been determined by X-ray crystallography and NMR spectroscopy (Donne et al., 1997; Gossert et al., 2005; López Garcia et al., 2000; Lysek et al., 2005; Riek et al., 1996; Zahn et al., 2000), revealing that it is highly conserved between species and that differences in amino acid sequence have little effect on the overall fold. The loop between the second β-strand and the second α-helix (the β2-α2 loop) is one region of sequence diversity among species (Fig. 3.1) and has been often cited as a region of interest in the conversion of PrP<sup>C</sup> to PrP<sup>res</sup> as well as in its interactions with other proteins (Kaneko et al., 1997). NMR measurements have shown that single amino acid changes in this loop can cause differential backbone mobility. Of more functional importance, they can also lead to the spontaneous onset of disease when expressed in an in vivo model (Gossert et al., 2005; Sigurdson et al., 2009). These latter observations suggest that interactions within the β2-α2 loop may play a key role in the conversion to the infectious form.
The investigation of the effect of PrP sequence differences on the mechanism of PrP conversion has been severely hampered by the great difficulty of obtaining samples of purified infectious PrP\textsuperscript{Sc} suitable for high-resolution structural studies. Using recombinant PrP, several groups have been able to generate PrP refolded into a β-sheet enriched, oligomeric state under low pH and mild denaturing conditions (β-oligomer or β-state) (Baskakov et al., 2001, 2002; Hornemann and Glockshuber, 1998; Jackson et al., 1999; Swietnicki et al., 1997). For some of those constructs, toxicity and infectivity could be established (Legname et al., 2004; Novitskaya et al., 2006). Recent work in our laboratories has shown that the propensity of PrP to form the β-state correlates with the susceptibility of that species to prion disease. Additionally we found that rabbits, a species with low susceptibility to prion disease possess PrP with a helix-capping motif in the β2-α2 loop, which appears to hamper the formation of the β-state.

In this chapter, I will demonstrate that the presence of either serine (rabbit) or asparagine (hamster) residues in positions 170 and 174 of PrP not only affect the secondary structure of the β2-α2 loop but also the propensity with which the prion protein misfolds into β-state-rich octamers.

3.2 Materials and Methods

3.2.1 Molecular biology

The construction of the expression vector followed the procedure described in chapter 2.1.1; see also (Khan et al., 2010). Site-directed mutagenesis was performed using QuikChange mutagenesis kits (Stratagene, La Jolla, CA, USA) as per manufacturer’s instructions.

3.2.2 Protein expression and purification

All wild-type and mutant constructs of hamster and rabbit PrP 121-231 were expressed using a pET28a vector (Novagen, Gibbstown, NJ, USA). Proteins were expressed as inclusion bodies in the \textit{E. coli} BL21 AI strain (Invitrogen, Carlsbad, CA, USA). PrP\textsuperscript{C} was then refolded and purified using the method described in chapter 2.2.1 (Zahn et al., 1997).
3.2.3 Crystallization

Purified mutant rabbit proteins were crystallized using microseeding techniques. Seeds were produced from crystals of wild-type rabbit PrP\(^C\) grown as described in Kahn et al. (Khan et al., 2010). The resultant crystals of the mutant rabbit proteins were crushed, diluted 10\(^4\)-10\(^6\) fold and used for an additional round of microseeding. Crystals were then grown using the hanging drop vapour diffusion method in a solution of sodium cacodylate pH 6.5 with 2.0 M - 3.0 M sodium chloride as precipitant. The crystals grew as large flat plates in space group P2\(_1\)2\(_1\)2\(_1\) and their unit cell axes did not differ by more than 0.1 Å from a = 29.6 Å, b = 86.2 Å, and c = 87.1 Å. The crystals were flash-frozen at 100 K using 30% (v/v) glycerol as cryoprotectant.

3.2.4 Crystal structure determination

Each diffraction data set was collected from a single crystal at beamline 08ID-1 at the Canadian Macromolecular Crystallography Facility (Canadian Light Source, Saskatoon, SK, Canada). The diffraction data were processed using XDS (Kabsch, 2010) and the phase problem was solved with the help of molecular replacement techniques using Phaser (McCoy et al., 2007) and employing the wild-type rabbit PrP\(^C\) structure (PDB ID: 3O79) as search model. The structure was refined using a combination of the program packages RefMac (Murshudov et al., 2011), PHENIX (Adams et al., 2010) and Coot (Emsley and Cowtan, 2004). Statistics for data collection and refinement are given in Table 3.1. Coordinates and structure factors have been deposited in the Research Collaboratory for Structural Bioinformatics PDB under accession codes 4HMR, 4HMM and 4HLS.

3.2.5 Unfolding curves

Purified PrP\(^C\) protein stocks of 100 μM were dialyzed into 50 mM sodium phosphate pH 7.0, 80 mM NaCl, 0.5 mM EDTA for urea denaturation experiments at pH 7.0. PrP stocks were dialyzed in 50 mM sodium acetate, pH 5.0 or pH 4.5, 80 mM, 0.5 mM EDTA for β-state propensity measurements. Stocks were then diluted 10-fold to a final concentration of 10 μM into increasing concentrations of urea from 0 M - 9 M in buffer solutions identical to their respective dialysis buffers, followed by incubation at room temperature for a minimum of 3 days to allow them to reach equilibrium.
Table 3.1: Data collection and refinement statistics for the crystal structures of S170N, S174N and S170N/S174N mutants of rabbit PrP<sup>C</sup> 121-230. <sup>a</sup>Values in parentheses are for the outermost shell. 
<sup>b</sup>\( R = \frac{\sum_{hkl} \sqrt{\left| I(hkl) \right|} - \left| \sum_{i} I(hkl) \right|}{\sum_{hkl} \sqrt{\left| I(hkl) \right|}} \), where \( I(hkl) \) is the intensity of reflection \( hkl \), \( \sum_{hkl} \) is the sum over all reflections and \( \sum_{i} \) is the sum over \( i \) measurements of reflection \( hkl \). 
<sup>c</sup>\( R = \frac{\sum_{hkl} \sqrt{\left| F_{\text{obs}}(hkl) \right|} - \left| F_{\text{calc}}(hkl) \right|}{\sum_{hkl} \sqrt{\left| F_{\text{obs}}(hkl) \right|}} \), where \( F_{\text{obs}} \) and \( F_{\text{calc}} \) are the observed and calculated structure-factor amplitudes, respectively. \( R_{\text{free}} \) is calculated for a randomly chosen 5% of reflections that were not used for structure refinement and \( R_{\text{work}} \) is calculated for the remaining reflections.

<table>
<thead>
<tr>
<th>Data Statistics</th>
<th>S174N</th>
<th>S170N</th>
<th>DBL</th>
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<td>P2&lt;sub&gt;1&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;</td>
<td>P2&lt;sub&gt;1&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;2&lt;sub&gt;1&lt;/sub&gt;</td>
</tr>
<tr>
<td>Cell constants</td>
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<td></td>
</tr>
<tr>
<td>a (Å)</td>
<td>29.6</td>
<td>29.5</td>
<td>29.5</td>
</tr>
<tr>
<td>b (Å)</td>
<td>86.3</td>
<td>86.1</td>
<td>86.4</td>
</tr>
<tr>
<td>c (Å)</td>
<td>87.1</td>
<td>87.0</td>
<td>87.1</td>
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<td>60 – 1.45 (1.50 – 1.45)</td>
<td>60 – 1.60 (1.70-1.60)</td>
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<td>300,690 (8,742)</td>
<td>216 428 (35,877)</td>
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<td>39,174 (2,798)</td>
<td>30,243 (4,938)</td>
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<td>7.7 (3.1)</td>
<td>7.2 (7.3)</td>
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<td>Completeness (%)</td>
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<td>97.3 (73.2)</td>
<td>99.7 (99.4)</td>
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<td>&lt;( I/\sigma I )</td>
<td>11.6 (46.0)</td>
<td>5.9 (53.8)</td>
<td>7.9 (37.2)</td>
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</table>

<table>
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<tr>
<th>Refinement Statistics</th>
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</thead>
<tbody>
<tr>
<td>Final ( R_{\text{cryst}} ) (%)</td>
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<td>14.6</td>
<td>15.5</td>
</tr>
<tr>
<td>( R_{\text{free}} ) (%)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.3</td>
<td>18.0</td>
<td>20.7</td>
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<td>Solvent (%)</td>
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<td>39.9</td>
<td>40.1</td>
</tr>
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<td>2</td>
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<tr>
<td>No. of all atoms</td>
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<td>1954</td>
<td>1892</td>
</tr>
<tr>
<td>No. of water molecules</td>
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<td>186</td>
<td>126</td>
</tr>
<tr>
<td>No. of sodium ions</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>No. of chloride ions</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Average B-factor (Å&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>15.8</td>
<td>18.2</td>
<td>20.8</td>
</tr>
<tr>
<td>Ramachandran plot</td>
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<td></td>
<td></td>
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<tr>
<td>Most favorable areas</td>
<td>99.5%</td>
<td>99.5%</td>
<td>98.9%</td>
</tr>
<tr>
<td>R.M.S.D. from ideal geometry</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lengths (Å)</td>
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<td>0.016</td>
<td>0.013</td>
</tr>
<tr>
<td>Angles (°)</td>
<td>1.532</td>
<td>1.731</td>
<td>1.505</td>
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Circular dichroism ellipticity was then measured using a 1mm path length in a quartz cuvette on a Jasco J-815 CD Spectrometer (Easton, MD, USA). Ellipticity values were measured at 220 nm and 229 nm every 0.5 s for 120 s and averaged. The free energy of unfolding ($\Delta G_{\text{unfolding}}$) of wild-type and mutant PrP at pH 7 were computed by fitting the data to a 2-state (N$\rightarrow$U) transition model. The proportion of $\beta$-state was determined using the method described by Khan et al. (Khan et al., 2010).

3.2.6 Time-resolved size exclusion chromatography and circular dichroism

Concentrated stocks (100 $\mu$M) of wild-type rabbit and hamster PrP$^C$ and the double mutants S170N/S174N rabbit PrP$^C$ and N170S/N174S hamster PrP$^C$ were dialyzed into 50 mM sodium acetate, pH 4.0, 80 mM NaCl, 0.5 mM EDTA. At $t = 0$, the concentrated protein stock was diluted to 10 $\mu$M protein and a final concentration of 4 M urea. A 200 $\mu$L sample was immediately injected onto a Superdex S200 10/30 column equilibrated in identical buffer and run at 0.5 ml/min while monitored using the absorbance at 280 nm. Additional samples of 200 $\mu$L were injected at indicated time intervals. At $t = 0$ and $t = 4$ hrs, CD wavelength scans were performed on the urea diluted samples using a 1mm path length in a quartz cuvette between 205 nm – 250 nm.

3.3 Results

3.3.1 Crystal structures of S174N, S170N and S170N/S174N mutants of rabbit PrP$^C$ 121-230

PrP from hamsters, a species quite susceptible to prion disease, has asparagines at residues 170 and 174 of its amino acid sequence, whereas rabbits, which are less susceptible to prion disease, incorporate serine at the equivalent positions (Fig. 3.1). In the previously determined structure of wild-type rabbit PrP$^C$ 121-230 (Khan et al., 2010), the side chain of S174 forms a hydrogen bond with the backbone of N171 and vice versa, creating a hydrophobic staple helix-cap motif (Presta and Rose, 1988). I hypothesized that these sequence differences affect the formation of
Figure 3.1: Alignment of hamster and rabbit PrP 121-231 amino acid sequences. The $\beta$2-$\alpha$2 helix-cap is highlighted in blue and the secondary structure locations are shown below.
the helix-cap and consequently the folding behavior of PrP, its refolding to the β-state, and thereby its conversion to the infectious form.

To test this hypothesis, I introduced the single mutations S170N and S174N as well as the double mutation S170N/S174N into rabbit PrP\(^C\) 121-230, successively changing the rabbit sequence in the β2-α2 loop to that of wild-type hamster PrP\(^C\). Similarly, the single mutants N170S and N174S as well as the double mutant N170S/N174S of hamster PrP\(^C\) 121-231 were constructed, to stepwise transform its β2-α2 loop to the wild-type rabbit PrP\(^C\) sequence.

The structures of the S170N, S174N and S170N/S174N mutants of rabbit PrP\(^C\) 121-230 were solved by x-ray crystallography to resolutions of 1.4 Å, 1.6 Å and 1.5 Å, respectively. Unfortunately, exhaustive attempts to crystallize wild-type hamster PrP\(^C\) 121-231 and its three helix-cap mutants did not meet with success.

The structures of the three rabbit PrP\(^C\) 121-230 mutants show the same dimeric arrangement of PrP in the asymmetric unit that was observed in the wild-type structure published previously. Electron density was observed for residues 126-230 in one chain, whereas only residues 126-220 were visible in the other, most likely the result of differences in crystal packing. The dimer interface buries 1620 Å\(^2\) of surface area and involves 17 intermolecular hydrogen bonds and 6 salt bridges. Although a dimeric PrP has not been unequivocally identified under “native” solution conditions, the crystallographic arrangement is predicted to be stable by the Protein Interfaces, Surfaces and Assemblies analysis program (PISA) (Krissinel and Henrick, 2007). The overall folds of the three rabbit PrP\(^C\) 121-230 mutants (S170N, S174N and S170N/S174N) are very similar to the wild-type structure (Figure 3.2). They all adopt the classic PrP\(^C\)-fold with three α-helices encompassing residues 143-157 (helix-1), 171-193 (helix-2), and 199-230 (helix-3) as well as a small two-stranded, anti-parallel β-sheet consisting of residues 128-130 (β-strand-1) and 162-164 (β-strand-2). Comparing the wild-type rabbit PrP\(^C\) 121-230 and the S170N, S174N and S170N/S174N mutants, the r.m.s.d. between all four structures is 0.51 Å for all backbone atoms.

The β2-α2 loops in the three mutant structures consist of residues P165-N171 and are followed by the first turn of helix-2 formed by residues Q172-F175. Similar to the wild-type, residues N167-169 form a 3\(_{10}\)-helical turn in all three mutants. In wild-type rabbit PrP, the side chain
Figure 3.2: Representative structures of S170N, S174N and S170/S174N mutants of rabbit PrP<sup>C</sup> 121-230. All three structures have the typical PrP fold of three α-helices and a small two-stranded β-sheet. All three structures displayed only 0.5 Å<sup>2</sup> root-mean-squared deviation between equivalent Cα positions when compared to each other and to wild-type. Insets: close-up views of the residues forming the helix-cap in the wild-type and their equivalents in the three mutant structures of rabbit PrP<sup>C</sup> 121-231. The reciprocal interactions between the backbone and side chains of S170 and S174 in the wild-type are ablated in the S174N and S170N/S174N mutant structures. The side chain of the mutant S170N is solvent exposed and disordered, but may weakly interact with the neighbouring N171.
carbonyl oxygen of N171 hydrogen bonds to the backbone amide of S174 and the side chain hydroxyl of S174 interacts with the backbone carbonyl oxygen of N171. These reciprocal interactions, flanked by the hydrophobic interactions of Y169 and F175, form the basis for the hydrophobic staple helix-cap. This arrangement is altered in the S170N rabbit PrP<sup>C</sup> structure with the side-chains of the mutant N170 and S174 no longer interacting with N171 to form the helix-cap. The electron density surrounding the N170 side chain is fairly weak suggesting it may be disordered (Fig. 3.2 and Fig. 3.3B). The side chain amide and backbone carbonyl of N171 can hydrogen bond with the side chain hydroxyl oxygen and backbone amide of S174, respectively. In the crystal structure of the rabbit S174N mutant PrP<sup>C</sup>, the mutated N174 no longer hydrogen bonds with the backbone of N171, ablating the reciprocal interactions forming the helix-cap and leaving the backbone of N171 exposed to solvent (Fig 3.2 and Fig. 3.3C). The structure of the rabbit S170N/S174N double mutant PrP<sup>C</sup> shows the features of both mutants with N170 remaining disordered as it is in the S170N mutant and N174 no longer hydrogen bonding to N171 (Fig 3.2 and Fig. 3.3D), adding up to the same loss of helix-cap-forming interactions found in the single S174N rabbit PrP<sup>C</sup> mutant. Changing residues in the β2-α2 loop in rabbit PrP<sup>C</sup> to those of hamster PrP<sup>C</sup> causes key interactions in the helix-cap to be lost. Our inability to crystallize hamster PrP carrying mutations to the corresponding rabbit amino acids prevented us from testing whether a helix-cap could be introduced into hamster PrP<sup>C</sup>. However, we did seek to determine if these N170S, N174S, and N170S/N174 mutations would affect the stability and β-state refolding behavior of hamster PrP.

### 3.3.2 Urea induced unfolding of wild-type and mutants of rabbit and hamster PrP<sup>C</sup>

The presence of helix-caps at the N-termini of α-helices compensates for the decreased stability caused by exposure of backbone amide groups to solvent. Therefore, I hypothesized that the presence or absence of the helix-cap in rabbit and hamster PrP<sup>C</sup> 121-231 would have a corresponding effect on their free energies of unfolding. I performed urea melts on the wild-type PrP<sup>C</sup> 121-231 of rabbit and hamster as well as on the three helix-cap mutants of each of the two proteins (Fig. 3.4). We found that wild-type rabbit PrP<sup>C</sup> 121-230 is significantly more stable than hamster PrP<sup>C</sup> with free energies of unfolding of 6.51 kJ/mol and 5.6 kJ/mol, respectively.
Figure 3.3: $2\text{F}_O-\text{F}_C$ electron density map at 1.5 $\delta$ overlaid on the $\beta_2-\alpha_2$ loops from respective structures of (A) wild-type rabbit PrP 121-230 (B) S170N mutant rabbit PrP 121-230, (C) S174N mutant rabbit PrP 121-230 and (D) S170N/S174N mutant rabbit.
Figure 3.4: Urea-induced unfolding curves of wild-type and helix-cap mutants of rabbit and hamster PrP<sup>C</sup> 121-230. Samples of wild-type and helix-cap mutants of hamster and rabbit PrP<sup>C</sup> 121-230 were diluted to 10 µM in 50 mM sodium phosphate pH 7.0 with indicated concentrations of urea and incubated at room temperature for 72 hours. The proportion folded was then determined by measuring ellipticity by circular dichroism at 220 nm and normalizing between folded and unfolded baselines. (A) In Hamster PrP<sup>C</sup>, the mutations of N170S and N174S caused a moderate increase in the free energy of unfolding of hamster PrP<sup>C</sup> whereas the N170S/N174S double mutation showed an additive effect. (B) In Rabbit PrP<sup>C</sup>, the mutation of S170N caused a small drop in the free energy of unfolding whereas for the mutations of S174N and S170N/S174N the decrease was more significant.
Table 3.2: Free energy of unfolding of wild-type rabbit and hamster PrP<sup>C</sup> 121-231 as well as S170N, S174N, S170N/S174N mutants of rabbit PrP<sup>C</sup> 121-230 and N170S, N174S, and N170S/N174S mutants of hamster PrP<sup>C</sup>.

<table>
<thead>
<tr>
<th>Species / Mutation</th>
<th>ΔG&lt;sub&gt;unfolding&lt;/sub&gt;</th>
<th>ΔΔG vs. WT</th>
<th>m</th>
<th>Urea ½</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hamster WT</td>
<td>5.68 ± 0.12</td>
<td>-1.12 ± 0.02</td>
<td>5.09 ± 0.15</td>
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<tr>
<td>Hamster N170S</td>
<td>6.08 ± 0.20 + 0.4</td>
<td>-1.11 ± 0.04</td>
<td>5.50 ± 0.26</td>
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<tr>
<td>Hamster N174S</td>
<td>6.41 ± 0.22 + 0.7</td>
<td>-1.10 ± 0.04</td>
<td>5.77 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Hamster DBL</td>
<td>6.84 ± 0.21 + 1.1</td>
<td>-1.13 ± 0.03</td>
<td>6.05 ± 0.26</td>
<td></td>
</tr>
<tr>
<td>Rabbit WT</td>
<td>6.18 ± 0.22</td>
<td>-1.02 ± 0.04</td>
<td>6.04 ± 0.31</td>
<td></td>
</tr>
<tr>
<td>Rabbit S170N</td>
<td>6.05 ± 0.21 - 0.2</td>
<td>-1.06 ± 0.04</td>
<td>5.73 ± 0.28</td>
<td></td>
</tr>
<tr>
<td>Rabbit S174N</td>
<td>5.60 ± 0.13 - 0.6</td>
<td>-1.08 ± 0.02</td>
<td>5.16 ± 0.17</td>
<td></td>
</tr>
<tr>
<td>Rabbit DBL</td>
<td>5.89 ± 0.15 - 0.3</td>
<td>-1.13 ± 0.03</td>
<td>5.23 ± 0.19</td>
<td></td>
</tr>
</tbody>
</table>
The S170N single mutation reduced the rabbit PrP\textsuperscript{C} ΔG of unfolding to 5.9 kJ/mol, while the S174N change and the S170N/S174N double mutant displayed ΔGs of 5.45 kJ/mol and 5.7 kJ/mol, respectively (Fig 3.4A). Conversely, asparagine to serine mutations in the hamster PrP\textsuperscript{C} sequence (Fig 3.4B) increased the free energies of unfolding to 5.9 kJ/mol and 6.5 kJ/mol for mutants N170S and N174S, respectively. The N170S/N174S double mutant showed an additive effect with a ΔG of 6.8 kJ/mol (Table 3.2). These data support my hypothesis that introducing serine residues into positions 170 and 174 of hamster PrP\textsuperscript{C}, residues that are involved in the helix-cap motif in rabbit PrP\textsuperscript{C}, increase the protein’s stability; conversely, their replacement with asparagine residues in the rabbit PrP\textsuperscript{C} sequence will decrease the stability of this protein.

3.3.3 β-State propensity measurements

Previous work in our lab has shown that species differences in the sequence of PrP can affect the propensity of PrP to populate the β-state upon incubation at low pH in the presence of urea. I decided to test whether the mutations we had made in the β2-α2 helix-cap also had these effects in rabbit and hamster PrP (121-231).

Wild-type rabbit PrP (121-230) does not form the β-state at pH 5.0 (Fig. 3.5A) and forms a maximum of 42.8% β-state at pH 4.5 (Fig. 3.5B); compared to wild-type hamster PrP, which reaches 96 % and 100% β-state at pH 5.0 and pH 4.5, respectively (Fig. 3.5C and D).

Introduction of the single mutations S170N and S174N and the double mutation S170N/S174N into rabbit PrP each cause respective increases in the β-state propensity of PrP. At pH 5.0, no β-state forms in the S170N mutant, whereas the S174N rabbit PrP begins to form the β-state to a maximum of 12.6% and the S170N/S174N double mutant forms to a maximum of 37% (Fig. 3.5A). At pH 4.5, the S170N and S174N single mutants form 54% and 66.6% β-state and the double mutant sees a larger increase to 84.9% β-state (Fig. 3.5B).

Conversely, introduction of mutations into hamster PrP cause a decrease in the population of β-state PrP at low pH. While the wild type hamster PrP displays 100% β-state at pH 5, the single N170S and N174S mutations show a decrease to 76.4% and 66.9% β-state respectively, whereas the double mutant N170S/N174S reaches a maximum of 61.3% (Fig. 3.5C). At pH 4.5, all three hamster PrP mutants reach 100% β-state but there are slight differences in the urea concentration.
**Figure 3.5**: β-state propensity measurements of wild-type and helix-cap mutants of rabbit and hamster PrP<sup>C</sup> 121-230. Samples were diluted to 10 µM in sodium acetate buffer at pH 5.0 or pH 4.5 with indicated concentrations of urea. After 72 hr incubation, the proportion of β-state was determined using the two-wavelength CD method (see Materials and Methods). (A) In hamster PrP at pH 5.0, the helix-cap mutations cause a decrease in the β-state population from 96% in the wild-type to 76%, 67%, and 61% in the N170S, N174S and the N170S/N174S mutants, respectively. (B) At pH 4.5, the wild-type and helix-cap mutants all eventually populate the β-state to 100%, but to reach it they require increased urea concentrations. (C) In Rabbit PrP at pH 5.0, only the S174N and S170N/S174N mutants begin to populate the β-state to 15% and 35%, respectively. (D) At pH 4.5, the helix-cap mutations S170N, S174N and S170N/S174N cause an increase in the maximum β-state populations to 54%, 67%, and 85%, respectively, compared to 43% in the wild-type. Overall error of 4.5% was estimated from the difference between observed and fitted values.
at which the β-state begins to form indicating differences in the stability of the PrP<sup>C</sup> state of these mutants or their ability to form the β-state (Fig 3.5D). These results demonstrate that single site mutations in this helix-cap motif in PrP affect the β-state propensity of PrP, and the mutations have an additive effect.

3.3.4 Time-resolved size-exclusion chromatography and circular dichroism

Previously, I showed that the β-state of PrP consists of a mixture of β-state monomers and β-state octamers (Khan et al., 2010). In order to determine whether species differences and mutations in PrP have an effect on the formation of β-state monomers and octamers, I monitored their proportions kinetically using circular dichroism and size-exclusion chromatography (SEC). Proteins at 100 μM concentration were dialyzed into their respective buffers at pH 4.0 without urea. At t = 0, they were diluted to 10 μM in identical buffer but with 4M urea and injected onto the columns at the indicated intervals.

Upon diluting the concentrated stock into 4 M urea at pH 4.0, CD wavelength scans of both the hamster wild-type and N170S/N174S double mutant show them converting immediately to primarily β-sheet secondary structure (Fig. 3.6). SEC elution profiles of both show nearly 100% monomer at t = 0 which converts to a maximum 78% octamer in the wild-type and 65% octamer in the hamster double mutant after 4 hours; no intermediate species were detected (Fig 3.7A). The extent of octamer assembly is slightly lower in the double mutant compared to the wild-type hamster PrP. The CD-signals for both the wild-type hamster and double mutant do not change significantly over the course of 4 hours, indicating constant β-structure (Fig. 3.6).

Similar to hamster PrP, both the wild-type and S170N/S174N rabbit PrP convert to primarily β-sheet secondary structure immediately after dilution to 10 μM PrP in 4 M urea (Fig. 3.6). However, the SEC elution profiles indicate that the assembly of β-state monomers into β-octamers is significantly slower in rabbit wild-type and double mutant compared to the hamster constructs (Fig. 3.7B). Both rabbit wild-type and double mutant at t = 0 remained monomeric, indicating that initially these both exist as β-state monomers. Over time the proportion of octamers in both the wild-type and double mutant increases, to a maximum of 33% after 4 hours; again no intermediate species were detected. In contrast to what had been seen with wild-type
Figure 3.6: Circular dichroism wavelength scans of (A) wild-type hamster PrP 121-231, (B) wild-type rabbit PrP 121-230, (C) hamster PrP 121-231 N170S/N174S, (D) rabbit PrP 121-230 S170N/S174N. At $t = 0$, samples of 100 µM PrP in 50 mM sodium acetate pH 4.0, 80 mM NaCl were diluted to a final PrP concentration of 10 µM and 4 M urea in identical buffer. Circular dichroism wavelength scans were then performed at $t = 0$ and $t = 4$ hrs between 205-250 nm at 0.1 nm intervals. The CD spectrum of $\alpha$-helical PrPC is included for comparison.
Figure 3.7: Time-resolved size exclusion chromatography analysis of wild-type and double helix-cap mutants of rabbit and hamster PrP. At $t = 0$, samples were diluted to a final PrP concentration of 10 µM in target buffer and immediately injected onto an S200 10/30 column. Fractional concentrations of monomer and octamer were calculated; no intermediate species were detected.
and mutant hamster PrP, rates and extent of β-octamer formation did not differ for wild-type and S170N/S174N rabbit PrP. These results confirm our earlier observation that the β-state of PrP consists of mixtures of β-sheet-rich monomer and octamer. In addition, we observed that the extent of assembly of β-state monomers into octamers differs between species and that mutations in the sequence of PrP can influence these transformations.

3.4 Discussion

3.4.1 Comparison of the crystal structures of rabbit PrP 121-231 to other PrP\(^{C}\) structures

The crystal structures of the S170N, S174N, and S170N/S174N mutants of rabbit PrP\(^{C}\) (121-231) show that hydrogen bonding interactions observed in the helix-cap of wild-type rabbit PrP\(^{C}\) can be removed by mutating the serine residues involved. Few mammalian species have serine at position 174 of their PrP sequence; pigs are one of them and, interestingly, also display a low susceptibility to prion disease. Many susceptible species have asparagine at positions 170 and 174; by mutating the rabbit PrP\(^{C}\) serines to asparagines I have shown that interactions involving S174 in the rabbit PrP\(^{C}\) helix-cap are disrupted suggesting that this motif may be absent in PrP\(^{C}\) from species that are more susceptible to prion disease. Supporting this idea are the results of Vorberg et al., who found that the N173S mutation in mouse PrP (equivalent to rabbit residue 174) imparts resistance against the RML strain of scrapie prions in a neuroblastoma cell culture model (Vorberg et al., 2003). However, the PrP sequence from elk, a species susceptible to chronic wasting disease, incorporates N170 and T174. Expression of the S170N /N174T mutant using a mouse model showed an increase in prion disease susceptibility (Sigurdson et al., 2009). This indicates that the presence or absence of S174 and its role in the helix-cap may play a role in infectivity.

Structural differences within the β2-α2 loop of various PrP\(^{C}\) proteins had already been observed when the first PrP\(^{C}\) structures were determined. Differences in the mobility involving the β2-α2 loop were described comparing the NMR structures of mouse and hamster PrP (Donne et al.,
1997; Riek et al., 1997), which contained mobile and rigid β2-α2 loops, respectively. Gossert et al. showed that the structure of elk PrP\(^C\) incorporated a rigid β2-α2 loop, which could be introduced into mouse PrP by mutating the mouse N173 to threonine (equivalent to 174 in other species)(Gossert et al., 2005). It was hypothesized that the differences in mobility could be caused by differences in hydrogen bonding within the β2-α2 loop but no specific bonds could be assigned. Subsequent structural work has suggested that the mobile or rigid loop may be a determinant in a species’ susceptibility to prion disease (Christen et al., 2008; Lysek et al., 2005). A recent NMR structure of rabbit PrP\(^C\) showed differences in stability and hydrogen bonding throughout the molecule when S174 is mutated to N174, but no specific change in interactions involving the residue where detected (Wen et al., 2010).

My structures and biophysical characterization of the wild-type and helix-cap mutants of rabbit and hamster PrP have identified a structural motif that clearly affects the folding behavior of PrP. Other recent studies have found additional features of rabbit PrP that may also contribute to the reduced susceptibility of rabbits to prion disease. Amino acid differences present in the C-terminus of rabbit PrP have been shown to interfere with PrP\(^Sc\) formation (Nisbet et al., 2010) and molecular dynamics simulations have suggested that salt bridges between D177-R163 and D201-R155 may increase global stability, preventing conversion to PrP\(^Sc\) (Zhang, 2010, 2011). Further understanding of the relationship between these features and a species’ susceptibility \textit{in vivo} would provide insight into the biophysical mechanism of the conversion of PrP to the infectious form and the pathogenesis of prion disease.

\textbf{3.4.2 \textit{Effect of mutations on β-state propensity}}

The result of our urea denaturation experiments involving wild-type rabbit PrP and several of its mutants show that disruption of the helix-cap reduces the folding stability of PrP\(^C\). Conversely, introduction of serine residues involved in the rabbit PrP\(^C\) helix-cap into hamster PrP\(^C\) increases the latter’s stability. Although the helix-cap could not be directly observed in the hamster PrP mutants due to my inability to crystallize it, the observed increase in stability similar to that of the wild-type rabbit protein makes its presence probable. Interestingly, the scale of ΔΔG caused by the mutations (Table 2) is approximately the scale expected by the gain or loss of a hydrogen bond, giving further weight to the idea that this helix-cap motif contributes to global stability. In addition, the presence of residues involved in the helix-cap also affects the propensity of rabbit
and hamster PrPC (121-230) to populate the β-state. Under conditions that promote β-state formation, amino acid changes in the S170N, S174N and S170N/S174N mutants of rabbit PrPC cause successive increases in the population of the β-state at equilibrium, whereas the reverse mutations in hamster PrP (N170S, N174S and N170S/N174S) cause successive decreases in β-state population. This indicates that the helix-cap plays a role in preserving the α-helical fold of PrPC and limits misfolding to the β-state. Without a detailed structure of the β-state, it is difficult to speculate on how the helix-cap would affect its structure and formation. However, in the urea-induced unfolding of PrP from various species, the β-sheet portion of PrPC unfolds first, followed by helices-2 and -3 (Julien et al., 2009). The helix-cap is present at the junction between these two parts of PrPC and could in some way inhibit their dissociation and the formation of the β-state.

Our data provide clues to how sequence differences affect the biophysical behavior of PrP. Determining the effects of mutations and species differences on prion infectivity has been hampered by the lack of detailed structural information regarding the infectious form, PrPSc. To circumvent this, many groups have studied the misfolding of PrP in vitro using the recombinant β-state of PrP. To date, β-state PrP has not been shown to be infectious on its own although complex procedures have been published that achieve infectivity but require additional cofactors (Raymond et al., 2012; Wang et al., 2010). So, it is still unclear how the β-state relates to the infectious form in vivo. However, several similarities between the β-state and the infectious form have been observed. The β-state is β-sheet rich and oligomeric (Baskakov et al., 2001, 2002; Horne mann and Glockshuber, 1998; Swietnicki et al., 1997) as is the most infectious form of PrPSc. It can also be protease-resistant and toxic to cells in vitro (Jackson et al., 1999; Novitskaya et al., 2006). Previous work in our lab has shown that the propensity to refold into the β-state also correlates to prion disease susceptibility between species and we have now linked this finding to single amino acid differences in the sequence of PrP, similar to what was observed in vivo (Priola et al., 1994; Prusiner et al., 1990). Although the β-state probably does not represent an absolute replica of PrPSc, the observed similarities argue that the β-state is well suited for study of PrP folding behaviour in vitro providing a basis to decide which observations are the most promising candidates for testing in in vivo models.
3.4.3  Kinetics of formation of the β-state and the transmission barrier

The β-state of PrP proteins consists of β-sheet-rich monomers that assemble into octamers (Khan et al., 2010). Under β-state-forming conditions, both PrPC 121-230 proteins from rabbit and hamster rapidly convert to the β-state monomeric form. However, the rate and extent of assembly of β-state monomers into octamers differs between species and is also affected by single amino acid changes in the helix-cap. This suggests that PrP from susceptible species assembles into octamers more quickly and to a greater extent than PrP from a resistant species, further supporting a connection between the β-state propensity and the susceptibility of a given species to prion disease.

Hypotheses regarding the transmission barrier have proposed that the rate of conversion of PrPC to PrPSc plays an important role in prion disease pathogenesis. In a recent publication, Sandberg et al. report that prion disease pathogenesis occurs in two stages, an accumulation of prion titer to a plateau phase followed by the onset of clinical symptoms (Sandberg et al., 2011). Our data now demonstrate that the PrP sequence from a given species can affect the rate and extent of misfolding of PrP to the β-state and its assembly into oligomers. This suggests that not only the level of expression but also the rate of conversion of PrPC to PrPSc and the latter’s accumulation may affect the pathogenesis of prion disease. PrPC from less susceptible species may have sequence and structural features, such as the helix-cap motif we have observed, that reduce the rate and extent to which conversion occurs, allowing the cell to compensate through proteolysis and clearance. While PrPC from susceptible species, lacking such structural motifs, may convert rapidly and efficiently to PrPSc, reaching concentrations that bring about clinical symptoms.

Additional factors must also be taken into consideration. A recent publication has demonstrated that although rabbits are less susceptible to infection with PrPSc from other species, they are still susceptible to prion disease albeit with a much lower attack rate and longer initial presymptomatic incubation periods (Chianini et al., 2012). Additionally, infectious material isolated from infected rabbits is able to re-infect other rabbits with a much higher attack rate and shorter incubation time in an example of prion adaptation. This demonstrates that although the sequence and structural features of PrP can lower prion disease susceptibility, they do not lead to complete immunity.
The effect of altered interactions within the helix-cap region on the conversion of PrP\textsuperscript{C} to the β-state suggests that this region plays an important role in the mechanism of conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc}. In the search for therapeutics and methods to prevent the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{Sc} it should be beneficial to focus efforts on regions such as the β2-α2 loop that have been shown to affect susceptibility to disease transmission.
Chapter 4

Discussion and Future Directions
4.1 Prion disease susceptibility of species correlates with PrP β-state propensity

According to the transmission barrier hypothesis, the susceptibility of a species to prion disease is dependent on its PrP sequence and strain of the infecting prion. Historically, some species, such as hamster and bank voles, appear to be more susceptible to prions from a variety of sources and strains (Bessen and Marsh, 1992a; Gibbs and Gajdusek, 1973; Kimberlin and Walker, 1977; Nonno et al., 2006), whereas other species such as rabbits, pigs and dogs have proven to be far less susceptible and have required larger infectious doses (Ryder et al., 2000) or pre-adaptation of prions using in vitro methods (Chianini et al., 2012) to achieve pathogenesis.

To account for this, we hypothesized that the observed differences in susceptibility were related to the ability of PrP from a given species to adopt the conformations necessary to facilitate its conversion to PrP\textsuperscript{res}. The conformational selection hypothesis states that each prion strain represents a stable conformation of PrP\textsuperscript{res} and the ability of a given species to be infected is dependent on the ability of its own PrP to stably adopt the PrP\textsuperscript{res} conformation of that strain. It stands to reason that if PrP from a species is capable of adopting many stable PrP\textsuperscript{res} conformations, then that species will be susceptible to many different prion strains. Vice versa, if a species has a PrP sequence that is compatible with only a few stable PrP\textsuperscript{res} conformations, then that species will be less susceptible.

Studying the ability of PrP from different species to adopt PrP\textsuperscript{res} conformations was impractical because making PrP\textsuperscript{res} \textit{in vitro} still remains very difficult, although some progress has been made. Therefore as a surrogate, I studied the ability of recombinant PrP to adopt the β-state, a misfolded form of PrP that is rich in β-sheet secondary structure and oligomeric (Baskakov et al., 2001; Hornemann and Glockshuber, 1998; Swietnicki et al., 1997). Using a novel method of two-wavelength circular dichroism measurements, I was able to determine the propensity of recombinant PrP from a variety of species to adopt the β-state conformation under conditions of low pH and moderate concentrations of urea. Species that historically have had a higher
susceptibility to prion infection populated the β-state to a higher level relative to those that are less susceptible.

Further characterization of β-state PrP and validation of the two-wavelength CD method using analytical ultracentrifugation and size exclusion chromatography revealed that the β-state did not consist solely of a β-sheet rich oligomer as was believed previously, but rather an equilibrium of β-sheet enriched monomer and octamer. Furthermore, the β-state is toxic to cells in culture.

Rabbits as a species have been historically known to be resistant to prion infection (Gibbs and Gajdusek, 1973). Studies into the source of rabbits’ resistance to prion infection revealed that rabbit PrP\textsuperscript{C} possesses several residues which inhibit its conversion to PrP\textsuperscript{res} when expressed and challenged in mouse neuroblastoma cells (Vorberg et al., 2003). To investigate the structural basis of these residues, I determined the crystal structure of rabbit PrP\textsuperscript{C} 121-230. This structure revealed that S174, a residue that has been shown to impart conversion resistance to rabbit PrP\textsuperscript{C}, forms critical hydrogen bonding contacts in a helix-cap motif at the N-terminus of helix-2 of PrP\textsuperscript{C}.

These observations support my hypothesis that prion disease susceptibility is related to β-state propensity. The relation of propensity and the concentration of β-octamer aligns well with the predictions of the conformational and kinetic selection model (Collinge and Clarke, 2007). The kinetic selection model of prion toxicity proposes a hypothetical toxic species PrP\textsuperscript{L} that is responsible for prion disease pathogenesis and has properties similar to that of the β-state octamer. In susceptible species, upon prion infection PrP\textsuperscript{res} interacts with the host PrP\textsuperscript{C} and converts it into PrP\textsuperscript{L}. As PrP\textsuperscript{L} accumulates, it becomes toxic and eventually matures into nontoxic PrP\textsuperscript{res} amyloid. In a resistant species, the resultant PrP\textsuperscript{L} and PrP\textsuperscript{res} interactions are unstable and both forms are cleared by proteolytic mechanisms, never allowing them to accumulate to toxic levels.

The hypothesized PrP\textsuperscript{L} species has been proposed to be oligomeric, similar to what has been seen with other protein misfolding diseases such as Alzheimer's Disease (Haass and Selkoe, 2007). Although it is tempting to speculate that the recombinant PrP β-octamer is representative of PrP\textsuperscript{L}, there is little direct evidence to support this claim as PrP oligomers have not been isolated and characterized \textit{in vivo}. The β-state octamer is rich in β-sheet, oligomeric and toxic, similar to what has been proposed for PrP\textsuperscript{L}. The ability to adopt the β-state octamer may
eventually be related to the propensity of PrP from a given species to adopt the PrP\(^{L}\) conformation.

It must be noted that rabbits are not completely immune to prion disease and can be infected with prions that were “pre-adapted” to infect rabbits using PMCA (Chianini et al., 2012). Upon adaptation by serial transmission in rabbits, it was found that the resultant strain had a high attack rate against rabbits and was able to overcome what is believed to be their natural low susceptibility to prion disease. These results do not explicitly contradict my hypothesis, but demonstrate that it is possible to find a PrP\(^{res}\) conformation that rabbit PrP can stably adopt. The finding that it required excessive rounds of PMCA to adopt this conformation, agrees with my proposal that the subset of stable PrP\(^{res}\) conformations available to rabbit PrP\(^{C}\) is significantly smaller than that of more susceptible species. As a result, and the authors agree, “it is unlikely there will be an outbreak of “mad rabbit disease,” and consumers of rabbit meat face much less of a risk than consumers of cattle or sheep products”.

Although my work has shown that the relationship between β-state propensity and species susceptibility appears to hold true with several mammalian species, it is hardly an exhaustive list. There are several other interesting mammalian species that are not known to have ever been infected with prion disease, such as horses, and in contrast other species that appear to be highly susceptible, such as mink and elk. Prion disease also appears to be exclusively a mammalian disease although other species are also known to express PrP and PrP homologues. Interestingly, when mouse or hamster PrP is expressed in fruit flies, they succumb to a disease with aggregates present in their neural tissue but they can be rescued by expression of PrP from less susceptible species such as rabbits (Fernandez-Funez et al., 2010). Species such as birds, reptiles and fish also produce PrP homologues; it would be interesting to see which of these - if any - can be converted to the β-state and whether any of these non-mammalian species are also susceptible to prion infection.

It would also be of value to determine whether β-state octamer is actually analogous to the hypothesized toxic intermediate, PrP\(^{L}\). Recently, a number of antibodies have been produced that are able to detect PrP oligomers \textit{in vivo} and \textit{in vitro}. These antibodies could be tested against the recombinant β-state octamer as well as the PrP oligomer. If the antibodies are able to bind both, then the epitopes could be mapped and compared for similarity. Furthermore,
antibodies that recognize the β-state and PrP^L could be used to monitor the pathogenesis of prion disease to determine if the accumulation of PrP^L and PrP^{res} occurs according to the kinetic selection model. By comparing the rates of PrP^L accumulation between species in vivo, one could see if there is a difference in the kinetic selection mechanisms between resistant and susceptible species.

4.2 Helix-cap in helix-2 modulates β-state propensity in hamster and rabbit PrP.

The transmission barrier is caused by differences in the amino acid sequence of the host PrP^C and the infecting strain of PrP^{res}. Even a single amino acid difference can give rise to a transmission barrier (Priola et al., 1994). Similarly, single amino acid changes in the sequence of PrP^C can confer resistance to PrP^C from conversion to PrP^{res}. Rabbit PrP^C is resistant to PrP^{res} conversion and specific residues in rabbit PrP^C confer resistance when introduced into equivalent positions of mouse PrP^C and expressed in mouse neuroblastoma cells (Vorberg et al., 2003). In the structure of wild-type rabbit PrP^C, I observed that the residue S174, which is one of the aforementioned residues, forms reciprocal hydrogen bonds with residue N171 and the protein backbone. These reciprocal interactions form a helix-cap at the N-terminus of helix-2.

In my second study, I proposed that these contacts contribute to the observed lowered propensity of rabbit PrP^C to refold into the β-state and that by mutating these residues to mimic those of a more susceptible species, hamster, would ablate these contacts leading to a higher rate of conversion.

To explore this hypothesis, I determined the structures of rabbit PrP^C 121-230 mutants S170N, S174N and S170N/S174N. These structures showed that the contacts in the helix-cap were indeed ablated by the mutations and these mutants not only had a lower stability at pH 7.0 but also populated the β-state to a higher level compared to wild-type rabbit PrP^C 121-230. Conversely, introducing the analogous rabbit residues into hamster PrP^C by mutating N170S, N174S and N170S/N174S increased its unfolding stability and lowered its β-state propensity.
Testing the effects of these mutations on the kinetics of β-state conversion revealed that both wild-type and mutant rabbit and hamster PrPs convert rapidly to the β-state monomer upon dilution into urea at low pH. However, the mutations in hamster PrP cause the β-state octamer to form more slowly and to a lesser extent when compared to the wild-type. In rabbits, the mutations did not affect the rate or extent of β-state octamer formation.

Previous studies of the effect of amino acid changes on the folding stability of PrP have shown that most polymorphisms that do not induce disease itself only have mild effects (Rezaei et al., 2002). My study showed that the mutations in the helix-cap not only affect the folding stability of recombinant rabbit and hamster PrP but also the propensity to adopt the β-state. This agrees with my hypothesis that PrP from susceptible species should be able to misfold and more readily adopt conformations that convert to PrP\textsuperscript{res} than that of resistant species. The conformational selection model proposes that it is interactions between PrP protomers in stable conformations of PrP\textsuperscript{res} that causes the differences between species and strains in the conformations of PrP. My data suggest that it is not only the conformations of PrP\textsuperscript{res}, but also the folding properties of PrP\textsuperscript{C} itself that can affect prion disease susceptibility. A highly stable PrP\textsuperscript{C} is more likely to impart general resistance to prion disease than resistance to specific strains as a stable PrP\textsuperscript{C} molecule may resist conversion from many different strain conformations. This is not to say that they are completely immune, but that it requires a more specific prion strain to achieve conversion.

The observed differences in the rates of assembly of the β-state monomer and octamer between rabbit and hamster PrP also agrees well with the kinetic selection model. Our previous work showed that the β-state describes an equilibrium of β-state monomers and octamers. In hamster, a susceptible species, the β-state monomers assemble quickly into β-state octamers. When mutations corresponding to the amino acids found in rabbit PrP are introduced, this rate is slowed and the final extent of formation is lower than wild-type, indicating that the β-state octamer formation has been effected. In the rabbit, the proportions of β-state octamer and monomer are not affected.

This adds further depth to the argument that the β-state octamer could represent the toxic species PrP\textsuperscript{L}. In the kinetic selection hypothesis, the rate of PrP\textsuperscript{L} formation must be fast enough to allow it to accumulate and reach a toxic threshold even against the competing mechanisms of maturation into non-toxic PrP\textsuperscript{res} fibrils and degradation by proteolytic mechanisms. Therefore
mutations that slow the rate of oligomer formation may decrease a species' susceptibility to prion disease pathogenesis.

One of the most interesting findings in my studies of rabbit PrP is that the helix-cap mutations did not affect the rate of octamer assembly or the final level of the monomer-octamer equilibrium, contrary to what had been observed with hamster PrP. However, we do know that these mutations affect the propensity of total β-state (monomer and dimer) formation as was observed with the two-wavelength method. This implies that in rabbit PrP, these particular mutations do not affect the assembly of β-state octamers from β-state monomers, but increase the formation of the β-state. This implies that rabbit PrP may impart a more blanket resistance to prion disease infection.

Although our data from the two-wavelength CD analysis of the hamster PrP 121-231 wild-type and mutants suggested that the helix-cap had formed as indicated by the increase in folding stability and decrease in β-state propensity, determining the structures would definitively show whether or not this is the case. Knowledge of these structures would have the additional benefit of elucidating whether hamster PrP\textsuperscript{C} forms a similar dimeric arrangement in the asymmetric unit as was observed with rabbit PrP\textsuperscript{C}. Exhaustive attempts were made to crystallize hamster PrP\textsuperscript{C}, but with no success. The sequences of hamster and rabbit PrP are very similar, and it is intriguing that one would crystallize with relative ease and the other would not at all. One area of PrP that could be responsible for this difference is the C-terminus. In the structures of rabbit PrP\textsuperscript{C}, this portion makes some contacts with symmetry mates in the crystal lattice. In order to crystallize hamster PrP\textsuperscript{C}, some mutations could be made in this area to correspond with rabbit PrP before further crystal trials would go ahead.

Although I have shown that these mutations affect the β-state propensity of recombinant PrP in vitro, it would be very interesting to see what effect they would have on a species’ prion disease susceptibility in vivo. To test this, similar mutations could be made in mouse PrP and expressed transgenically in mice. Similar work was done to determine the effect of the N174T mutation on mouse PrP to mimic the sequence that is present in elk PrP (Sigurdson et al., 2009). It was found that these mutations caused the spontaneous pathogenesis of a disease that was very similar to prion disease. If similar results could be obtained introducing rabbit and hamster mutations in
mouse than “rabbitized” mice may show a decrease in susceptibility and vice versa with “hamsterized” mice.

Alternatively, rather than using laborious and expensive transgenic methods, protein misfolding cyclical amplifications (PMCA) using recombinantly produced PrP mutants and infectious PrP<sub>res</sub> could be employed instead (Wang et al., 2010). It was recently shown that wild-type rabbit PrP<sup>C</sup> can be converted to infectious PrP<sup>res</sup> using several rounds of PMCA (Chianini et al., 2012). Using a similar method but with recombinant wild-type and mutant rabbit and hamster PrP, one could determine whether the number of rounds of PMCA required to convert PrP<sup>C</sup> to PrP<sup>res</sup> as well as the structure and/or infectious properties of the resultant PrP<sup>res</sup> are affected.
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Appendix A

Isolation and characterization of a PrP\(^C\) dimer and β-state octamer by chemical cross-linking in solution.
A.1. Introduction

The primary event in prion disease pathogenesis is the conversion of monomeric, mainly α-helical PrP\textsuperscript{C}, to the aggregated, primarily β-sheet infectious form, PrP\textsuperscript{res}. Major progress has been made in understanding the interactions of host PrP\textsuperscript{C} and the infectious PrP\textsuperscript{res}, but a detailed structure of the infectious form and a specific mechanism of conversion is still lacking (Diaz-Espinoza and Soto, 2012).

A great deal of effort has been spent to determine the structural details of the infectious form, PrP\textsuperscript{res}. Several models of PrP\textsuperscript{res} have been proposed using data from low-resolution methods, like electron microscopy and X-ray fiber diffraction, combined with other structural constraints obtained from biochemical and biophysical methods. The β-helix model derived from homology modeling and data obtained from two dimensional crystals of scrapie prions shows a symmetrical, trimeric arrangement of β-helices formed from residues 89-176 at the centre of the fibril and α-helices-2 and -3 on the outside of the fibril (Govaerts et al., 2004; Wille et al., 2002). The β-spiral model is derived from molecular dynamics calculations and structural restraints obtained from a depth of biochemical experiments. This model shows PrP protomers associating via extended β-sheets formed from the native β-strands of PrP\textsuperscript{C} at a 45° angle to the filament axis (DeMarco and Daggett, 2004). Studies with recombinant PrP have also revealed that PrP can form an in-register cross-β-sheet similar to what had been observed with yeast prions (Cobb et al., 2007; Lu et al., 2007).

However, much of the structural focus so far has been on the larger structures of amyloid fibrils. Comparatively little is known about the mechanism of how PrP\textsuperscript{res} interacts with PrP\textsuperscript{C} and the initial stages of conversion into the smallest infectious and toxic forms, which have been shown to be the most virulent (Silveira et al., 2005).

PrP\textsuperscript{res} is known to physically interact with PrP\textsuperscript{C} (Chesebro et al., 2005; Prusiner et al., 1990) and a dimeric association has been proposed to be the primary interaction in the mechanism of conversion (Tompa et al., 2002). PrP\textsuperscript{C} forms a monomer-dimer equilibrium in solution (Meyer et al., 2000). When a fusion protein of PrP-FK506 binding protein is induced to form dimers with the addition of AP20187, they rapidly convert to aggregates rich in β-sheet (Roostae et al., 2009).
After this interaction, the next step in the pathogenic mechanism is proposed to be the formation and accumulation of a smaller, oligomeric toxic species which then matures into amyloid fibrils of PrP\textsuperscript{res} (Collinge and Clarke, 2007). Small oligomers have been implicated in other protein misfolding diseases, and recombinant PrP forms small oligomers under mild denaturing conditions similar to those that may be found in the cellular endosomal pathway (Baskakov et al., 2001; Hornemann and Glockshuber, 1998; Swietnicki et al., 1997).

The previously determined structures of wild-type rabbit PrP\textsuperscript{C} 121-230 and several mutants all showed a dimeric arrangement of protomers in the asymmetric unit. The dimeric association buries a relatively large surface area of approximately 1600 Å\textsuperscript{2}, involves many polar and ionic interactions and is predicted to be stable in solution by PISA (Krissinel and Henrick, 2007). Several contacts were of particular interest, including a salt bridge that forms between E204 and K185 and close association and hydrogen bonding between the symmetry-related N197 residues of both promoters (Fig. A.1)

I hypothesized that if the observed salt bridge were to form a stable association in solution, then it would be possible to crosslink these residues using EDC. The second association, between residues N197 of both protomers, is the closest of the asymmetric unit dimer. The two strands between α-helices-2 and -3 of both protomers are only 3-4 Å apart at their nearest approach, almost close enough to form anti-parallel strands of β-sheet. If these strands were crosslinked together then it may induce closer association between the protomers and affect the conversion of PrP to the β-state.

To test these hypotheses, I designed crosslinking protocols using EDC, which crosslinks a primary amine to a carboxylate. I also attempted to achieve disulfide crosslinking using a N197C mutant of rabbit PrP\textsuperscript{C} 121-230 under oxidizing conditions. Using EDC, I was able to crosslink dimers in solution using both hamster and rabbit PrP\textsuperscript{C}. Control experiments showed that this crosslink was specific, dependent on the concentration of PrP, and resulted in a dimer with the similar secondary structure to and gross tertiary structure of PrP\textsuperscript{C}. A site-specific disulfide crosslink using an N197C mutant of rabbit PrP\textsuperscript{C} resulted in a dimer that had similar secondary structure but lower thermal stability of unfolding when compared to the PrP\textsuperscript{C} monomer.
Figure A.1: (A) The non-crystallographic dimer found in the asymmetric unit of crystals of wild-type rabbit PrP\textsuperscript{C} 121-230 and mutants. (B) Close interactions between the conserved threonine-rich loops between helices-2 and -3 centered around N197 of the dimer subunits. (C) Hydrophobic contacts exclude water from the interface and an intermolecular salt-bridge between K185 - E207. (D) Hydrogen bonds and salt-bridges in cleft formed by rigid loops.
Using similar crosslinking protocols, I also was able to crosslink an octamer of hamster PrP 121-231 under conditions that populate the β-state. The octamer crosslinking reaction is dependent on the concentrations of both PrP and EDC, and mutation experiments showed that probably more than one of the lysine or N-terminal amines are involved in the reaction.

A.2. Materials and Methods

A.2.1 Molecular biology

Rabbit PrP 121-230 and hamster PrP 121-231 where cloned as described in section 2.2.1. The vectors coding for the K185R, K194R, K204R and K220R mutants of hamster PrP 121-231 were generated using a Quik-Change mutagenesis kit (Stratagene) according to manufacturer's instructions.

A.2.2 Protein expression and purification

All PrP constructs were expressed as inclusion bodies in *E. coli* BL21 AI and purified by on-column refolding as described in section 2.2.2.

One modification was made in the purification protocol of the N197C mutant of rabbit PrP* C* 121-230. After the on-column refolding step, 1 mM DTT was added to all buffers to keep the exposed cysteine reduced.

A.2.3 EDC crosslinking

1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Pierce, Rockford, IL, USA) crosslinking stock solutions were prepared in water to 100-fold above stated concentrations for reactions. At \( t = 0 \), crosslinker solution was diluted 100-fold into protein samples to the final reaction concentration. Reactions were then incubated for 2 hours at 37ºC. The crosslinking reaction was stopped either by adding SDS-PAGE sample buffer or Tris, pH 8.0 to a final concentration of 1mM.
A.2.4 Disulfide crosslinking

Immediately prior to crosslinking, DTT was removed from samples using buffer exchange into 20 mM Tris, pH 8.0 with 3 rounds of 10-fold spin concentration and dilution using Amicon Ultra centrifugal Filter Units (EMD Millipore, Billerica, MA, USA). In PrP samples at 50 μM, formation of the crosslinking disulfide bridges were started with CuSO₄ to a final concentration of 50 μM and incubated at room temperature for 1 minute. Reactions were quenched by the addition of a 100-fold excess of N-ethyl maleimide (5 mM final concentration) to block unreacted cysteines.

A.2.5 SDS-PAGE

Samples were diluted with a minimum 1:1 ratio with 2 x SDS-PAGE sample buffer (2% SDS, 20% glycerol, 20 mM Tris, pH 6.8, 2 mM EDTA, 160 mM DTT and 0.1 mg/ml Coomassie G-250 dye (Note: Non-reducing samples contained no DTT) to a final protein content of 1.0-1.5 μg/ml. 10 μL samples (10-15 μg total protein content) were loaded onto 15% or 18% Tris-glycine SDS-PAGE gels as stated, and run at 200 V. Gels were stained by soaking overnight in Coomassie Brilliant Blue solution (40% methanol, 10% acetic acid, 0.025% Coomassie Brilliant Blue R-250) and destained in destaining solution (40% methanol, 10% acetic acid).

A.2.6 Circular Dichroism Spectroscopy

All circular dichroism (CD) measurements were performed using a Jasco J-815 CD Spectrometer (Easton, MD, USA) and a 1 mm quartz cuvette. Wavelength scans were performed in triplicate and averaged between 200-250 nm with 10 nm/min and a bandwidth of 1 nm. Thermal scans were performed using the same cuvette and a Peltier temperature control unit (Jasco PTC 423S/15) with a heating rate of 1°C/min; ellipticity was monitored at 220 nm.

A.2.7 Size-exclusion chromatography

Size-exclusion chromatography experiments were performed using an ÄKTA FPLC system (GE Biosciences, Uppsala, Sweden) on an S75 16/60 column. Samples (1mL) were injected and run at 1 mL/min in 20 mM Tris, pH 8.0, 150 mM NaCl buffer.
A.2.8  *Bis-ANS fluorescence*

Fluorescence emission wavelength scans were performed using protein samples at 10 µM in 20 mM Tris, pH 8.0, at room temperature. Bis-ANS (4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt) (Sigma-Aldrich, St. Louis, MO, USA) was added to a final concentration of 4 µM and emission spectra were scanned between 400-600 nm (excitation 365 nm) with 2 mm slit widths using a Kari fluorimeter. Pure buffer was used as blanks.

A.2.9  *Urea-induced β-state conversion*

To induce β-state conversion, hamster PrP samples at 50 µM, were dialyzed into 50 mM MES pH 4.5, 80 mM NaCl. Urea solution was then added to the samples to a final concentration of 4 M and samples were incubated at room temperature for a minimum of 48 hours and a maximum of 7 days. Samples were then crosslinked as described above.

A.3.  *Results*

A.3.1  *A homodimer of rabbit and hamster PrPC 121-230 is crosslinked by EDC*

Although PrP dimers have long been hypothesized, in both the normal function of PrP<sup>C</sup> and the conversion of PrP<sup>C</sup> to PrP<sup>Ces</sup>, they have been notoriously difficult to detect due to their seemingly transient nature. In order to overcome this problem, I utilized chemical crosslinking with EDC to stabilize the interaction. EDC is a “zero-length” crosslinker that forms a covalent bond between a carboxylate and primary amine, resulting in an iso-peptide bond. EDC reacts first with the carboxylate forming an unstable O-acylisourea intermediate that very quickly reacts either with a nearby primary amine forming an isopeptide bond and the isourea byproduct, or the intermediate is quickly hydrolyzed by water in the surrounding buffer. Therefore, for an EDC crosslink to form efficiently, the association must have a fairly stable carboxylate-amine interaction.

To test whether dimers of PrP<sup>C</sup> could be crosslinked in solution, rabbit and hamster PrP<sup>C</sup> 121-230 were reacted in solution at 50 µM protein concentration with an 80-fold molar excess (4 mM final concentration) of EDC. SDS-PAGE of the EDC positive reactions of hamster and rabbit PrP<sup>C</sup> show small amounts of crosslinked dimer with a molecular mass of 27 kDA, twice the
molecular weight of the monomeric PrP$^C$ (13.5 kDa) (Fig A.2). Under in vitro solution conditions, recombinant human PrP$^C$ is known to form disulfide linked, domain swapped dimers (Knaus et al., 2001) and I have observed similar disulfide linked dimers in preparations of recombinant rabbit and hamster PrP$^C$. To eliminate the possibility that our observed dimers were the result of misfolding during protein preparation, I performed negative controls of the reaction without EDC added and did not observe dimers, neither with nor without DDT.

Lysozyme solutions were also included in the cross-linking reactions as a non-PrP protein control. Lysozyme is able to form dimers (Booth et al., 1997) in solution, but under our conditions, no EDC-crosslinked dimers were observed. This suggests that the transient interaction of PrP in solution is more stable than what has been observed previously with lysozyme.

**A.3.2 PrP$^C$ dimer crosslinking is specific and dependent on tertiary structure**

With crosslinking reactions, there is the possibility of crosslinking occurring due to non-specific, random interactions in solution. With protein-protein interactions, if the interaction is specific, then it is dependent on the tertiary structure of the proteins in question. To establish the specificity of the observed PrP$^C$ homodimeric crosslink, an unfolded protein control was used to determine if the crosslinking reaction was dependent on the tertiary structure of PrP$^C$.

Crosslinking reactions were prepared in the absence and presence of 8 M urea, 9 M urea, and 9 M urea with 5mM β-mercaptoethanol. In the absence of urea, dimers are clearly visible when the solution was incubated with EDC (Fig. A.3). After incubation with 8 M, 9 M urea and with β-ME to unfold the protein, no crosslinked dimers were observed. This indicates that the dimerization interaction is dependent on the tertiary structure of folded PrP$^C$ and is not a non-specific crosslinking reaction.

**A.3.3 PrP$^C$ dimer crosslinking is dependent on PrP concentration**

Previous analysis suggested that the hypothesized dimeric interaction of PrP$^C$ is based on an equilibrium (Meyer et al., 2000). If this is the case, then the concentration of dimer would be dependent on the concentration of PrP$^C$ and governed by an equilibrium constant, $K_{dimer}$. In order to test this, I crosslinked PrP$^C$ at increasing protein concentrations to see if the proportion of dimer increases relative to the amount of monomer.
Figure A.2: Crosslinking of lysozyme, rabbit and hamster PrP\textsuperscript{C} with EDC. 50 μM lysozyme, rabbit PrP\textsuperscript{C} 121-231 and hamster PrP\textsuperscript{C} 121-231 in 50 mM MES, pH 6.0 were crosslinked (+) with an 80:1 EDC to protein ratio. No EDC was added in negative (-) controls. SDS-PAGE of samples was performed with reducing (R) or non-reducing (NR) sample buffer.

Figure A.3: Tertiary structure dependence of EDC crosslink of PrP\textsuperscript{C}. 50 μM rabbit PrP\textsuperscript{C} 121-230 in 50 mM MES, pH 6.0 was incubated without or with 8 M Urea, 9 M Urea and 9 M urea with 5 mM β-ME for 72 hours. Samples were then crosslinked (+) with an 80:1 ration of EDC to protein. No EDC was added to negative controls (-).
Rabbit and hamster PrP\(^C\) 121-230 were crosslinked at concentrations ranging from 25 \(\mu M\) to 125 \(\mu M\) with a constant 80-fold excess of EDC (Fig. A.4). After quenching, crosslinking reactions were then diluted to equivalent protein concentrations of 25 \(\mu M\) before loading onto SDS-PAGE gels so that the proportions of monomer and dimer could be effectively compared. Crosslinked dimer was observed with both the hamster and rabbit PrP at the lowest protein concentration of 25 \(\mu M\) and increased in proportion relative to the monomer up to 125 \(\mu M\). This indicates that the dimer interaction of PrP\(^C\) is equilibrium based.

I was also interested to see what would happen if the PrP\(^C\) concentration was increased beyond 125 \(\mu M\). The PrP\(^C\) concentration dependence reactions were repeated with rabbit and hamster PrP\(^C\) at protein concentrations ranging from 50-250 \(\mu M\) (Fig. A.5). At 50 \(\mu M\), one can see the crosslinked PrP dimer that was observed in previous experiments, but at protein concentrations of 100 \(\mu M\) and above, a higher molecular mass corresponding to a trimer of PrP is observed.

**A.3.4 The EDC crosslinked PrP\(^C\) dimer is properly folded**

Chemical crosslinking can be a harsh process and due to enforcing distance constraints can lead to distorted tertiary structure and/or partial denaturation. If this does occur, then artifactual interactions can result as proteins begin to aggregate and crosslink non-specifically. In order to determine whether EDC crosslinking resulted in a distorted structure of PrP\(^C\), I sought to purify EDC crosslinked monomers and dimers of rabbit PrP\(^C\) and determine whether their three-dimensional structures were maintained.

Rabbit PrP\(^C\) was batch-crosslinked at 50 \(\mu M\) with an 80-fold excess of EDC (4 mM final concentration). The crosslinking reaction was quenched with 20 mM Tris buffer, pH 8, and the sample was concentrated down to 1 mL. The concentrated sample was then loaded onto an S200 16/60 column. The elution profile showed distinct peaks for the monomer and dimer (Fig. A.6A). The monomer peak was isolated, but in order to further purify the dimer peak, the peak fractions were combined and concentrated and rerun (Fig. A.6B). SDS-PAGE of the peak showed that the peak was relatively pure (Fig. A.6C).
**Figure A.4:** Protein concentration dependence (0 – 125 µM) of EDC crosslink of rabbit and hamster PrP\(^C\). Protein samples at stated concentrations in 50 mM MES, pH 6.0 were crosslinked with an 80:1 EDC to protein ratio. No EDC was added to negative controls (-).

**Figure A.5:** Protein concentration dependence (0 – 250 µM) of EDC crosslink of rabbit and hamster PrP\(^C\). Protein samples at stated concentrations in 50 mM MES, pH 6.0 were crosslinked with an 80:1 EDC to protein ratio. No EDC was added to negative controls (-).
In order to determine whether the secondary structure of PrP<sup>C</sup> was maintained, circular dichroism spectra of the crosslinked monomer and dimer were performed. The uncrosslinked control of rabbit PrP<sup>C</sup> showed the characteristic α-helical CD spectrum (Fig A.7A). Both the crosslinked monomer and dimer had identical CD spectra, indicating that the secondary structure of the PrP<sup>C</sup> was unaltered by the crosslinking.

Even if the secondary structure is intact, perturbation caused by crosslinking could expose internal hydrophobic residues, generating possible artifactual sites for association and potential aggregation. To test for this, I performed bis-ANS fluorescence to test for differences in the exposure of hydrophobic residues in the crosslinked monomer and dimer (Fig. A.7B). The fluorescence spectra show no significant increase in the bisANS fluorescence indicating that there is no increase in exposure of hydrophobic residues caused by the EDC-crosslinking.

A.3.5  *Rabbit mutant N197C also forms dimers via disulfide crosslink*

The area of closest interaction between the monomers of the asymmetric unit seen in the rabbit PrP crystal structure is centered on residue N197 in the loop between helix-2 and helix-3 with a Cα-Cα distance of 4.1 Å (Fig. A.1). If the observed arrangement is indeed the one crosslinked by EDC, then it should also be possible to form a similar dimer by oxidatively crosslinking an N197C mutant.

To test this hypothesis, I changed N197 in rabbit PrP to cysteine. The N197C mutant protein was then expressed, purified and crosslinked using copper sulfate. The reaction was then stopped by injecting the solution onto an S75 column 16/60 equilibrated with 50mM Tris, pH 8 (Fig. A.8A). The elution profile showed two distinct peaks; samples of each peak were then run on SDS-PAGE under reducing and non-reducing conditions (Fig. A.8B). The reduced samples showed only monomeric PrP, whereas the non-reduced samples showed a mixture of monomer and dimer in the loaded samples. The presence of reducible crosslinked dimer shows that I was successful in inducing a disulfide bridge stabilizing an existing dimer in solution.

As had been done with the EDC crosslinked dimer, I then measured the CD spectra of both the purified N197C monomer and the crosslinked dimer to ensure that the secondary structures of
Figure A.6: Purification of EDC-crosslinked dimer of rabbit PrP<sup>C</sup> 121-231. (A) SEC purification of EDC-crosslinked rabbit PrP<sup>C</sup> 121-230. Sample was run on an S200 16/60 column in 20 mM Tris, pH 8.0, 150 mM NaCl. (B) Dimer peak was isolated and re-run under the same conditions. (C) SDS-PAGE of peak fractions.
Figure A.7: Structural characteristics of the purified EDC-crosslinked rabbit PrP\(^C\) dimer. (A) Circular dichroism spectra of crosslinked monomer (xMono), dimer (xDimer) and uncrosslinked (UnXlinked) rabbit PrP\(^C\) 121-230. (B) bis-ANS fluorescence of crosslinked and uncrosslinked species. Crosslinked monomer, dimer and uncrosslinked rabbit PrP\(^C\) 121-231 all show similar \(\alpha\)-helical CD spectra and similar ANS fluorescence indicating similar overall structure.
Figure A.8: (A) Size exclusion chromatography of refolded rabbit PrP$^C$ 121-231 N197C on an S75 16/60 column in 20mM Tris, pH 8.5, 125 mM NaCl. Sample was crosslinked with CuSO$_4$ and quenched with a 100-fold excess of N-ethyl maleimide. (B) Peak fractions were run on SDS-PAGE to determine whether the dimer was disulfide-crosslinked. Reducing samples contained 5mM β-mercaptoethanol.
Figure A.9: Structural characteristics of disulphide-crosslinked N197C rabbit PrP<sup>C</sup> 121-230. (A) Circular dichroism wavelength scans of purified, disulfide-crosslinked rabbit PrP<sup>C</sup> 121-230 N197C dimer and uncrosslinked purified monomer. (B) Temperature melt (2°C/minute) of purified, disulfide crosslinked rabbit PrP<sup>C</sup> 121-230 N197C dimer and uncrosslinked purified monomer monitored by CD at 220 nm.
both forms were undisturbed by the N197C mutation and/or the crosslinking. The spectra of both the N197C monomer and crosslinked dimer both showed the same α-helical CD spectrum, indicating that neither the mutation nor crosslinking affected the overall structure of PrP\(^C\) (Fig. A.9A)

Curious to determine whether crosslinking had any significant effect on the thermal stability of the PrP\(^C\) monomer or dimer, I used a temperature-controlled CD spectrometer to monitor the ellipticities of the monomer and the crosslinked dimer at 220 nm while heating the sample from 25 °C to 90 °C (Fig. A.9B). The result showed the monomer had a partially reversible thermal denaturation curve with a melting point at roughly 75 °C. The crosslinked dimer on the other hand had a completely irreversible denaturation curve with a lower melting point at approximately 65 °C. This indicates that the disulfide crosslink at N197C, although not changing the overall final structure, does appear to affect the folding/unfolding behaviour of rabbit PrP\(^C\), reducing the thermal stability of the protein.

A.3.6 EDC crosslinks the β-state octamer of hamster PrP

As was shown with hamster and rabbit PrP in previous papers, under mild denaturing conditions and low pH, PrP will form the β-state octamer that is rich in β-sheet. If the contacts that are hypothesized in the dimer interaction are carried over into the octamer, then it should also be possible to crosslink the β-state octamer using EDC as well.

Hamster PrP\(^C\) 121-231, was converted to the β-state by incubating 50 μM samples at pH 4.5 with increasing concentrations of urea from 0 M - 8 M. After incubation, the samples were crosslinked with EDC and run on SDS-PAGE (Fig. A.10). At lower concentrations from 0 M - 2 M urea, the majority of crosslinked PrP\(^C\) remains monomeric, indicating that the β-state octamer had not formed under these conditions. From 3 M - 5 M urea, the crosslinked β-state octamer is clearly visible at the calculated molecular mass of an octamer of PrP 121-231 of approximately 110 kDa. There also appears to be a small amount of higher molecular weight species, possibly dimers of β-state octamers. From 6 M - 8 M urea, no octamer is observed, only monomer remains. The presence of the crosslinked β-state octamer from 3 M - 5 M agrees well with the observed propensity of hamster PrP 121-231 to form the β-state octamer under these conditions (see chapter 3, Fig. 3.5).
Figure A.10: Urea titration and EDC crosslinking of β-state hamster PrP 121-231. Hamster PrP at 50 µM was incubated in 50 mM MES, pH 4.5, 72 mM NaCl and stated concentration of urea for 72 hours. Samples were then crosslinked with an 80:1 EDC to protein ratio and run on 15% SDS-PAGE.
A.3.7 Crosslinker concentration dependence

To determine the ratio of β-state crosslinking as a function of the concentration of EDC, samples of 50 μM PrP hamster PrP 121-231 were converted to β-state and then crosslinked, increasing the molar ratios of EDC:protein from 5:1 to 400:1 (Fig. A.11). In the EDC-free, negative control, no crosslinked species were visible; however, even under conditions as low as a 5-fold molar excess of EDC, a crosslinked dimer was observed. As the EDC to PrP ratio was increased to 10:1, larger species are observed such as trimers at 10:1 and tetramers at 50:1. At a ratio of 200:1, the crosslinked octamer was accompanied by tetramer, trimer and dimer forms. There is a conspicuous absence of crosslinked species corresponding to molecular weights ranging from a pentamer to septamer, suggesting a structural constraint limiting the crosslinking of such species. At 400:1 EDC to protein ratio, the β-state octamer is the major species present and the proportions of smaller molecular weight species are drastically reduced. However again, there are also some larger molecular species present.

A.3.8 Protein concentration dependence

Samples of hamster PrP$^C$ 121-231 were prepared with increasing concentrations of protein from 25 to 200 μM and then converted to β-state by incubation for 48 hours at pH 4.5 in 4 M urea with 80 mM NaCl (Fig. A.12). All β-state converted samples were then crosslinked with a 200-fold excess of EDC except for the negative control. In the EDC-free, negative control, no crosslinked species were observed. At 25 μM PrP concentration, the majority of crosslinked species is dimeric with some trimers and tetramers present but no octamers. At 50 μM, the proportion of dimer is reduced and some octamer is observed. As the protein concentration is increased, the proportion of dimer and higher molecular mass species is reduced and the proportion of oligomer appears to increase. Again there does not appear to be a species of molecular mass corresponding to the pentameric to septameric forms.

A.3.9 Identification of crosslinking sites

EDC crosslinks primary amines, either from a lysine residue or the N-terminus, with a carboxylate group, either from the C-terminus or from an aspartate or glutamate residue. There
are only four lysines in the primary sequence of hamster PrP 121-231, reducing the number of amines available for crosslinking to five, including the N-terminus. Determining which of these primary amines is responsible for the EDC crosslink would give valuable data on which parts of the PrP molecule make the protein-protein interactions that form the β-state octamer.

In order to deduce which, if any of the lysines are involved in the EDC crosslinks, a series of single K to R mutations of each of the hamster PrP 121-231 lysines were produced. Each of these lysine mutants: K185R, K194R, K204R and K220R were refolded to the β-state at pH 4.5 with 4 M urea and crosslinked with a 200-fold molar excess of EDC. The SDS-PAGE analysis showed that none of these mutations had a noticeable impact on the crosslinking pattern of the β-state octamer (Fig. A.13). This indicates that either more than one of the lysines in hamster PrP 121-231 are responsible for the EDC crosslink, or that the N-terminus is involved.

A.4. Discussion

A.4.1 Rabbit and hamster PrP\textsubscript{C} 121-231 form a semi-stable dimer in solution

Although large aggregates of amyloid have been identified in nerve tissue infected with prion disease for years, more recently the roles of smaller structures of PrP have been of some interest. Although PrP\textsubscript{C} is believed to be normally monomeric \textit{in vivo}, it has been proposed that dimeric interactions are involved in either the function of PrP\textsubscript{C}, the mechanism of conversion to PrP\textsubscript{Sc}, or both (Biasini et al., 2012; Tompa et al., 2002). Also, similar to other protein misfolding diseases such as Alzheimer’s Disease, oligomers of PrP rather than large aggregates have been implicated as the disease-causing agent in prion disease (Huang et al., 2013).

In an interesting observation, I found that the asymmetric units of crystals of wild-type and mutants of rabbit PrP\textsubscript{C} 121-230 contain dimers of PrP\textsubscript{C}. The amount of buried surface (~1600 Å\textsuperscript{2}) and number of ionic and hydrogen bonding interactions at the dimer interface suggested that the observed arrangement might well be stable in solution. Analysis of the interface using PISA also predicted that the asymmetric unit dimer may be stable in solution. Based on interactions involving the observed salt-bridge between residues E207 of one subunit and K185 of the other as well as close contacts between symmetry related N197 residues, I proposed utilizing chemical
Figure A.11: Crosslinker concentration dependence of EDC crosslinking of β-state hamster PrP. Samples of 50 μM hamster PrP were incubated in 4 M urea, pH 4.5, 80 mM NaCl for 72 hours. EDC was then added at the excess stated and incubated at 37°C for 2 hours. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min.

Figure A.12: Protein concentration dependence of EDC crosslinking of β-state hamster PrP. Samples of hamster PrP at the stated concentrations were incubated in 4 M urea, 50 mM MES, pH 4.5, 80 mM NaCl and incubated for 72 hours. A 200-fold excess of EDC was then added and incubated at 37°C for 2 hours. The reaction was stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min.
Figure A.13: The effect of single K to R mutations on the crosslinking of β-state hamster PrP 121-231. Samples of each lysine mutant at 50 μM were incubated in 4 M urea, 50 mM MES, pH 4.5, 80 mM NaCl for 72 hours. A 200-fold excess of EDC was then added and incubated at 37°C for 2 hours. The reactions were stopped by the addition of SDS-PAGE sample buffer and boiling for 5 min.
crosslinking with either EDC or disulfide-bridge-forming cysteine mutants to capture this interaction in solution.

I was able to crosslink dimers of both hamster PrP\textsuperscript{C} 121-231 and rabbit PrP\textsuperscript{C} 121-230 in solution using the crosslinker EDC. Chemical crosslinking of proteins can often produce non-specific results due to simple random collisions in solution. However, the conditions and relative concentrations of EDC and PrP used in these reactions are similar to those that are generally used for crosslinking specific interactions in protein complexes (Marekov, 2007). The crosslinking reaction of EDC must occur rapidly in aqueous conditions or the crosslinker will be hydrolyzed by water. The two protomers of the PrP dimer must interact in solution long enough for the crosslinking reaction to occur, suggesting a semi-stable association.

To further rule out the possibility of non-specific crosslinking, I included lysozyme as a protein control as it is known to self-associate and form dimers in solution (Maroufi et al., 2008). Under conditions that produced crosslinked dimers of hamster and rabbit PrP\textsuperscript{C}, no crosslinked dimers of lysozyme were observed, indicating that the conditions were mild enough to rule out non-specific crosslinking of PrP\textsuperscript{C} due to random collision. In high concentrations of urea, the majority of secondary and tertiary structure in PrP is destroyed. Under these conditions, any potential crosslinks can be assumed to be non-specific. As no crosslinks were observed in these unfolded samples, I can safely assume that the crosslinks that I observed in the folded samples are specific and dependent on tertiary structure.

Previous studies have hypothesized that PrP\textsuperscript{C} may interact with itself in solution, but this is the first time that a stable association has been captured under non-denaturing conditions. Previous work has proposed that PrP may associate in a weak monomer-dimer equilibrium (Meyer et al., 2000), but dimers of PrP could only be isolated under denaturing conditions such as when exposed to SDS (Jansen et al., 2001; Kaimann et al., 2008) or when engineered as protein fusions (Roostae et al., 2009) to force the interaction to occur. In contrast, EDC and disulfide formation of PrP do not initiate dimer formation but only capture a pre-existing dimeric state and make it durable. In addition, there is only a short time window for the crosslinking reaction to occur due to the rapid hydrolysis of EDC. My findings support the idea that PrP may weakly or transiently associate in solution long enough for a crosslink to occur, but not in a stable complex that would allow the isolation of the PrP dimer.
A.4.2 The EDC crosslinking reaction is dependent on the concentration of PrP

Previous work has shown that PrP may associate as a dimer in solution, albeit at nearly undetectable levels (Meyer et al., 2000). It was proposed that this was the result of an equilibrium between monomeric and dimeric PrP, with a small equilibrium constant. If this were the case, performing the reaction at increasing concentrations of PrP should produce a proportional increase in the amount of dimer, exactly what was observed in my experiments.

Interestingly, as the PrP concentration is increased to 250 µM, larger crosslinked species begin to appear, including trimers. This suggests that PrP<sup>C</sup> is not only capable of forming dimers, but larger oligomers as well. Since the interface that we have proposed is symmetrical over a two-fold axis, then to form larger oligomers, additional contacts would have to be made at additional sites.

In the crosslinking solution, PrP is free to assume any orientation and its interactions are governed by Brownian motion. However, in vivo PrP<sup>C</sup> is a membrane anchored protein that is sequestered in DRMs (Peters et al., 2003). This greatly restricts its freedom of movement from three dimensions to two, further constrained by the size of the DRM, increasing its effective concentration dramatically. The stronger interaction that we observed as PrP concentration increased, suggests that at the effective concentrations possible in its native environment, PrP may very well interact with itself.

A.4.3 The crosslinked dimer maintains its native-like PrP<sup>C</sup> structure but alters its unfolding behaviour

If the observed dimeric association of PrP is related to the mechanism of conversion of PrP<sup>res</sup>, then it may be possible that the association coincides with some structural changes in the PrP<sup>C</sup> protomers, such as an increase in β-sheet content. The purification and characterization of the crosslinked dimer of PrP gave evidence that the secondary and tertiary structures of PrP<sup>C</sup> are maintained during crosslinking.

The ability of PrP to self-associate suggests that consideration of dimer formation should not be restricted to the discussion of mechanisms of pathogenicity or conversion to PrP<sup>res</sup> but may well be a part of the protein’s normal physiological function.
To further explore the effect of dimerization on the behaviour of PrP\textsuperscript{C}, I introduced a cysteine mutation at position 197, the point of closest contact in the asymmetric unit dimer of rabbit PrP\textsuperscript{C}. This mutation itself did not appear to alter the structure of the PrP, neither in its monomer nor dimer form. However, a distinct difference was observed in the unfolding behavior of the dimer, which underwent irreversible unfolding at a lower temperature when compared to the monomer, which underwent partially reversible unfolding.

This change in the unfolding behaviour of dimeric PrP\textsuperscript{C} relative to the monomer suggests that self-association plays a critical role in the folding of PrP. Under normal circumstances, monomeric PrP may undergo folding changes and maintain its relative stability and not convert to a misfolded form, however, when associated with other PrP, it undergoes irreversible unfolding. PrP\textsuperscript{res} may exploit this behavior in the mechanism of conversion of PrP\textsuperscript{C}.

Monitoring the interactions of PrP in a membrane-like environment showed that PrP does interact with other monomers and does undergo some secondary structure change (Elfrink et al., 2008). The crosslinking of the PrP dimer did not alter the gross structural characteristics of the dimer, but did change the thermal stability of unfolding. This agrees with what was observed with the engineered dimer which quickly aggregated upon dimerization (Roostae et al., 2009). These findings suggest that although dimerization of PrP may not be determinant in the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{res}, it may well represent a first step.

### A.4.4 β-State octamer is crosslinked by EDC

Although the crosslinking results suggest that PrP weakly self-associates in solution, it is unclear if this is relevant to the mechanism of conversion of PrP\textsuperscript{C} to PrP\textsuperscript{res}. It has been suggested that dimerization of PrP is involved in the conversion of PrP\textsuperscript{C} to PrP\textsuperscript{res} (Tompa et al., 2002). In the kinetic selection model, PrP\textsuperscript{C} is first converted into toxic oligomers (Collinge and Clarke, 2007). If the dimer that we have observed from EDC crosslinking is representative of an early associative state in the formation of the proposed toxic oligomers, then it stands to reason that these contacts may be maintained in the PrP oligomer.

To determine whether similar contacts were present in the β-state, I crosslinked hamster PrP 121-231 with EDC at low pH (4.5) with increasing concentrations of urea from 0 M – 8 M. A crosslinked species was observed in the samples between 3 M – 5 M urea with an estimated
molecular mass of ~110 kDa (Fig. A.10), similar to the predicted molecular mass of an octamer of hamster PrP 121-231 (~108 kDa). This confirms that there is a relatively stable amine-carboxylate interaction within the β-state octamer and gives further evidence towards my hypothesis that the contacts resulting in the crosslinking of the hamster PrP\textsuperscript{C} dimer in solution may also be present in the β-state octamer.

**A.4.5 β-state crosslinking is EDC and protein concentration dependent.**

By varying the concentration of hamster PrP 121-231 and holding the proportion of EDC constant, I was able to show that crosslinking of the β-octamer is protein concentration dependent. At lower protein concentrations (i.e. 25 μM) there is not a significant population of crosslinked β-octamer, but there is significant populations of crosslinked dimer and trimer (Fig A.12). The molar ratio of protein:EDC is held constant in these reactions, so it can be assumed that the probability of a crosslink occurring is independent of crosslinker concentration. The presence of crosslinked dimer and trimer at lower protein concentrations disagrees with our previous data that show that β-state PrP consists of a monomer-octamer equilibrium. However, it is possible that at low PrP concentrations, the rate of exchange between β-state monomer and octamer is increased. This would result in a lower probability of coincidence of the formation of the octamers and the 7 EDC crosslinks necessary to crosslink the octamer completely. The incomplete crosslinking of the octamer would then result in the observed crosslinked species of intermediate size.

Crosslinking of the β-state octamer is also dependent on the proportion of EDC to protein. By maintaining a constant protein concentration while increasing the proportion of EDC, I demonstrated that at low EDC to protein ratios (from 5 to 100:1), intermediate crosslinked species of dimer, trimer and tetramer appear while at higher EDC to protein ratios (200 and 400:1) crosslinked β-octamer and some larger species are present.

At the protein concentration and buffer conditions used, hamster PrP 121-230 is known to populate to β-state octamer to nearly 100%. As the proportion of EDC increased, the proportions of the small crosslinked species diminished coinciding with the appearance of the crosslinked β-octamer, indicating that the observed intermediate crosslinked species are likely the result of incomplete crosslinking of the octamer. To achieve complete crosslinking of the β-state octamer, at least seven crosslinks must occur between the eight protomers of the β-octamer.
Interestingly, as the proportion of EDC was increased, there were no intermediate crosslinked species larger than a tetramer together with octamers. This may indicate an asymmetry between the crosslinks within the β-octamer and that the crosslinks required to form the dimer, trimer and tetramer are more favorable whereas the crosslink forming the octamer requires higher concentrations of EDC.

**A.4.6 EDC crosslinking of β-state octamer involves multiple residues**

Zero length crosslinking with EDC has the advantage of providing some low-resolution structural data or constraints in the form of telling which residues are in close proximity to one another. By determining which residues are being crosslinked in the β-state octamer, I hoped to be able to determine where in the primary structure of hamster PrP that the protomers are interacting with one another and also perhaps some basic symmetry information if the crosslinks are all the same.

In the sequence of hamster PrP 121-231, there are only 4 lysine residues, making it possible to mutate these residues individually to arginine which has similar biochemical properties, but is unreactive to EDC.

Four lysine to arginine mutants of hamster PrP 121-231 were produced: K185R, K194R, K204R and K220R. When converted to the β-state and crosslinked with EDC, each mutant showed a crosslinking pattern indistinguishable from the wild-type. This result indicates that no individual lysine residue is solely responsible for the EDC crosslink in the β-oligomer. This leaves the possibility that the N-terminus may be involved, or that multiple lysine residues are capable of forming crosslinks, or a combination of the two.

**A.4.7 Future directions**

Although I was able to produce a crosslinked dimer of PrP<sub>C</sub> in solution, it remains unclear whether dimeric PrP<sub>C</sub> is physiologically relevant. Without an established functional mechanism of PrP<sub>C</sub> or a mechanism of conversion for PrP<sub>C</sub> to PrP<sub>res</sub>, it is unclear what role dimeric PrP<sub>C</sub> plays (if any) or whether it is an *in vitro* artifact of a highly purified, recombinant fragment of PrP<sub>C</sub>.
Several experimental approaches could address these concerns. The first would be to analyze the structure and self-association of membrane-anchored PrP<sup>C</sup>. Previous structural analyses of membrane-anchored PrP<sup>C</sup> did indicate a change in secondary structure (Elfrink et al., 2008). I observed that dimerization of PrP<sup>C</sup> is concentration dependent. In a membrane environment, GPI-anchored PrP<sup>C</sup> is restricted to movement in two dimensions, resulting in an effective concentration that may be much higher than in solution. If one factors in the sequestration of PrP<sup>C</sup> in detergent-resistant microdomains, the effective concentration could be orders of magnitude higher than in solution. To test this, crosslinking experiments and further structural characterizations could be performed with PrP<sup>C</sup> anchored in a membrane. A more ambitious approach would be to attempt to crosslink PrP<sup>C</sup> in cellulo or even in vivo and detect dimeric PrP by Western blot or mass spectrometry. Such experiments would determine whether dimeric PrP plays a physiological role.

Using site-specific mutagenesis or mass spectrometry, the residues that form the EDC crosslinks in both the dimer and octamer could be identified and used to provide some structural constraints such as distances between residues and subunit arrangements. Preliminary experiments that I performed using single lysine to arginine mutants failed to identify individual residues that formed crosslinks, but by producing multiple mutants with only a single lysine and the N-terminus remaining may be able to more effectively identify which residues are involved. Furthermore, mass spectrometry has been used to identify the sites of EDC crosslinks by identifying peptide fragments that contain multiple N- and C-termini produced by the isopeptide bond formed by EDC (Marekov, 2007).

Identifying residues in close proximity raises the possibility of producing site-specific disulfide crosslinks with cysteine mutants such as was shown with the N197C mutant of rabbit PrP<sup>C</sup>. These kinds of site-specific disulfide crosslinks are more specific than those produced with other crosslinkers, as there is only a single position where they can occur. This leads to more uniform samples and more reproducible results.

This level of specificity makes it possible to purify and study them with higher resolution structural methods such as X-ray crystallography or NMR. Determining the structure of the crosslinked dimer of N197C would determine whether the crosslink has introduced any structural changes that lead to the observed differences in the thermal stability between the monomer and
the dimer. Furthermore, a high resolution structure of an infectious and/or toxic prion is the “final frontier” of prion research (Diaz-Espinoza and Soto, 2012) but the disordered and aggregated nature of prions has impeded high resolution structural studies. Producing a site-specific crosslinked form of the β-octamer would possibly stabilize its structure enough to pursue crystallographic or NMR experiments.

It would also be informative to determine whether these types of associations are occurring in vivo. Samples of infectious prions could be crosslinked with EDC and then analyzed with mass spectrometry to determine whether the crosslinks that are formed are the same as that observed with recombinant PrP.
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