Structure and Dynamics of the Y145Stop Variant of the Human Prion Protein Studied by Magic-Angle Spinning Solid State NMR

DISSERTATION

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Abstract

Transmissible spongiform encephalopathies (TSEs), a group of neurodegenerative diseases including scrapie in sheep, bovine spongiform encephalopathy in cattle, and Creutzfeldt-Jakob disease in humans, are caused by the conversion of the monometric prion protein to highly ordered, amyloid aggregates. These amyloid aggregates are believed to be the infectious agent, accounting for the transmissibility of TSEs.1-3 In this work, we used solid state NMR methods to determine the structure and dynamic properties of the Y145Stop mutant of the human prion protein, huPrP23-144,4,5 in its fibril state. This variant is readily converted under physiologically relevant buffer conditions from soluble monomeric form to amyloid fibrils and provides an experimentally tractable model which reproduces in vitro some of the most fundamental aspects of prion propagation, including the phenomena of ‘species barrier’ and prion strains.6,7 Understanding the atomic level, three-dimensional structure and conformation dynamics of these fibrils is fundamental to understanding their functions in living organisms, and may in time lead to the development of effective medical treatments for TSEs and other amyloidal diseases.

The insoluble, non-crystalline nature of amyloid fibrils makes studying these molecules difficult by traditional high-resolution structural techniques, however solid state NMR has been used in the past decade to study a number of proteins in their amyloid state.8-22 In this work, we apply the solid state NMR techniques used in these
studies as well as a number of experiments designed by our group to study huPrP23-144 fibrils.

Surprisingly, our initial studies\textsuperscript{23} revealed that huPrP23-144 fibrils contains a highly compact, relatively rigid and β-sheet-rich core region of ~30 amino acids located near the C-terminus. Concurrently, the remaining residues, corresponding primarily to the large N-terminal domain, exhibit significant conformational flexibility within the fibril lattice. An analysis of the conformational flexibility of huPrP23-144 fibrils by detecting directly, at ambient conditions, the highly dynamic segments using J-coupling based MAS SSNMR methods, found the presence of all amino acid types outside the core region of fibrils. Experiments which probed the molecular motions occurring on the NMR timescale within the relatively rigid amyloid core domain, using site-resolved SSNMR measurements of backbone dipolar order parameters, found little variation on the sub-μs timescale. Similar experiments which examined the transverse spin relaxation rate constants found significant variations from motions on the μs-ms timescale which likely underlie the large variation in peak intensities seen in earlier 3D correlation experiments.\textsuperscript{24}

Using a series of \textsuperscript{15}N-\textsuperscript{13}C correlation experiments on uniform, dilute and mixed isotopically labeled samples, huPrP23-144 molecules were found to align in-register and parallel to form fibrils with an approximate inter-fibril distance of 4.4 Å. These experiments also detected the presence of a number of long-range structural restraints which were verified and expanded in a series of time resolved correlation experiment to provide a number of \textsuperscript{15}N-\textsuperscript{13}C and \textsuperscript{13}C-\textsuperscript{13}C distance restraints. When combined with the
results from three dipole correlation experiments, which provide restraints on the protein backbone torsion angles, an atomic level structural model of huPrP23-144 amyloid fibrils is beginning to form.
Dedication

To my wife, may our life together be filled with adventures
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sister- and brother-in-law. I look forward to the years ahead and for the arrival of Chloe.

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**Fields of Study**

Major Field: Chemical Physics
# Table of Contents

Abstract ........................................................................................................................................... ii

Dedication ......................................................................................................................................... v

Acknowledgments ............................................................................................................................... vi

Vita ................................................................................................................................................... ix

List of Tables ...................................................................................................................................... xvii

List of Figures ................................................................................................................................... xviii

Chapter 1 .......................................................................................................................................... 1

Introduction to solid state NMR spectroscopy .................................................................................. 1

1.1 Introduction .................................................................................................................................. 1

1.2 Nuclear Spin Physics and NMR ................................................................................................... 6

1.3 Matter and Spin ............................................................................................................................ 6

1.4 Quantum Description of Nuclear Spin ......................................................................................... 8

1.5 Nuclear Spin Interactions ........................................................................................................... 11

1.5.1 Zeeman .................................................................................................................................... 11

1.5.2 Chemical Shift ....................................................................................................................... 12

1.5.3 Dipole-dipole ....................................................................................................................... 14
1.5.4 J-coupling .................................................................................................................. 15
1.5.5 Radio Frequency Pulses .............................................................................................. 16
1.5.6 Relaxation ..................................................................................................................... 17
1.6 Magic-angle Spinning ...................................................................................................... 18
1.7 Multidimensional NMR .................................................................................................. 21
Chapter 2 ................................................................................................................................ 23
Molecular conformation and dynamics of the Y145Stop variant of the human prion protein in an amyloid fibril .................................................................................................................. 23
2.1 Introduction ....................................................................................................................... 23
2.2 Results ............................................................................................................................... 26
2.2.1 Solid-State NMR Spectra Identify a Highly Ordered, Compact Amyloid Core with a Predominantly β-Strand Conformation ............................................................................. 26
2.2.2 Variable Temperature NMR Studies of Conformational Dynamics in huPrP23-144 Amyloid ........................................................................................................................................... 35
2.3 Discussion ........................................................................................................................... 42
2.4 Materials and Methods .................................................................................................... 47
2.4.1 Expression and Purification of huPrP23-144. ............................................................... 47
2.4.2 Preparation of huPrP23-144 Amyloid Fibrils ................................................................. 48
2.4.3 Solid-State NMR Spectroscopy ..................................................................................... 48
Chapter 3 ........................................................................................................................................... 53
Conformational Flexibility of Y145Stop Human Prion Protein Amyloid Fibrils Probed by Solid-State Nuclear Magnetic Resonance Spectroscopy ......................................................... 53

3.1 Introduction .................................................................................................................................. 53

3.2 Experimental Section .................................................................................................................... 56

3.2.1 Preparation of $^{13}$C, $^{15}$N-Labeled huPrP23-144 Amyloid Fibrils .................................... 56

3.2.2 Solid-State NMR Spectroscopy ............................................................................................... 56

3.2.3 Data Processing and Simulations ........................................................................................... 65

3.2.4 Dipolar Coupling Error Estimation ....................................................................................... 67

3.3 Results and Discussion .................................................................................................................. 68

3.3.1 Direct Detection of Highly Flexible Segments of huPrP23-144 Amyloid ....................... 68

3.3.2 Probing the Sub-Microsecond Timescale Backbone Dynamics in the Core Region of huPrP23-144 Amyloid ................................................................................................ 76

3.3.3 Transverse Nuclear Relaxation Rates of huPrP23-144 Amyloid Core Residues. ........... 90

3.4 Conclusions .................................................................................................................................. 99

Chapter 4 ........................................................................................................................................... 103
Intermolecular Alignment of Y145Stop Human Prion Protein Amyloid Fibrils Probed by Solid-State Nuclear Magnetic Resonance Spectroscopy .................................................. 103

4.1 Introduction .................................................................................................................................. 103
4.2 Results and Discussion................................................................. 104

4.2.1 2D ZF-TEDOR Experiments.................................................. 104

4.2.2 Time Resolved 1D BS-TEDOR and ZF-TEDOR experiments...... 113

4.3 Materials and Methods ............................................................. 115

4.3.1 Solid-State NMR Spectroscopy............................................. 115

4.3.2 Data Processing and Simulations. .......................................... 116

Chapter 5 ......................................................................................... 120

Torsion Angle Measurement............................................................. 120

5.1 Introduction ................................................................................ 120

5.2 Projection Angle Measurements .............................................. 120

5.3 $^1\text{H}_i-^{15}\text{N}_i-^{13}\text{C}_\alpha-^1\text{H}_\alpha$ Experiment.......................... 125

5.4 $^1\text{H}_{i+1}-^{15}\text{N}_{i+1}-^{13}\text{C}_\alpha-^1\text{H}_\alpha$ Experiment..................... 132

5.5 $^{15}\text{N}_{i+1}-^{13}\text{C}_\gamma-^{13}\text{C}_\alpha-^{15}\text{N}_i$ Experiment .................. 138

5.6 Comparison to TALOS Prediction............................................ 145

5.7 Materials and Methods ............................................................. 148

Chapter 6 ......................................................................................... 151

Distance Measurements ..................................................................... 151

6.1 Introduction .............................................................................. 151

6.2 TEDOR Measurements ............................................................. 151
List of Tables

Table 1.1 Table of Abbreviation used in this work................................................................. 5
Table 2.1 $^{15}$N and $^{13}$C chemical shifts for residues in the amyloid core region of huPrP23-144 fibrils. ......................................................................................................................... 33
Table 3.1 Tentative $^{1}$H and $^{13}$C resonance assignments for the flexible residues in huPrP23-144 fibril. .................................................................................................................. 73
Table 3.2 Scaled backbone dipolar couplings and order parameters for residues in the amyloid core region of huPrP23-144 fibrils. ..................................................................................... 89
Table 5.1 Summary of huPrP23-144 experimentally measured projection angles and the corresponding projections angles predicted from TALOS. ......................................................... 147
Table 6.1 $^{13}$C-$^{15}$N distances measured by the 3D ZF-TEDOR experiment on diluted 1,3-glycerol huPrP23-144 fibrils........................................................................................................ 157
Table 6.2 Listing of $^{13}$C-$^{13}$C cross peaks from long range contacts detected in the DARR spectrum of uniform 1,3-glycerol huPrP23-144. .............................................................. 164
Table 6.3 Listing of $^{13}$C-$^{13}$C cross peaks from long range contacts detected in the DARR spectrum of uniform 2-glycerol huPrP23-144.............................................................................. 165
List of Figures

Figure 1.1 Simulated $^{15}$N NMR spectrum of N-acetyl-L-valine at variable MAS spinning rates ............................................................ 20
Figure 1.2 2D NC$\alpha$ spectrum and of GB3 with 1D $^{15}$N and $^{13}$C projections ............... 22
Figure 2.1 Two-dimensional NCO (A) and NCA (B) spectra of huPrP23-144 fibrils, acquired at 11.111 kHz MAS rate and 0°C .................................................. 27
Figure 2.2 Small regions from a two-dimensional $^{13}$C-$^{13}$C correlation spectrum of huPrP23-144 fibrils ........................................................................... 28
Figure 2.3 Small regions from a 2D $^{15}$N-$^{13}$C$'$ spectrum of huPrP23-144 fibrils showing the A117, G124 and P137 correlations and the corresponding 1D $^{13}$C traces ............... 30
Figure 2.4 Representative strips from 3D CONCA, NCACX and NCOCX spectra of huPrP23-144 fibrils showing sequential backbone connectivity for residues A118-V122. ........................................................................................................... 31
Figure 2.5 Secondary structure of the amyloid core in huPrP23-144 fibrils predicted by PSSI (A). Secondary chemical shifts for $^{13}$C$'$ (B), $^{13}$C$\alpha$ (C) and $^{13}$C$\beta$ (D) ............... 34
Figure 2.6 Variable temperature 1D $^{13}$C cross-polarization (CP) (A-H) and $^{13}$C direct polarization (DP; i.e., 90°-acquire) (I-P) spectra of huPrP23-144 fibrils recorded at 11.111 kHz MAS. ........................................................................................................ 37
Figure 2.7 Variable temperature 2D $^{15}$N-$^{13}$C$\alpha$ and $^{13}$C-$^{13}$C spectra of huPrP23-144 fibrils. ........................................................................................................ 40
Figure 2.8 Two-dimensional NMR spectra of huPrP23-144 fibrils recorded at 0°C (red contours) and -30°C (blue contours). ................................................................. 41

Figure 2.9 Representative atomic force microscopy images of the huPrP23-144 amyloid fibril sample used for solid-state NMR measurements ................................................................. 52

Figure 3.1 (A) Semi-constant time refocused INEPT pulse scheme\textsuperscript{113, 116} used to record the 2D $^1$H-$^{13}$C and $^1$H-$^{15}$N correlation spectra in Figure 3.5 ................................................................. 59

Figure 3.2 3D dipolar-chemical shift pulse schemes used to measure the $^1$H-$^{15}$N, $^1$H-$^{13}$C, $^1$H-$^{13}$C', $^1$H-$^{15}$N, $^1$H-$^{13}$C, and $^1$H-$^{13}$C' one-bond dipolar couplings in huPrP23-144 fibrils (see Figures 3.9-3.14) .................................................................................................................. 62

Figure 3.3 3D SSNMR pulse schemes used to measure the $^1$H, $^{13}$C, and $^{15}$N R\textsubscript{1p} and $^{15}$N R\textsubscript{2} relaxation rates in huPrP23-144 fibrils in residue specific fashion (see Figures 3.15-3.20) .................................................................................................................. 64

Figure 3.4 Amino acid sequence of huPrP23-144. .................................................................................................................. 71

Figure 3.5 Two-dimensional $^1$H-$^{13}$C (A) and $^1$H-$^{15}$N (B) correlation spectra of huPrP23-144 fibrils recorded at 30°C and 11.111 kHz MAS rate ................................................................. 72

Figure 3.6 2D $^{13}$C-$^{13}$C' CTUC-COSY\textsuperscript{115} spectra of huPrP23-144 fibrils recorded at 11.111 kHz MAS rate ................................................................. 75

Figure 3.7 Cross-peak intensities for residues in huPrP23-144 amyloid core .................................................................................................................. 78

Figure 3.8 Representative 2D $^{15}$N-$^{13}$C planes from 3D dipolar chemical shift SSNMR experiments on huPrP23-144 amyloid fibrils .................................................................................................................. 82

Figure 3.9 Backbone dipolar couplings in the amyloid core region of huPrP23-144 fibrils. .................................................................................................................. 83
Figure 3.10 (Left) Experimental $^1\text{H}^N-^{15}\text{N}$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D T-MREV dipolar-chemical shift correlation scheme (Figure 3.2A). .......................................................... 84

Figure 3.11 Experimental $^1\text{H}^{\alpha}-^{13}\text{C}^{\alpha}$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D T-MREV dipolar-chemical shift correlation scheme (Figure 3.2A). .......................................................... 85

Figure 3.12 Experimental $^{15}\text{N}-^{13}\text{C}^{\alpha}$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D band-selective TEDOR scheme (Figure 3.2B). ........................................................................................................ 86

Figure 3.13 Experimental $^{15}\text{N}-^{13}\text{C}'$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D band-selective TEDOR scheme (Figure 3.2B). ........................................................................................................ 87

Figure 3.14 Backbone order parameters in the amyloid core region of huPrP23-144 fibrils derived from measurements of $^1\text{H}^N-^{15}\text{N}$ (A), $^1\text{H}^{\alpha}-^{13}\text{C}^{\alpha}$ (B), $^{15}\text{N}-^{13}\text{C}^{\alpha}$ (C) and $^{15}\text{N}-^{13}\text{C}'$ (D) one-bond dipolar couplings........................................................................................................ 88

Figure 3.15 Selected site-resolved measurements of transverse relaxation rate constants, $R_1$ and $R_2$, for residues in the amyloid core region of huPrP23-144 fibrils. ........................................ 92

Figure 3.16 Experimental $^1\text{H} R_1$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. ................................................................................................. 93

Figure 3.17 Experimental $^{13}\text{C}^{\alpha} R_1$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. ................................................................................................. 94
Figure 3.18 Experimental $^{15}$N R$_{1p}$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. ................................................................. 95

Figure 3.19 Experimental $^{15}$N R$_{2}$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. .................................................................................. 96

Figure 3.20 Site-specific $^1$H R$_{1p}$ (A), $^{13}$C$_{\alpha}$ R$_{1p}$ (B), $^{15}$N R$_{1p}$ (C) and $^{15}$N R$_{2}$ (D) relaxation rate constants plotted as a function of residue number for the huPrP23-144 amyloid core region. ........................................................................................................... 97

Figure 4.1 NCO region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 1,3-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. .............................. 109

Figure 4.2 NC$_{\alpha}$ region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 2-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. ......................... 110

Figure 4.3 NCx region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 1,3-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. ......................... 111

Figure 4.4 Amino acid sequence of the amyloid core of huPrP23-144 fibrils with contacts consistent with an in-register parallel intermolecular alignment underlined as seen in the NCO (red), NCx (green) and NC$_{\alpha}$ (blue) ZF-TEDOR spectra...................................................... 112

Figure 4.5 Trajectories and fibril models for inter-fibril distance measurements. ........ 114

Figure 4.6 ZF-TEDOR and BS-TEDOR pulse sequences. ........................................ 119

Figure 5.1 Model of the peptide backbone. ................................................................. 124

Figure 5.2 $^1$H$_r$-$^{15}$N$_r$-$^{13}$C$_{\alpha}$-$^1$H$_{\alpha}$ pulse sequence........................................ 127

Figure 5.3 Dependence of the projection angle, $\Theta$, measured in the $^1$H$_r$-$^{15}$N$_r$-$^{13}$C$_{\alpha}$-$^1$H$_{\alpha}$ experiment on the molecular torsion angle $\phi$. ............................................................... 128
Figure 5.4 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ experiment. ........................................ 129

Figure 5.5 Simulated $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ dephasing curves. .................................................. 130

Figure 5.6 Sample experimental and best-fit simulated $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ dephasing curves. .......................................................................................................................... 131

Figure 5.7 $^1H_{i+1}-^{15}N_{i+1}-^{13}C_i^\alpha-^1H_i^a$ pulse sequence.................................................. 134

Figure 5.8 Dependence of the projection angle, $\Theta$, measured in the $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ experiment on the molecular torsion angle $\psi$. .................................................. 135

Figure 5.9 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ experiment .................................................. 136

Figure 5.10 Sample experimental and best-fit simulated $^1H_i-^{15}N_i-^{13}C_i^\alpha-^1H_i^a$ dephasing curves .......................................................... 137

Figure 5.11 $^{15}N_{i+1}-^{13}C_i^\alpha-^{13}C_i^\alpha-^{15}N_i$ pulse sequence .................................................. 140

Figure 5.12 Dependence of the projection angle $\Theta$ measured in the $^{15}N_{i+1}-^{13}C_i^\alpha-^{15}N_i$ experiment on the molecular torsion angle $\psi$. .................................................. 141

Figure 5.13 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^{15}N_{i+1}-^{13}C_i^\alpha-^{15}N_i$ experiment .................................................. 142

Figure 5.14 Simulated $^{15}N_{i+1}-^{13}C_i^\alpha-^{15}N_i$ dephasing curves .................................................. 143

Figure 5.15 Sample experimental and best-fit simulated $^{15}N_{i+1}-^{13}C_i^\alpha-^{15}N_i$ dephasing curves .......................................................... 144

Figure 5.16 Correlation plots between the experimental measured projections angles verses those predicted by TALOS. .................................................. 146

xxii
Figure 6.1 Sample 2D NCx regions of the 3D ZF-TEDOR experiment. ....................... 154
Figure 6.2 Representative ZF-TEDOR experimental trajectories and best-fit simulations. ........................................................................................................................................................................... 155
Figure 6.3 ZF-TEDOR trajectories and best-fit simulations for all non-trivial distances in the 3D ZF-TEDOR experiment. ........................................................................................................................................................................... 156
Figure 6.4 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 1,3-glycerol huPrP23-144 with 500 ms DARR mixing. ........................................................................................................................................................................... 161
Figure 6.5 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 1,3-glycerol huPrP23-144 with 375 ms DARR mixing. ........................................................................................................................................................................... 162
Figure 6.6 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 2-glycerol huPrP23-144 with 500 ms of DARR mixing. ........................................................................................................................................................................... 163
Chapter 1
Introduction to solid state NMR spectroscopy

1.1 Introduction

Misfolded proteins, particularly those in the amyloidal state, have recently been linked to a number of human disorders including Alzheimer’s, Parkinson’s and Huntington’s diseases as well as type II diabetes.\textsuperscript{25} Amyloid fibrils, which are insoluble, fibrous, extracellular protein aggregates, share a number of physicochemical properties including elongated morphology, the ability to bind Congo Red dye in a manner leading to characteristic apple-green birefringence, and a so-called ‘cross-β’ fiber diffraction pattern indicative of the presence of β-sheets with individual β-strands arranged perpendicular to the fibril axis.\textsuperscript{26} Understanding the atomic level, three-dimensional structure and conformation dynamics of these fibrils is fundamental to understanding their functions in living organisms and may in time lead to the development of effective medical treatments for amyloidal diseases. The focus of this dissertation will be on the structure and dynamics of a single misfolded protein aggregate, the Y145Stop mutant of the human prion protein, referred to in this work as huPrP23-144, in its amyloid fibril state which is associated with prion protein hereditary amyloidosis known as PrP cerebral amyloid angiopathy.\textsuperscript{27}
Of the over 67,000 protein structures deposited into the Protein Data Bank\textsuperscript{28} as of May 2011, nearly 88\% percent of the structures were solved using X-ray crystallography. This method uses a beam of high-energy X-rays focused on a single crystal to determine the atomic structure with a resolution, in favorable cases, below 2.0 Å.\textsuperscript{29} A high-quality crystal is required for this technique. Such crystals are often difficult to obtain and precludes the use of this method for studying non-crystalline protein structures such as amyloid fibrils. Solution-state NMR, nuclear magnetic resonance, accounts for all but less than a half percent of the remaining structures in the Protein Data Bank (11.5\%). In this method ~0.2-1.0 mM concentrations of soluble, isotopically labeled $^{13}$C, $^{15}$N and in some cases $^2$H enriched protein samples are placed in a large homogeneous magnetic field where a series of radio-frequency pulses are used to control the nuclear spins in the sample.\textsuperscript{30, 31} As its name suggests, a suitable solvent must be found which dissolves the proteins and allows for the rapid tumbling of the molecule, a requirement which cannot be found for proteins in their amyloidal state without disrupting the structure of interest. For this reason, solid-state NMR techniques have been used for a number of years to study amyloid fibrils\textsuperscript{8-22} and other insoluble, non-crystalline protein structures\textsuperscript{32-34}.

In solid-state NMR studies of proteins, isotopic $^{13}$C and $^{15}$N enrichment is necessary, which can be readily accomplished through robust biosynthetic methods.\textsuperscript{35} Each magnetically non-equivalent, spin-active nuclei in the protein has a unique resonance frequency which contributes to the spectra. In static powdered samples, the anisotropic nature of the chemical shift tensor as well as dipole-dipole interactions with neighboring nuclei lead to broad peaks. This broad ‘powder pattern’ limits the number of unique
nuclei which can be reasonably resolved in static experiments and would preclude the studies of uniformly labeled proteins using solid-state NMR in this manner. With the introduction of magic-angle spinning (MAS) by Andrew et al.\textsuperscript{36} and Lowe\textsuperscript{37} by which samples are spun at an angle of \(\sim 54.74^\circ\) with respect to the magnetic field resulting in the averaging of the anisotropic portion of the chemical shift and dipole-dipole tensors, spectra with sharp resonance lines can be collected of solid protein samples. Cross polarization (CP)\textsuperscript{38}, a technique where magnetization from abundant nuclei is transferred to dilute nuclei, and the introduction of two-dimensional (2D) and higher dimensional NMR experiments\textsuperscript{39-41} provided the necessary resolution and sensitivity to resolve individual peaks for small to medium sized proteins.

The combination of cross polarization, magic angle spinning and robust high-power broadband proton decoupling sequences such as TPPM\textsuperscript{42}, two pulse phase modulation, and SPINAL\textsuperscript{43}, small phase incremental alteration, allow for routine studies of biological solids. Over the last three decades, the use of cross polarization, magic-angle spinning (CPMAS)\textsuperscript{44,45} methods has risen and work on designing radio-frequency pulse sequences for recoupling the dipole-dipole and chemical shift anisotropic interactions removed by magic-angle spinning has been fruitful. This work has lead to experiments for measuring a number of structurally interesting parameters in solid biological samples.

This dissertation will address the methods and results of a series of solid-state NMR experiments probing the structure and dynamic properties of the Y145Stop variant of the human protein (huPrP23-144). The remainder of this chapter will introduce the necessary nuclear spin physics required to understand the magic-angle spinning solid-
state NMR experiments presented in the later chapter of this work. Chapter 2 presents
the chemical shift assignment of huPrP23-144 from a series of 2D and 3D $^{13}$C and $^{15}$N
correlation experiments. From this chemical shift data, the protein was found to contain a
small (~30 amino acid), rigid amyloid core region from residue 112-141 consisting of
three β-sheets with the remaining regions of the protein being conformationally flexible.
This chapter also examines how temperature changes affect the quality of the spectra
collected. Chapter 3 examines the flexible region of huPrP23-144 using J-based
polarization transfer experiments as well as exploring the dynamic nature of the amyloid
core. This work found that the chemical shifts of the flexible region of the protein found
using J-based transfer experiments were in agreement with a random coil conformation.
A quantitative study of the dynamics of the amyloid core region found little variation in
this region on the sub-μs timescale but significant differences in the dynamics on the μs
to ms timescale. Chapter 4 presents TEDOR, transfer echo double resonance, based
measurements on mixed $^{13}$C and $^{15}$N labeled samples to determine that the fibrils align
with neighboring fibrils in-register and parallel to each other. Additional time resolved
TEDOR experiment found that the inter-fibril distance is approximately 4.4 Å. Chapters
5 and 6 collectively examine the methods and results of a number of experiments which
provide structural restraints needed to determine the structure of a single huPrP23-144
fibril. Chapter 5 focuses on three experiments which restrain the backbone torsion angles
of the amyloid core. Chapter 6 focuses on $^{13}$C-$^{13}$C and $^{15}$N-$^{13}$C distance restraints found
using DARR/RAD, dipolar assisted rotational resonance/RF assisted diffusion, and
TEDOR experiments. To aid reading a table of abbreviation is supplied in Table 1.1.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMRB</td>
<td>Biological Magnetic Resonance Bank</td>
</tr>
<tr>
<td>BS-TEDOR</td>
<td>Band-selective Transverse Echo Double Resonance</td>
</tr>
<tr>
<td>CJD</td>
<td>Creutzfeldt-Jakob Disease</td>
</tr>
<tr>
<td>CP</td>
<td>Cross Polarization</td>
</tr>
<tr>
<td>CPMAS</td>
<td>Cross Polarization Magic-Angle Spinning</td>
</tr>
<tr>
<td>CTUC-COSY</td>
<td>Constant Time Uniform-sign Cross-Peak Correlation Spectroscopy</td>
</tr>
<tr>
<td>CW</td>
<td>Continuous Wave</td>
</tr>
<tr>
<td>DP</td>
<td>Direct Polarization</td>
</tr>
<tr>
<td>DARR</td>
<td>Dipolar Assisted Rotational Resonance</td>
</tr>
<tr>
<td>DSS</td>
<td>Dimethyl-Silapentane-Sulfonate</td>
</tr>
<tr>
<td>GSS</td>
<td>Gerstmann-Straussler-Scheinker disease</td>
</tr>
<tr>
<td>INEPT</td>
<td>Insensitive Nuclei Enhanced by Polarization Transfer</td>
</tr>
<tr>
<td>MAS</td>
<td>Magic-Angle Spinning</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>POST-C7</td>
<td>Permutationally Offset Stabilized C7</td>
</tr>
<tr>
<td>RAD</td>
<td>RF Assisted Diffusion</td>
</tr>
<tr>
<td>REDOR</td>
<td>Rotational Echo Double Resonance</td>
</tr>
<tr>
<td>RF</td>
<td>Radio Frequency</td>
</tr>
<tr>
<td>RFDR</td>
<td>Radio Frequency-Driven Recoupling</td>
</tr>
<tr>
<td>RMS</td>
<td>Root Mean Square</td>
</tr>
<tr>
<td>r-SNOB</td>
<td>Refocusing Selective excitation for Biochemical application</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>SPECIFIC-CP</td>
<td>Spectrally Induced Filtering In Combination with Cross Polarization</td>
</tr>
<tr>
<td>SPINAL</td>
<td>Small Phase Incremental Alteration</td>
</tr>
<tr>
<td>SSNMR</td>
<td>Solid State Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>TEDOR</td>
<td>Transfer Echo Double Echo</td>
</tr>
<tr>
<td>T-MREV</td>
<td>Transverse Mansfield-Rhim-Elleman-Vaughn sequence</td>
</tr>
<tr>
<td>TPPM</td>
<td>Two Pulse Phase Modulation</td>
</tr>
<tr>
<td>TSE</td>
<td>Transmissible Spongiform Encephalopathies</td>
</tr>
<tr>
<td>ZF-TEDOR</td>
<td>Z-filtered Transverse Echo Double Resonance</td>
</tr>
</tbody>
</table>

Table 1.1 Table of Abbreviation used is this work.
1.2 Nuclear Spin Physics and NMR

Through the next few sections a theoretical description of nuclear spin physics will be developed to explain the phenomenon of nuclear magnetic resonance first discovered by the group of Purcell\(^46\) and Bloch\(^47,48\). The goal here is not to explain in detail all of the physics needed to describe NMR but rather to provide the reader with a brief overview of the interactions and theoretical descriptions used in the ensuing chapters. For a more thorough background of nuclear magnetic resonance, the reader is encouraged to consult one of many excellent texts on the topic\(^31,41,49-52\), especially those focusing specifically on the study of solids\(^53-56\).

1.3 Matter and Spin

Each atomic isotope has a unique number of protons and neutrons in the nucleus which give rise to a property know as nuclear spin. Although nuclei can be in excited nuclear spin states, the energy required for such transitions is large and typically not available in normal laboratory settings. For this reason we concern ourselves only with the ground state nuclear spin. Since both neutrons and protons have a spin of $\frac{1}{2}$, which are combined as angular momenta, the possible ground-state nuclear spin values are integers ($0, 1, 2,...$) and half integers ($1/2, 3/2, ...$). Although rules do exist for predicting which nuclear spin state is the ground state, for our purposes, we can consider the ground-state nuclear spin as an empirical property of each isotope. In addition, we will limit ourselves to considering only isotopes with ground state nuclear spins of $1/2$. 
When a spin active particle is placed in a magnetic field, the interaction between the particle’s magnetic moment and the applied field appears in the Hamiltonian as the Zeeman interaction:

\[ \hat{H} = -\hat{\mu} \cdot \mathbf{B} \]

where \( \hat{\mu} \) the magnetic moment of the particle and \( \mathbf{B} \) is the applied magnetic field. Taking the field along the z-axis and writing the magnetic moment in terms of its magnetogyric ratio (\( \gamma \)) and angular momentum operator (\( \hat{I}_z \)) we find:

\[ \hat{H} = -\gamma \hbar B_0 \hat{I}_z \]

Here \( B_0 \) is the strength of the applied magnetic field in the z-direction. For a spin \( \frac{1}{2} \) particle, \( \hat{I}_z \) has two eigenvalues, +1/2 and -1/2, resulting in an energy splitting of the two eigenstates of \( \gamma \hbar B_0 \). The Boltzmann equation provides us with the relative populations of the two energy states (\( n_+ \) and \( n_- \)) for an ensemble of spin \( \frac{1}{2} \) particles at given temperature \( T \):

\[ \frac{n_+}{n_-} = \exp\left( -\frac{\Delta E}{kT} \right) = \exp\left( -\frac{\gamma \hbar B_0}{kT} \right) \]

In this equation \( k \) is the Boltzmann constant. For magnetic fields typical in NMR experiments and temperature above a few fractions of a degree Kelvin, the argument in the exponential is extremely small and the “high-temperature approximation” can be made by expanding the exponential and keeping only the first two terms:

\[ \frac{n_+}{n_-} \approx 1 + \frac{\gamma \hbar B_0}{kT} \]

Rearrange this to express the fractional population in the lower energy state is:
At room temperature and typical NMR fields, this fractional excess is only on the order of \( \sim 1 \times 10^{-5} \) indicative of the low sensitivity found in NMR experiment. Nevertheless this small population differences gives rise to a signal which can be detected by today’s NMR spectrometers on solid samples only a few \( \mu \text{L} \) in volume.

1.4 Quantum Description of Nuclear Spin

As the last section indicated, NMR requires the use of quantum mechanics to fully describe the spin physics. In this section we will describe the typical approximations and modification to the Schrödinger equation used in NMR experiments with the next section detailing a number of interactions in the nuclear spin Hamiltonian. The time dependent Schrödinger equation describes the evolution of a quantum wave function \( \Psi \) over time under a given Hamiltonian as:

\[
\iota \frac{\partial}{\partial t} |\Psi\> = \hat{H} |\Psi\>
\]

This can be recast into the Liouville-von Neumann equation:

\[
\iota \frac{\partial \rho}{\partial t} = [\hat{H}, \rho]
\]

Where the density matrix, \( \rho \), is defined as:

\[
\rho = \sum \rho_i |\Psi_i\> <\Psi_i|
\]
If the Hamiltonian is time-independent, the general solution to the Liouville-von Neumann equation for the density matrix at time $t$ is:

$$\rho(t) = e^{-i\hat{H}t} \rho(0) e^{i\hat{H}t}$$

Introducing the time propagation operator, $\hat{U}(t)$, this becomes:

$$\rho(t) = \hat{U}(t) \rho(0) \hat{U}^+(t)$$

Where the time propagator is defined as:

$$\hat{U}(t) = \exp(-i\hat{H}t)$$

The full time propagator can be decomposed into a product of time propagators with uniform or non-uniform time steps:

$$\hat{U}(t, 0) = \hat{U}_n \hat{U}_{n-1} \ldots \hat{U}_1 \equiv \hat{U}\left(t, \frac{(n-1)t}{n}\right) \ldots \hat{U}\left(\frac{t}{n}, 0\right)$$

The density matrix at time $t$ can then be determined according to:

$$\rho(t) = \hat{U}_n \hat{U}_{n-1} \ldots \hat{U}_1 \rho(0) \hat{U}_1^+ \ldots \hat{U}_{n-1}^+ \hat{U}_n^+$$

The expectation value for operator $\hat{A}$ at time $t$ is calculated from the trace:

$$<\hat{A}(t)> = Tr\{\hat{A} \rho(t)\} = Tr\{\hat{A} \hat{U}(t) \rho(0) \hat{U}^+(t)\}$$

In the case of a time-dependent Hamiltonian the above equations hold provided the propagator is a solution to the following operator differential equation:

$$\frac{\partial}{\partial t} \hat{U}(t) = \hat{H}(t) \hat{U}(t)$$

The most compact solution to this differential equation is the Dyson expression

$$\hat{U}(t) = \hat{T} \cdot \exp(-i \int_0^t \hat{H}(t) dt)$$

9
Here $\hat{T}$ is the Dyson time ordering operator. For an ensemble of magnetically equivalent, isolated spin $\frac{1}{2}$ particles in a magnetic field along the z-direction at thermal equilibrium, the initial density matrix is related to the Boltzmann distribution presented in section 1.3. Specifically working in the Zeeman basis, the off-diagonal elements are zero and the diagonal elements are given by:

$$\rho_{ii} = \frac{\exp\left(-\frac{E_i}{kT}\right)}{\sum_s \exp\left(-\frac{E_s}{kT}\right)}$$

where the sum runs over both eigenstates. Calculating these diagonal elements in the same manner as in section 1.3, the initial density matrix for an ensemble of spin $\frac{1}{2}$ particles is:

$$\rho(0) = \begin{pmatrix}
\frac{1}{2} + \frac{1}{4} \frac{\gamma h B_0}{kT} & 0 \\
0 & \frac{1}{2} - \frac{1}{4} \frac{\gamma h B_0}{kT}
\end{pmatrix}$$

It is often convenient to express the density matrix in terms of the angular momentum operators. For an isolated spin $\frac{1}{2}$ system matrix representation of $\hat{I}_x$, $\hat{I}_y$ and $\hat{I}_z$ are:

$$\hat{I}_x = \frac{1}{2} \begin{pmatrix} 0 & 1 \\ 1 & 0 \end{pmatrix}$$

$$\hat{I}_y = \frac{1}{2i} \begin{pmatrix} 0 & 1 \\ -1 & 0 \end{pmatrix}$$

$$\hat{I}_z = \frac{1}{2} \begin{pmatrix} 1 & 0 \\ 0 & -1 \end{pmatrix}$$

Additionally the unit operator is:

$$\frac{1}{2} \mathbb{I} = \frac{1}{2} \begin{pmatrix} 1 & 0 \\ 0 & 1 \end{pmatrix}$$
The initial density matrix can therefore be written in terms of its angular momentum operators as:

\[ \rho(0) = \frac{1}{2} \hat{1} + \frac{1}{2} \gamma \hbar B_0 \frac{1}{kT} \hat{I}_z \]

This is often referred to as \( \hat{I}_z \) for simplicity. Density matrix of larger spin systems can be constructed from the direct product of the individual spin density matrices. For the angular momentum description subscripts are added, ie: \( \hat{I}_{1z} \hat{I}_{2z} \).

1.5 Nuclear Spin Interactions

In this section, the terms of the nuclear spin Hamiltonian relevant to solid state NMR of biological solids are discussed. Again this should not be taken as a complete description of all possible nuclear spin interactions in solids as a number of important interactions are not discussed, most notably the electric quadrupolar coupling, as they do not contribute to the experiments described in later chapters.

1.5.1 Zeeman

The interaction of a spin \( \frac{1}{2} \) nuclei with an applied magnetic field is referred to as the nuclear Zeeman interaction. As discussed in section 1.3 the Hamiltonian for this interaction is given by:

\[ \hat{H} = -\gamma \hbar B_0 \hat{I}_z = \omega_0 \hat{I}_z \]

where \( \omega_0 \) is the nuclear Larmor frequency. The result of this interaction is the precession of the spin around the applied magnetic field at the Larmor frequency. This can be seen
by solving the time-dependent Schrödinger equation in the absence of all other interactions:

\[ i \frac{d}{dt} |\psi(t)\rangle = \omega_0 \hat{I}_z |\psi(t)\rangle \]

The solution to this differential equation is:

\[ |\psi(t)\rangle = \exp(-i\omega_0 t\hat{I}_z) |\psi(0)\rangle \]

Here the exponential operator is a rotation operator processing the spin around the z-axis with frequency \( \omega_0 \). In most solid state NMR experiments on spin \( \frac{1}{2} \) particles, this is by far the largest term in the Hamiltonian and as such the Zeeman eigenstates, \( |S,m_S\rangle = |1/2,+1/2\rangle \) and \( |1/2,-1/2\rangle \), are used as the preferred basis set. In addition, to a good approximation, only the secular terms of the Hamiltonian must be considered in this basis. These secular terms will be given in the following sections when discussing additional terms in the nuclear spin Hamiltonian.

### 1.5.2 Chemical Shift

The external magnetic field also affects the electrons in the molecule being studied by inducing a current. This in turn generates small magnetic field variations which are felt by nearby nuclei. This induced magnetic field is given the name chemical shift and can be expressed in a manner similar to the Zeeman interaction:

\[ \hat{\mathcal{H}} = -\hat{\mathbf{\mu}} \cdot \mathbf{B}_{\text{induced}} = -\hat{\mathbf{\mu}} \cdot \delta \cdot \mathbf{B}_0 \]

where \( \mathbf{B}_{\text{induced}} \) is the induced magnetic field, and \( \delta \) is the chemical shift tensor which is a tensor of rank two. In this expression and the discussion below, the “deshielding” convention is used where the resulting shift to the Larmor frequency in an isotropic liquid...
is $+\delta_{iso}$\textsuperscript{52}. A similar derivation using the “shielding” conversion can be made using a chemical shielding tensor, $\mathbf{\sigma}$, with opposite sign as the chemical shift tensor. The chemical shift tensor, $\mathbf{\delta}$, in Cartesian coordinates takes the form:

$$
\mathbf{\delta} = \begin{pmatrix}
\delta_{xx} & \delta_{xy} & \delta_{xz} \\
\delta_{yx} & \delta_{yy} & \delta_{yz} \\
\delta_{zx} & \delta_{zy} & \delta_{zz}
\end{pmatrix}
$$

Since this interaction is small compared to the Zeeman interaction to good approximation we can keep only the secular term:

$$\hat{\mathcal{H}} = -\gamma \delta_{zz}^{LAB}(\theta) B_0 \hat{I}_z$$

Here $\delta_{zz}^{LAB}(\theta)$ is the zz element of the chemical shift tensor in the laboratory frame which depends on the particular molecular orientation, $\theta$, of a given crystallite in the powder sample. This orientation dependence implies that all three principle components ($\delta_{xx}$, $\delta_{yy}$, $\delta_{zz}$) of the chemical shift tensor can contribute to $\delta_{zz}^{LAB}$ when the appropriate Wigner rotations$^57$ are applied to translate the molecule from the principle axis frame into the laboratory frame. This interaction can be broken down into an isotropic portion which does not depend on the molecular orientation and an anisotropic portion that is orientation dependent. The isotropic portion is given by:

$$\delta_{iso} = \frac{1}{3}(\delta_{xx} + \delta_{yy} + \delta_{zz})$$

A number of conventions are used to relate the principle components of the chemical tensor to anisotropic parameters of the chemicals shift. The most common convention used by Haeberlen$^53$ are the anisotropy $\delta_{aniso}$ and asymmetry, $\eta$, parameters defined as follows.

$$\delta_{aniso} = \delta_{zz} - \delta_{iso}$$
In a powder, the different molecular orientation of each crystallite has a unique chemical shift which slightly shifts the resonance condition. This leads to a broad powder pattern caused by the superposition of the sharp peaks from each crystallite. This anisotropy can be removed through magic-angle spinning resulting in a single sharp peak at the isotropic chemical shift.

1.5.3 Dipole-dipole

As nuclear spins are in close proximity to neighboring spins, it is not surprising that that these spins can interact with each other. The first of these interactions is the direct interaction between the magnetic fields of the two nuclei, given the name dipole-dipole coupling. This is a through-space interaction as opposed to the J-coupling which is mediated through the electron cloud which will be discussed in the next section. The Hamiltonian for the interaction is given by:

$$\mathcal{H} = b_{ij}(3(\vec{I}_i \cdot \vec{e}_{ij})(\vec{I}_j \cdot \vec{e}_{ij}) - \vec{I}_i \cdot \vec{I}_j)$$

where $\vec{e}_{ij}$ is the unit vector parallel to the line joining the two spins. The leading term $b_{ij}$ is dipole-dipole coupling constant given by:

$$b_{ij} = \frac{\mu_0 \gamma_i \gamma_j}{4\pi r_{ij}^3}$$

Here $\gamma_i$ and $\gamma_j$ are the magnetogyric ratios of the two interacting spins, $\mu_0$ is the permeability constant, and $r_{ij}$ is the distance between the spins. This distance dependence is used frequently in solid-state NMR to determine structural restraints by measurement.
of the dipole-dipole coupling constant. As with the chemical shift, only the secular portion of this interaction must be considered which takes the form:

\[ \mathcal{H} = d_{ij} (3\vec{I}_{iz} \vec{I}_{jz} - \vec{I}_i \vec{I}_j) \]

Here \( d_{ij} \) is the secular dipole-dipole coupling which is given by:

\[ d_{ij} = b_{ij} \frac{1}{2} (3\cos^2 \theta_{ij} - 1) \]

where \( \theta_{ij} \) is the angle between the vector \( e_{ij} \) and the external magnetic field. For nuclei of different isotopic species, heteronuclear, and for nuclei of the same isotopic species, homonuclear, in the “weak-coupling limit”\(^{41} \) where the chemical shift difference between the coupled spins greatly exceeds the dipole coupling constant, the above equation can be further truncated to:

\[ \mathcal{H} = d_{ij} 2\vec{I}_{iz} \vec{I}_{jz} \]

As with the chemical shift anisotropy, the angular dependence of the dipole-dipole interaction can be exploited and removed by magic-angle spinning.

1.5.4 \( J \)-coupling

The second nuclear interaction between nearby spins is the \( J \)-coupling, which is caused by bonding electrons indirectly coupling spins. The interaction has a Hamiltonian of the form:

\[ \mathcal{H} = 2\pi \vec{I}_i \cdot \vec{J}_{ij} \cdot \vec{I}_j \]
where $J_{ij}$ is the J-coupling tensor. Although theoretically the J-coupling may have an anisotropic portion it is extremely small and can be ignored. The resulting secular Hamiltonian is:

$$\hat{\mathcal{H}} = 2\pi J_{ij}\hat{I}_i\hat{I}_j$$

As was seen for the dipole-dipole coupling, for heteronuclear spins and in the “weak-coupling limit” for homonuclear spins this can be further truncated to:

$$\hat{\mathcal{H}} = 2\pi J_{ij}\hat{I}_{iz}\hat{I}_{jz}$$

The well-known J-coupling multiplet patterns seen in solution state NMR are typically not observed in the solid state as J-couplings between $^{13}$C and $^{15}$N nuclei are typically small (<100 Hz) and the rapid relaxation of signals results in broad resonance lines which cover these small splittings.

1.5.5 Radio Frequency Pulses

The final nuclear spin Hamiltonian term considered is different than the others presented thus far, as it is under the control of the experimenter. In a typical NMR experiment, a number of radio-frequency pulses (rf-pulses or just pulses) are applied, which contribute to the nuclear-spin Hamiltonian while the pulse is active, resulting in the evolution of the density matrix toward a particular desired state. For a radio frequency pulse with frequency $\omega_{rf}$, amplitude $B_{rf}$ and phase $\phi_{rf}$, the relevant Hamiltonian introduced into the system when the pulse is active is:

$$\hat{\mathcal{H}} = -\frac{1}{2}\gamma B_{rf}\left(\cos(\omega_{rf}t + \phi_{rf})\hat{I}_x + \sin(\omega_{rf}t + \phi_{rf})\hat{I}_y\right)$$
Pulsed NMR techniques in solids allow experimenters to remove unwanted interaction or to reintroduce a desired interaction in a controlled way. One of the most common interactions controlled by rf-pulses is the dipole-dipole interaction. In Section 1.6, magic-angle spinning is shown to remove this interaction from spinning samples resulting in narrow lines, but this interaction can be reintroduced through a number of pulse elements including those designed to transfer polarization between different isotopes, heteronuclear dipole recoupling, with pulse sequence elements such as cross polarization (CP)\textsuperscript{38} or TEDOR\textsuperscript{58, 59}. Similarly, the dipole-dipole interactions can be introduced between spins of the same isotope, homonuclear dipole recoupling, with pulse sequence elements such as RFDR\textsuperscript{60}, DARR/RAD\textsuperscript{61, 62} and POST-C7\textsuperscript{63}.

1.5.6 Relaxation

Relaxation of nuclear polarization is typically approached in a phenomenological manner, as will be the case in this section. Two types of nuclear relaxation are typically considered in NMR experiments. The spin-lattice relaxation or longitudinal relaxation causes magnetization parallel to the applied magnetic field, along the z-axis, to return to the thermal equilibrium value established by the Boltzmann equation in an exponential manner. The time constant associated with this relaxation is $T_1$, with typical values in biological solids in the millisecond to second range. The second relaxation effect concerns polarization perpendicular to the applied magnetic field, in the xy-plane. This transverse or spin-spin relaxation results in the transverse plane polarization
exponentially decaying to zero with a time constant $T_2$. For solids, typical $T_2$ rates are a few milliseconds.

### 1.6 Magic-angle Spinning

Early in the development of NMR, mechanical rotation of the sample was used to reduce the effect of the magnetic field inhomogeneity. In the late 1950’s Andrew et al.\textsuperscript{36} and Lowe\textsuperscript{37} introduced magic-angle spinning, in which samples are rotated at an angle of $\sim54.74^\circ$ with respect to the magnetic field. This eliminates the chemical shift anisotropy and dipole-dipole interaction. A full description of the physics involved in this technique is given by Maricq and Waugh\textsuperscript{64} and is discussed in a number of excellent texts on solid-state NMR\textsuperscript{53-56}. Briefly, the chemical shift anisotropy and dipole-dipole interactions can be recast into rank 2 spherical tensors. These tensors must be transformed from the principal axis frame, where the tensor is diagonal, to the laboratory frame where the detection of the NMR signal is performed. This tensor transformation is achieved by performing a series of Wigner rotations. In magic-angle spinning, the final Wigner rotation performed accounts for the mechanical rotation of the sample. The Euler angles\textsuperscript{57} for the transformation are $(0, \Theta, \omega_r t)$, where $\Theta$ is the angle between the axis of rotation and external magnetic field, and $\omega_r t$ takes into account the spinning of the rotor at $\omega_r$. When this Wigner rotation is applied to the chemical shift tensor and dipole-dipole tensor the majority of terms have coefficient which are time dependent according to $\exp(i \cdot n \cdot \omega_r \cdot t)$ where $n$ is 1 or 2 which average to zero at integer and half-integer multiples of the rotor cycle. In the case of the dipole-dipole interaction the only remaining term
receives a coefficient of $3 \cdot \cos^2(\Theta) - 1$ from the Wigner rotation, which evaluates to zero when $\Theta$ is set to the “magic-angle” of $\sim 54.74^\circ$. The remaining terms of the chemical shift tensor after Wigner rotation into the laboratory frame correspond to the isotropic chemical shift which is unaffected by magic-angle spinning.

The effects of magic-angle spinning on the simulated $^{15}$N spectrum of N-acetyl-L-valine are shown in Figure 1.1. The broad static powder pattern in panel A separates as the spinning speed increases in panels B and C until a single peaks at the isotopic chemical shift is present in panel D. Spinning sidebands at rotor multiples of the spinning speed are present in the spectrum as a result of incomplete averaging, but do not cause issues with the interpretation of the spectrum.
Figure 1.1 Simulated $^{15}$N NMR spectrum of N-acetyl-L-valine at variable MAS spinning rates. Static (A) and spinning simulations at MAS rates of 1.0 kHz (B), 2.5 kHz (C), and 5.0 kHz (D) were calculated using SIMPSON$^{65}$ with chemical shift tensor parameters as determined by Hou et al.$^{66}$ Specifically, the simulation parameters were $\delta_{\text{iso}}=126.3$ ppm, $\delta_{\text{aniso}}=103$ ppm, $\eta=0.25$. 200 Hz Lorentzian apodization was applied to the static spectrum and 50 Hz Lorentzian apodization applied to the spinning spectra.
1.7 Multidimensional NMR

The sensitivity achievable on modern superconducting magnets using magic-angle spinning, cross polarization and high-power proton decoupling is sufficient to perform a number of interesting experiments on biological solid samples. On the other hand, the resolution under these conditions is typically insufficient to resolve peaks for a majority of the spin-active nuclei in the sample. As proposed by Jean Jeener\textsuperscript{39} and first shown experimentally by Richard Ernst and co-workers\textsuperscript{40}, correlating multiple spins in a 2D or higher dimensional spectrum can increase the resolution of NMR experiments. Today these multi-dimensional experiments have been developed to the point where individual resonances from a solid protein sample can be resolved. For example, a 2D $^{15}$N-$^{13}$C\textalpha (NC\textalpha) correlation spectrum of the protein GB3 is shown in Figure 1.2. It can be seen in the projections on the top and right side of the 2D spectrum that the 1D $^{13}$C or $^{15}$N spectrum have insufficient resolution to identify all the residues in the protein, whereas a majority of the peaks in the 2D spectrum are well separated and can be assigned to $^{15}$N-$^{13}$C spin-pairs in the protein.
Figure 1.2 2D NC$\alpha$ spectrum and of GB3 with 1D $^{15}$N and $^{13}$C projections.
Chapter 2

Molecular conformation and dynamics of the Y145Stop variant
of the human prion protein in an amyloid fibril

Adapted from J.J. Helmus, K. Surewicz, P.S. Nadaud, W.K. Surewicz, C.P. Jaroniec,
Proceedings of the National Academy of Sciences of the USA, 2008, 105, 6284-6289.

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2.1 Introduction

Polymerization of normally soluble proteins into amyloid deposits underlies a number of dreaded human disorders, including Alzheimer’s, Parkinson’s and Huntington’s diseases, and type II diabetes. Regardless of the amino acid sequence and three-dimensional structure of amyloidogenic precursor proteins, mature amyloid fibrils share many physicochemical properties including elongated morphology, the ability to bind Congo Red dye in a manner leading to characteristic apple-green birefringence, and a so-called ‘cross-β’ fiber diffraction pattern indicative of the presence of β-sheets with individual β-strands arranged perpendicular to the fibril axis. With a notable exception of extensively studied Aβ peptide, however, higher-resolution data for these biologically important structures are very scarce and incomplete. This is largely due to difficulties in applying classical methods of structural biology to study high molecular
weight protein aggregates such as amyloid fibrils. Recent crystallographic studies have provided atomic-level insight into amyloid-like microcrystals formed by a number of short (4-7 residue) peptides, revealing a common ‘steric zipper’ motif where pairs of β-sheets form a dry interface with interdigitation of side chains. Remarkably, these basic steric-zippers can be organized in distinct molecular architectures, suggesting structural complexity of amyloids formed by larger, biologically relevant proteins. Direct crystallographic studies for such larger proteins are, however, not yet feasible.

The conversion of a monomeric protein to highly ordered, amyloid-like aggregates is also associated with the pathogenesis of transmissible spongiform encephalopathies (TSEs), a group of neurodegenerative disorders such as Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep, and bovine spongiform encephalopathy in cattle. The protein of interest here is a ~209 amino acid glycoprotein, dubbed prion protein (PrP). Most remarkable, the aggregates formed upon conversion of the normal form of mammalian prion protein (PrP^C) to the misfolded conformation (PrP^Sc) are believed to be infectious, accounting for the transmissibility of TSEs. Once highly controversial, the hypothesis that a disease can be transmitted by a ‘protein-only’ mechanism is now gaining acceptance, especially in view of recent progress in generating infectious PrP^Sc in vitro. Furthermore, the notion that proteins alone can be infectious is strongly supported by a plethora of findings regarding protein conformation-based inheritance in fungi.

Despite recent advances, our understanding of the biophysical mechanisms and structural basis of mammalian prion protein conversion to the amyloid state is relatively
poor and of low resolution. Recently, we and others have shown that certain aspects of this process can be conveniently studied using the recombinant Y145Stop mutant of the human prion protein, huPrP23-144\textsuperscript{4, 5}. This C-truncated protein, associated with prion protein hereditary amyloidosis known as PrP cerebral amyloid angiopathy\textsuperscript{27}, undergoes very efficient autocatalytic conformational conversion under physiologically relevant buffer conditions\textsuperscript{4, 5}; a similar reaction for recombinant full-length prion protein or PrP90-231 has been reported only in the presence of relatively high concentrations of chemical denaturants\textsuperscript{73, 74}. Most importantly, using this experimentally tractable model we were able to reproduce \textit{in vitro} some of the most fundamental aspects of prion propagation, including the phenomena of ‘species barrier’ and prion strains\textsuperscript{6, 7}. One of the main lessons from these studies was that both the seeding specificity as well as strain variability of PrP amyloid are fully encoded in the fibril conformation. However, the techniques used in previous conformational analysis were of intrinsically low resolution, precluding molecular-level description of PrP23-144 amyloid structure and the structural basis of strain specificity.

Here we have analyzed the molecular conformation and dynamics of huPrP23-144 amyloid fibrils using high-resolution magic-angle spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) spectroscopy. MAS SSNMR is one of very few techniques that can provide site-specific information about proteins in non-crystalline solid state; therefore, this approach is uniquely suited for structural studies of amyloid fibrils. Indeed, some of the most successful recent applications of SSNMR have been aimed at the atomic-level characterization of amyloidogenic peptides and proteins, including Aβ\textsubscript{8}, α-
synuclein\textsuperscript{10, 11}, fungal prion proteins HET-s, Sup35p and Ure2p\textsuperscript{20-22}, as well as peptide fragments of β2-microglobulin\textsuperscript{14} and transthyretin\textsuperscript{75, 76}. Our SSNMR measurements performed using uniformly \textsuperscript{13}C, \textsuperscript{15}N-labeled protein, allowed us to identify a compact (~28-residue), rigid core of the huPrP23-144 amyloid, as well as determine the location of β-strands within this core region.

2.2 Results

2.2.1 Solid-State NMR Spectra Identify a Highly Ordered, Compact Amyloid Core with a Predominantly β-Strand Conformation

The fingerprint 2D \textsuperscript{15}N-\textsuperscript{13}C′ (NCO), \textsuperscript{15}N-\textsuperscript{13}C\textalpha (NCA) and \textsuperscript{13}C-\textsuperscript{13}C (CC) chemical shift correlation spectra of uniformly \textsuperscript{13}C, \textsuperscript{15}N-labeled huPrP23-144 amyloid fibrils at 0°C are shown in Figure 2.1 and Figure 2.2. Essentially identical spectra (i.e., |Δδ|\textsubscript{avg} < 0.2 ppm) were obtained for two independently prepared fibril samples, indicating high reproducibility of the experiments. Remarkably, only ~20% of the cross-peaks (i.e., 28 out of a total of 125 or 126), expected in 2D NCO, NCA or CC spectra (C′-C\textalpha region) are observed under the present experimental conditions (note that huPrP23-144 used in this study contains 126 residues, including a four-residue N-terminal extension). Given that cross-polarization (CP), which requires the presence of substantial through-space magnetic dipole-dipole couplings\textsuperscript{38, 77}, was used to transfer magnetization between \textsuperscript{1}H, \textsuperscript{13}C and \textsuperscript{15}N nuclei, this clearly indicates the presence of a compact (~28-residue), relatively rigid ‘core’ region of the amyloid.
Figure 2.1 Two-dimensional NCO (A) and NCA (B) spectra of huPrP23-144 fibrils, acquired at 11.111 kHz MAS rate and 0°C. Each spectrum was recorded as a 768* (t₁, $^{15}$N) × 1400* (t₂, $^{13}$C) data matrix with acquisition times of 15.3 ms (t₁) and 28 ms (t₂), and a measurement time of 8.5 h. Cross-peaks are drawn with the lowest contour at ~10 times the root-mean-square (RMS) noise level and labeled by residue number according to the $^{15}$N frequency. Also shown are representative $^{13}$C and $^{15}$N 1D traces for residues A117 (A) and G119 (B).
Figure 2.2 Small regions from a two-dimensional $^{13}\text{C}-^{13}\text{C}$ correlation spectrum of huPrP23-144 fibrils. Regions correspond to (A) aliphatic-carbonyl and (B) aliphatic-aliphatic correlations. Data were acquired at 500 MHz, 11.111 kHz MAS rate and 0°C, with DARR $^{13}\text{C}-^{13}\text{C}$ mixing ($\tau_{\text{mix}} = 10$ ms) and $\sim 70$ kHz TPPM $^1\text{H}$ decoupling in $t_1$ and $t_2$. The spectrum was recorded as a 600 ($t_1$, $^{13}\text{C}$) $\times$ 1400* ($t_2$, $^{13}\text{C}$) data matrix with acquisition times of 9.0 ms ($t_1$) and 28 ms ($t_2$), and a measurement time of 20 h. Cross-peaks are drawn with the lowest contour at $\sim 5$ times the RMS noise level.
In contrast to this rigid core region, the remainder of the protein backbone in huPrP23-144 fibrils shows a high degree of conformational flexibility (*vide infra*). The existence of such rigid and dynamic segments has been previously reported for amyloid fibrils formed by other proteins, including α-synuclein\textsuperscript{10,11} and HET-s\textsuperscript{16,20}.

Site-specific \textsuperscript{13}C and \textsuperscript{15}N NMR linewidth measurements further reaffirm that the amyloid core of huPrP23-144 fibrils is highly ordered on the atomic level. Indeed, as demonstrated by representative \textsuperscript{13}C and \textsuperscript{15}N 1D traces shown in Figure 2.1, for most residues 2D NCO and NCA spectra display relatively narrow and symmetric resonances. Quantitative measurements for a set of 20 well-resolved peaks in the 2D NCA spectrum (processed with no apodization) gave the average \textsuperscript{15}N and \textsuperscript{13}C widths at half-height of 0.98 ± 0.18 and 0.63 ± 0.13 ppm, respectively. These linewidths, comparable to those recently reported for amyloids formed by a synthetic fragment of transthyretin\textsuperscript{75,76} and HET-s protein\textsuperscript{20}, approach the limit of narrowness observed for peptides and globular proteins in a highly ordered microcrystalline state\textsuperscript{78}. Moreover, a 2D NCO spectrum recorded in the presence of \textsuperscript{1}H and \textsuperscript{15}N spin decoupling during \textsuperscript{13}C detection shows that for residues distributed throughout the amyloid core, the \textsuperscript{13}C spectral resolution is to a large extent limited by the presence of \textsuperscript{13}C′-\textsuperscript{13}C\textalpha J-couplings as seen in Figure 2.3. This further confirms high degree of order in this part of huPrP23-144.

To obtain the sequential \textsuperscript{13}C and \textsuperscript{15}N backbone and side-chain resonance assignments for the amyloid core region, a set of 2D and 3D chemical shift correlation spectra was recorded at 0°C (see Section 2.4, Materials and Methods).
Figure 2.3 Small regions from a 2D $^{15}$N-$^{13}$C' spectrum of huPrP23-144 fibrils showing the A117, G124 and P137 correlations and the corresponding 1D $^{13}$C traces. The spectrum was acquired using parameters analogous to those given in Figure 2.1, but with an acquisition time of 30 ms and in the presence of a rotor-synchronized $^{15}$N π-pulse train (~28 kHz rf field, $xy$-8 phase cycling, one pulse every 8 rotor cycles) to decouple the ~15 Hz $^{15}$N-$^{13}$C' J-couplings. No apodization was applied in the $^{13}$C dimension during data processing. The partially-resolved doublets in the $^{13}$C dimension (which are less pronounced in the absence of $^{15}$N-$^{13}$C' decoupling; c.f., Fig. 1) correspond to ~55 Hz $^{13}$C'-$^{13}$Cα J-couplings, and indicate that the amyloid core region of huPrP23-144 fibrils is highly ordered.
Figure 2.4 Representative strips from 3D CONCA, NCACX and NCOCX spectra of huPrP23-144 fibrils showing sequential backbone connectivity for residues A118-V122. Spectra were recorded at 11.111 kHz MAS rate and 0°C. Acquisition parameters, CONCA: 24* (t₁, ¹⁵N) × 24* (t₂, ¹³C′) × 1000* (t₃, ¹³C) data matrix with acquisition times of 8.3 ms (t₁), 8.3 ms (t₂) and 20 ms (t₃), measurement time 38.5 h; NCACX: 26* (t₁, ¹⁵N) × 28* (t₂, ¹³C) × 1400* (t₃, ¹³C) data matrix with acquisition times of 9.0 ms (t₁), 4.9 ms (t₂) and 28 ms (t₃), measurement time 48.5 h; NCOCX: 26* (t₁, ¹⁵N) × 24* (t₂, ¹³C′) × 1400* (t₃, ¹³C) data matrix with acquisition times of 9.0 ms (t₁), 8.3 ms (t₂) and 28 ms (t₃), measurement time 41.5 h. Cross-peaks are drawn with the lowest contour at ~8 times the RMS noise level. Strips are labeled by residue number according to the ¹⁵N (F₁) frequency, ¹³C F₂ frequencies are given inside each strip on the right, and the major cross-peaks corresponding to α, β and γ carbons, are identified (minor peaks from neighboring spectral planes are marked by asterisks).
Representative strips from 3D $^{13}$C′-$^{15}$N-$^{13}$C (CONCA), $^{15}$N-$^{13}$Cα-$^{13}$CX (NCACX), and $^{15}$N-$^{13}$C′-$^{13}$CX (NCOCX) spectra (Figure 2.4) show sequential backbone connectivity for residues A118-V122. The resonance assignments are listed in Table 2.1 and shown in the 2D spectra in Figure 2.1 and 2.2. Importantly, no signals from residues 23-111 and 142-144 were observed in any of the experiments, clearly indicating that the amyloid core of huPrP23-144 maps to the region encompassing residues 112 to 141. Resonances for residues 112, 130 and 141 exhibited notably reduced intensities, and no cross-peaks were observed for residues 128-129 (except M129 $^{13}$C′, via a weak correlation to L130 $^{15}$N in the NCO spectrum). This points to the presence within the 112-141 core of regions characterized by varying degrees of conformational flexibility, with a short segment Y128-M129 being especially highly dynamic. Note that the identification of residues within the 112-141 core region is unambiguous. This unambiguousness of assignment, confirmed by multiple 2D and 3D NMR spectra, was facilitated by the fact that several amino acid types with unique $^{13}$C chemical shift patterns (i.e., Ala, Ile, Val) are present only in this part of huPrP23-144.

It is well-established that NMR chemical shifts are highly sensitive reporters of protein secondary structure, both in solution and the solid phase. Here, the $^{13}$C and $^{15}$N shifts were used to predict the backbone conformation of the huPrP23-144 amyloid core region. The $^{13}$C′, $^{13}$Cα, and $^{13}$Cβ secondary shifts, $\Delta\delta$, (i.e., the differences between the experimental chemical shifts and the corresponding random-coil values) are plotted as a function of the residue number in Figure 2.5.
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Table 2.1 $^{15}$N and $^{13}$C chemical shifts for residues in the amyloid core region of huPrP23-144 fibrils. Chemical shifts are reported in ppm, and referenced relative to DSS using adamantane as a secondary standard, with the $^{13}$C chemical shift of 40.48 ppm for the downfield resonance $^{80}$. Resonances corresponding to residues 23-111, 128-129 (except M129 $^{13}$C') and 142-144 were not detected at 0°C, in NMR experiments which used cross-polarization techniques $^{38,77}$. This is due to increased conformational flexibility for these residues as discussed in the main text.
Figure 2.5 Secondary structure of the amyloid core in huPrP23-144 fibrils predicted by PSSI (A). Secondary chemical shifts for $^{13}$C’ (B), $^{13}$Cα (C) and $^{13}$Cβ (D). (E) Normalized PSSI probabilities of the secondary structure types: (●) β-strand, (▼) α-helix; (○) coil.
The inspection of these plots reveals that the $^{13}\text{C}'$ secondary shifts for all but two residues are negative, with an average value of $\Delta\delta_{\text{avg}} = -1.9 \pm 1.6$ ppm. Furthermore, most $^{13}\text{C}\alpha$ and $^{13}\text{C}\beta$ secondary shifts are negative ($\Delta\delta_{\text{avg}} = -0.6 \pm 1.5$ ppm) and positive ($\Delta\delta_{\text{avg}} = 1.5 \pm 1.4$ ppm), respectively. This immediately indicates that the majority of residues in the huPrP23-144 amyloid core region adopt a $\beta$-strand conformation. To further probe this issue, a semi-quantitative conformational analysis was performed using the program PSSI$^{81}$. The normalized probabilities of different secondary structure types (i.e., $\alpha$-helix, $\beta$-strand and coil) and the secondary structure classification are shown in Figure 2.5. Altogether, this analysis clearly indicates that most residues between 112-122 and 130-139 are in a $\beta$-strand conformation, whereas unordered structure is predicted for residues 140-141 at the edge of the amyloid core, as well as residues 123-129. The latter region contains four glycines and the highly flexible Y128-M129 segment.

2.2.2 Variable Temperature NMR Studies of Conformational Dynamics in huPrP23-144 Amyloid.

As noted above, most of the NMR signals, including those corresponding to the entire N-terminal segment 23-111, were not observed in 0°C experiments with cross-polarization. This suggests that large segments of huPrP23-144 in the amyloid state remain highly dynamic, attenuating nuclear dipole-dipole couplings required for efficient cross-polarization. To confirm the presence of this segmental dynamics, we performed systematic solid-state NMR studies as a function of temperature between -30 and 30°C. Figure 2.6 shows a series of 1D $^{13}\text{C}$ MAS spectra, where the initial $^{13}\text{C}$ magnetization
was generated by either $^1$H-$^{13}$C CP (i.e., the conditions that ‘filter out’ flexible parts of the protein) or direct polarization (DP) (i.e., a single 90° $^{13}$C pulse, which excites with equal efficiency nuclei in both rigid and flexible regions). These experiments may be summarized as follows. First, while the CP spectra between -20 and 30°C show only modest temperature-dependent differences, the -30°C spectrum is markedly different, displaying substantially broader signals and a ~2-fold increase in the total integrated intensity as compared with the spectrum recorded at -20°C. Second, the linewidths in the DP series of spectra progressively decrease as the temperature increases; a particularly striking example is the region corresponding to side-chains of His, Phe, Trp and Tyr (~100-140 ppm). Third, for all CP/DP pairs recorded between -20 and 30°C, significant differences in peak widths and intensities (i.e., apart from a uniform intensity scaling of the entire spectrum) are observed at each temperature. Only for the -30°C CP/DP pair is the CP spectrum an essentially 2-fold enhanced version of the DP spectrum, as would be expected in the rigid-lattice limit $^{38}$. Altogether, these variable temperature studies confirm the presence of non-uniform backbone and side-chain dynamics of huPrP23-144 molecules in the amyloid fibrils. While the amyloid core remains highly ordered at all temperatures probed, the entire region between the N-terminus and residue 112 exhibits significant conformational dynamics. This segmental flexibility is attenuated at low temperatures, becoming effectively ‘frozen out’ at approximately -30°C.
Figure 2.6
Figure 2.6 (previous page) Variable temperature 1D $^{13}$C cross-polarization (CP) (A-H) and $^{13}$C direct polarization (DP; i.e., 90°-acquire) (I-P) spectra of huPrP23-144 fibrils recorded at 11.111 kHz MAS. Temperatures are: (A,I) 30°C; (B,J) 20°C; (C,K) 10°C; (D,L) 0°C (initial condition); (E,M) 0°C (after warming to 30°C and cooling to -30°C); (F,N) -10°C; (G,O) -20°C; (H,P) -30°C. Data were recorded with 256 scans, 30 ms acquisition with 70 kHz TPPM $^1$H decoupling, and recycle delays of 2.5 s (CP) and 5 s (DP), processed using an 81°-shifted sine-bell window function, and zero-filled to 16384 points. All spectra are displayed on the same horizontal and vertical scales. $^{13}$C\' rotational sidebands are indicated by asterisks in the -30°C CP spectrum (H), and the arrow near 0 ppm in the -30°C DP spectrum (P) indicates a background natural abundance $^{13}$C resonance characteristic of the NMR probe used (observed in all $^{13}$C DP spectra). Tentative assignments of selected, well-resolved $^{13}$C resonances (based on the approximate spectral intensities and expected chemical shifts obtained from the BioMagResBank, http://www.bmrb.wisc.edu) are indicated in the 30°C DP spectrum (I). For the backbone Gly Cα and Thr Cβ resonances, the frequencies of the peak maxima (45.2 ppm for Gly Cα and 69.6 ppm for Thr Cβ) correspond closely to random-coil values (45.1 ppm for Gly Cα and 69.2 ppm for Thr Cβ).
In order to gain higher resolution insight into temperature-dependent changes in the segmental flexibility of huPrP23-144 fibrils, we recorded 2D NCA and CC spectra at -30, 0 and 20°C (Figure 2.7 and Figure 2.8). While the 0 and 20°C spectra are similar to each other, major changes in peak intensities and widths are evident at -30°C. In addition to the amyloid core signals, numerous additional peaks appear at -30°C in all spectral regions associated with backbone and side-chain $^{15}\text{N}-^{13}\text{C}$ and $^{13}\text{C}-^{13}\text{C}$ correlations. The appearance of these additional resonances indicates that at -30°C parts of the protein outside the amyloid core region become sufficiently rigid to enable efficient dipolar magnetization transfers between $^1\text{H}$, $^{13}\text{C}$ and $^{15}\text{N}$ nuclei, in complete agreement with the 1D $^{13}\text{C}$ spectra discussed above. In fact, quantitative analysis of the 2D NCA spectra reveals that most residues in huPrP23-144 become rigid at -30°C. Using the integrated cross-peak volumes it can be estimated that ~83-120 total residues and ~35-47 glycines contribute to the -30°C spectrum. Given the approximate nature of such an analysis, these estimates are in reasonable agreement with the expectation that 125 residues in huPrP23-144, including 40 glycines, should contribute to the spectrum in the rigid-lattice limit. Finally, it is notable that, in contrast to the amyloid core resonances, additional cross-peaks that appear at -30°C exhibit substantial inhomogeneous linebroadening. This suggests that the flexible N-terminal region of huPrP23-144 in amyloid fibrils likely exists as an ensemble of largely disordered states characterized by a random-coil-like conformation. This conclusion is further supported by characteristic random-coil chemical shifts observed for all Gly $^{13}\text{C}\alpha$ and Thr $^{13}\text{C}\beta$ resonances that can be uniquely identified in the 1D $^{13}\text{C}$ DP spectrum recorded at 30°C (Figure. 2.6).
Figure 2.7 Variable temperature 2D $^{15}$N-$^{13}$C$\alpha$ and $^{13}$C-$^{13}$C spectra of huPrP23-144 fibrils. (A-C) 2D $^{15}$N-$^{13}$C$\alpha$ spectra recorded at temperatures of 20°C (A), 0°C (B), and -30°C (C). Small regions from 2D $^{13}$C-$^{13}$C DARR spectra ($\tau_{\text{mix}} = 10$ ms) corresponding to aliphatic-carbonyl (D-F) and aliphatic-aliphatic (G-I) correlations, recorded at 20°C (D,G), 0°C (E,H), and -30°C (F,I). Acquisition, processing and display parameters are identical for each spectrum within the $^{15}$N-$^{13}$C$\alpha$ and $^{13}$C-$^{13}$C series (see Figures 2.1 and 2.2 for details of experimental parameters and resonance assignments, and Figure 2.8 for overlay views of $^{15}$N-$^{13}$C$\alpha$ and $^{13}$C-$^{13}$C spectra acquired at different temperatures).
Figure 2.8 Two-dimensional NMR spectra of huPrP23-144 fibrils recorded at 0°C (red contours) and -30°C (blue contours). Small regions from (A) NCA and (B-D) CC spectra corresponding to (B) aliphatic-aliphatic, (C) aliphatic-carbonyl and (D) aromatic-aromatic correlations (c.f., Figure 2.1, 2.2 and 2.7).
2.3 Discussion

In addition to cases arising sporadically and those acquired by infection, prion diseases may also have a genetic origin. Over twenty mutations in the PRNP gene have been identified to date that correspond to Gerstmann-Straussler-Scheinker disease (GSS), familial CJD and fatal familial insomnia. Unlike in some other forms of prion diseases, a consistent feature of GSS syndrome is extensive accumulation of amyloid fibrils. Systematic biochemical studies have revealed that the major component of this amyloid is the ~7 kDa PrP fragment spanning residues ~88 to ~146, although the exact position of N- and C-termini can vary between residues 81-88 and 144-153, respectively\textsuperscript{82, 83}. A similar fragment is also found in amyloid deposits associated with cerebral PrP amyloid angiopathy in individuals with the Y145Stop mutation\textsuperscript{27}. Furthermore, the region encompassing residues ~89-140 is an integral part of the minimal sequence shown to sustain prion replication in cell culture and transgenic mice\textsuperscript{84, 85}, suggesting that it plays an essential role in the PrP\textsuperscript{C} to PrP\textsuperscript{Sc} conversion.

The apparent importance of the ~89-144 region of PrP in the pathogenesis of different forms of prion diseases has prompted numerous biophysical and structural studies with synthetic peptides encompassing this sequence\textsuperscript{86-89}. However, despite considerable effort, structural characterization of amyloid fibrils formed by these peptides is incomplete, remaining at a relatively low resolution. To bridge this gap, we have recently initiated systematic structural studies of PrP amyloid using MAS solid-state NMR spectroscopy. Instead of resorting to short synthetic peptide fragments, our present work was performed using recombinant protein encompassing the entire sequence of a
disease-associated human prion protein variant Y145Stop (huPrP23-144). Apart from facilitating studies with a longer polypeptide of obvious biological relevance, another advantage of a recombinant protein is that it provides inexpensive means for the incorporation of $^{13}\text{C},^{15}\text{N}$-labeled amino acids. Solid-state NMR spectra of fibrils formed by uniformly $^{13}\text{C},^{15}\text{N}$-labeled huPrP23-144 were of surprisingly high resolution, allowing an unambiguous assignment of resonances corresponding to all amino acid residues showing a high degree of immobilization.

The key finding of the present study is that the relatively rigid and highly ordered core of huPrP23-144 amyloid maps to the C-terminal region encompassing residues 112-141, whereas the entire N-terminal sequence 23-111 and a short segment 142-144 are highly flexible, lacking any ordered organization. Chemical shift analysis indicates that the core region contains β-strands between residues 112-122 and 130-139, with residues 123-129 likely forming a partially flexible loop. The 112-122 segment may not consist of a single continuous strand, but rather two shorter strands (residues 112-115 and 118-122) separated by a short, rigid non-β segment 116-117 (Figure 2.5). Interestingly, while in the native structure of the full-length human prion protein monomer residues 128 and 129 are part of β-strand 128-131$^{90}$, in the huPrP23-144 amyloid structure these residues appear to be in a less ordered conformation. Although the present data are insufficient to deduce a high-resolution structural model, the general folding motif for huPrP23-144 in amyloid fibrils appears similar to the β-strand-bend-β-strand motif first identified in Aβ(1-40) amyloid$^8$ and later found in many other amyloid fibrils$^{14,20-22}$. 
Previously solid-state NMR was used to probe the structure of a 55-residue synthetic peptide corresponding to mouse PrP segment 89-143 with P101L mutation. The authors concluded that upon aggregation the peptide ‘appears to convert completely to an extended, β-sheet-like form’. However, this conclusion was based on relatively limited chemical shift data for only six residues (corresponding to G94, A115, V121, M129, A132, and G142 in the sequence of human PrP) and, thus, was rather general in nature. A subsequent, more detailed study focused specifically on residues 112-124 (113-125 in the human sequence), indicating β-sheet conformation in this region. A similar region has been also found to comprise the β-sheet core in fibrils formed by a short PrP fragment 106-126. While the present study with huPrP23-144 amyloid supports the existence of β-strand(s) between residues 112-122, our detailed spectroscopic data are clearly at odds with the notion that essentially the entire PrP90-144 sequence is part of an extended β-sheet structure. In fact, there is conclusive evidence that the regions spanning residues 23-111 (including G94) and 141-144 (including G142) are highly flexible, falling outside the ordered β-sheet core of the amyloid. This apparent discrepancy between the present data and conclusions reached in the previous solid-state NMR study with mouse P101L PrP89-143 may be due to intrinsically different conformational preferences of residues ~90-111 in the shorter peptide and PrP23-144 containing the entire N-terminal sequence, different sample preparation methods (P101L PrP89-143 aggregates were obtained by precipitation from an acetonitrile:acetate-buffered saline mixture and the solid material was completely dried before NMR measurements, whereas the present study was performed using highly homogenous population of fully hydrated
fibrils prepared using physiologically relevant buffer conditions), or very limited direct experimental data in the previous study for the region N-terminal to residue 113. The latter possibility is especially plausible given that the presence of β-structure in this region in P101L PrP89-143 was proposed solely on the basis of a single Cα chemical shift measurement for G94, with no information for other residues\(^8\). Furthermore, chemical shift-based analysis of secondary structure for glycines tends to be less reliable than for other residues. One could also argue that relatively small species-dependent sequence differences and/or the P101L (P102L in the human sequence) mutation dramatically affect conformational preferences of the 90-111 region of the prion protein. This is, however, highly unlikely given essentially identical amide I infrared spectra of amyloid fibrils for human, mouse, and the P102L variant of human PrP23-144\(^7,9\).

The PK-resistant core of the infectious PrP\(^{Sc}\) starts at residue ~90. Previous modeling efforts based on electron microscopic data for 2D crystals proposed that the entire sequence 90-175 forms left-handed β-helices\(^9\). A fundamentally different picture was deduced from molecular dynamics simulations at low pH, postulating a PrP\(^{Sc}\) model consisting of six short β-strands between residues 90-165 (five within the 90-144 region), with the entire α-helical domain of PrP\(^C\) being preserved\(^9\). The structure experimentally observed in the present study for huPrP23-144 fibrils is at odds with the motifs predicted for the 90-144 region by both of these theoretical models. Most intriguingly, recent studies with amyloid fibrils formed spontaneously by the recombinant full-length prion protein (PrP23-231) or PrP90-231 consistently indicate that the β-sheet core in these fibrils maps to the C-terminal region starting at residue ~160, with the entire N-terminal
portion of the protein lacking significant PK-resistance\textsuperscript{94, 95} and showing no evidence for β-structure in direct biophysical experiments\textsuperscript{95, 96}. Although further studies are needed to explain the conundrum of apparently different structural preferences for residues 90-144 in fibrils formed by C-terminally truncated huPrP23-144 and PrP variants containing the entire C-terminal domain, the present observations call for great caution in extrapolating data for fragments of amyloidogenic proteins to infer structural properties of same sequences within fibrils formed by respective full-length proteins. Given these caveats, as well as obvious questions as to whether the high-resolution structures of amyloid fibrils formed \textit{in vitro} are the same as those found in diseased brain, our study falls short of resolving the ongoing controversy regarding the structure of PrPr\textsubscript{Sc}. Nevertheless, detailed structural data for huPrP23-144 amyloid are of major interest in the context of familial prion diseases such as GSS syndrome and familial cerebral angiopathy with 145Y mutation, in which cases the C-truncated PrP fragments are of direct relevance to the disease pathogenesis.

Apart from its central role in some familial prion diseases, PrP23-144 has been recently shown to provide an invaluable model for studying basic mechanistic principles underlying the phenomenon of prion strains, as well as the molecular basis of barriers in prion propagation\textsuperscript{6, 7}. In particular, previous studies have demonstrated that the seeding specificity and strain characteristics of PrP23-144 are fully encoded in fibril conformation, and that these properties depend on the nature of amino acid residues at positions 138 and 139. However, the conformational correlates established in these studies were of intrinsically low resolution. The present work reveals that residues 138
and 139 in huPrP23-144 are located just at the edge of the C-terminal strand of the β-sheet core, a strategic location that may explain the role of these two residues in controlling fibril conformation as well their capacity for species- and strain-selective recruitment of the protein monomer. Further extension of this work to interatomic distance measurements—together with inclusion of PrP23-144 amyloid variants used in the previous study—should allow construction of atomic-resolution structural models, providing full understanding of the structural basis of PrP23-144 amyloid strains.

2.4 Materials and Methods

2.4.1 Expression and Purification of huPrP23-144.

The plasmid encoding huPrP23-144 with N-terminal linker containing His₆-tag and a thrombin cleavage site has been described previously. Uniformly $^{13}$C, $^{15}$N-labeled huPrP23-144 was expressed in *Escherichia coli* using a minimal medium with $^{13}$C-glucose (3 g/l) and $^{15}$NH₄Cl (1 g/l) (Cambridge Isotope Laboratories) as the sole carbon and nitrogen sources, respectively, and purified using a nickel-nitrilotriacetic acid agarose resin. The His₆-tag was cleaved using biotinylated thrombin (Novagen), the thrombin sequestered using streptavidin-agarose beads, and the residual His₆-tag removed by dialysis against ultrapure water. The purified 126-residue protein consisted of the huPrP23-144 sequence with an N-terminal Gly-Ser-Asp-Pro extension. The protein was stored as a lyophilized powder, and had a final purity of >95% as determined by SDS-PAGE.
2.4.2 Preparation of huPrP23-144 Amyloid Fibrils.

Lyophilized huPrP23-144 was dissolved in ultrapure water at a concentration of 400 μM, and fibrilization was initiated as described previously\textsuperscript{4, 6} by addition of potassium phosphate buffer, pH 6.4, to a final concentration of 50 mM. Fibrils were allowed to form at 25°C under the conditions of gentle rotation at 8 rpm. Prior to NMR analysis, amyloid fibril samples were characterized by atomic force microscopy (Figure 2.9; see references\textsuperscript{4, 7} for experimental details). Fibrils (~12 mg of \textsuperscript{13}C,\textsuperscript{15}N protein) were pelleted by centrifugation, washed several times with 50 mM potassium phosphate buffer, pH 6.4, and packed using low-speed centrifugation into a 3.2 mm limited-speed 36 μl Varian SSNMR rotor. The rotor was sealed using custom-made spacers to prevent sample dehydration during experiments.

2.4.3 Solid-State NMR Spectroscopy.

Spectra were recorded using a three-channel 500 MHz Varian spectrometer, equipped with a 3.2 mm BioMAS probe in the \textsuperscript{1}H–\textsuperscript{13}C–\textsuperscript{15}N configuration. The sample temperature was controlled by a stream of compressed air, delivered to the sample via a variable-temperature (VT) stack. All temperature values listed in the paper consistently refer to the temperature of the VT gas at the sample; under the experimental conditions used the average sample temperatures were 5 ± 2°C higher than the VT gas temperature, as determined by lead nitrate calibration.

The pulse schemes employed in this work are analogous to those used in previous SSNMR studies of proteins\textsuperscript{78}. The following 2D and 3D experiments were used to
establish sequential resonance assignments of the huPrP23-144 amyloid core region at 0°C: 2D $^{13}$C-$^{13}$C (CC), 2D $^{15}$N-$^{13}$Cα (NCA), 2D $^{15}$N-$^{13}$C’ (NCO), 2D $^{15}$N-(13Cα)-$^{13}$CX (N(CA)CX), 2D $^{15}$N-(13C’)-$^{13}$CX (N(CO)CX), 3D $^{13}$C’-$^{15}$N-$^{13}$Cα (CONCA), 3D $^{15}$N-$^{13}$Cα-$^{13}$CX (NCACX), and 3D $^{15}$N-$^{13}$C’-$^{13}$CX (NOCX). Where applicable, the $^{15}$N-$^{13}$Cα/$^{13}$C’ correlations were established using adiabatic SPECIFIC CP ($\tau_{mix,NCA}/\tau_{mix,NCO} = 3.5/6.0$ ms), and $^{13}$C-$^{13}$C magnetization transfer was achieved using dipolar assisted rotational resonance ($\tau_{mix} = 10$ ms for 2D CC, 15 ms otherwise). Two-pulse phase modulated proton decoupling at a field strength of ~70 kHz was used during all chemical shift evolution periods. Data were processed using NMRPipe. Unless otherwise noted in figure captions all spectra were acquired at 11.111 kHz MAS rate and 0°C.

The two-dimensional NCO and NCA spectra presented in Figure 2.1 were recorded as 768* ($t_1$, $^{15}$N) × 1400* ($t_2$, $^{13}$C) data matrices with acquisition times of 15.3 ms ($t_1$) and 28 ms ($t_2$), and a measurement time of 8.5 h. Cross-peaks were drawn with the lowest contour at ~10 times the root-mean-square (RMS) noise level and labeled by residue number according to the $^{15}$N frequency.

The two-dimensional $^{13}$C-$^{13}$C spectrum presented in Figure 2.2 was acquired with DARR $^{13}$C-$^{13}$C mixing ($\tau_{mix} = 10$ ms) and ~70 kHz TPPM $^1$H decoupling in $t_1$ and $t_2$. The spectrum was recorded as a 600 ($t_1$, $^{13}$C) × 1400* ($t_2$, $^{13}$C) data matrix with acquisition times of 9.0 ms ($t_1$) and 28 ms ($t_2$), and a measurement time of 20 h. Cross-peaks were drawn with the lowest contour at ~5 times the RMS noise level.
The two-dimensional NCO spectrum used in Figure 2.3 was acquired using parameters analogous to those given above for the NCO spectrum in Figure 2.1, but with an acquisition time of 30 ms and in the presence of a rotor-synchronized $^{15}$N $\pi$-pulse train (\(\sim 28\) kHz rf field, $xy$-8 phase cycling\(^79\), one pulse every 8 rotor cycles) to decouple the \(\sim 15\) Hz $^{15}$N-$^{13}$C$'$ J-couplings. No apodization was applied in the $^{13}$C dimension during data processing. The partially-resolved doublets in the $^{13}$C dimension (which are less pronounced in the absence of $^{15}$N-$^{13}$C$'$ decoupling; c.f., Fig. 2.1) correspond to \(\sim 55\) Hz $^{13}$C$'$-$^{13}$C$\alpha$ J-couplings, and indicate that the amyloid core region of huPrP23-144 fibrils is highly ordered.

Acquisition parameters for the 3D sequential resonance assignments slices in Figure 2.4 are as follows, CONCA: $24\times (t_1, ^{15}$N) $\times 24\times (t_2, ^{13}$C$') \times 1000\times (t_3, ^{13}$C) data matrix with acquisition times of 8.3 ms ($t_1$), 8.3 ms ($t_2$) and 20 ms ($t_3$), measurement time 38.5 h; NCACX: $26\times (t_1, ^{15}$N) $\times 28\times (t_2, ^{13}$C$\alpha) \times 1400\times (t_3, ^{13}$C) data matrix with acquisition times of 9.0 ms ($t_1$), 4.9 ms ($t_2$) and 28 ms ($t_3$), measurement time 48.5 h; NCOCX: $26\times (t_1, ^{15}$N) $\times 24\times (t_2, ^{13}$C$') \times 1400\times (t_3, ^{13}$C) data matrix with acquisition times of 9.0 ms ($t_1$), 8.3 ms ($t_2$) and 28 ms ($t_3$), measurement time 41.5 h. Cross-peaks were drawn with the lowest contour at \(\sim 8\) times the RMS noise level.

The one-dimensional spectra in Figure 2.6 were recorded with 256 scans, 30 ms acquisition with 70 kHz TPPM $^1$H decoupling, and recycle delays of 2.5 s (CP) and 5 s (DP), processed using an $81^\circ$-shifted sine-bell window function, and zero-filled to 16384 points. All spectra are displayed on the same horizontal and vertical scales. $^{13}$C$'$ rotational sidebands are indicated by asterisks in the -30°C CP spectrum, and the arrow near 0 ppm
in the -30°C DP spectrum indicates a background natural abundance $^{13}$C resonance characteristic of the NMR probe used (observed in all $^{13}$C DP spectra). Tentative assignments of selected, well-resolved $^{13}$C resonances (based on the approximate spectral intensities and expected chemical shifts obtained from the BioMagResBank, http://www.bmrb.wisc.edu) are indicated in the 30°C DP spectrum. For the backbone Gly C\textalpha{} and Thr C\textbeta{} resonances, the frequencies of the peak maxima (45.2 ppm for Gly C\textalpha{} and 69.6 ppm for Thr C\textbeta{}) correspond closely to random-coil values (45.1 ppm for Gly C\textalpha{} and 69.2 ppm for Thr C\textbeta{}).

Chemical shifts assignments for the amyloid core region of huPrP23-144 are listed in Table 2.1. The chemicals shifts are reported in ppm, and referenced relative to DSS using adamantane as a secondary standard, with the $^{13}$C chemical shift of 40.48 ppm for the downfield resonance$^{80}$. Resonances corresponding to residues 23-111, 128-129 (except M129 13C’) and 142-144 were not detected at 0°C, in NMR experiments which used cross-polarization techniques$^{38,77}$. 
Figure 2.9 Representative atomic force microscopy images of the huPrP23-144 amyloid fibril sample used for solid-state NMR measurements. The fibrils were formed by incubating the protein (400 µM) at 25°C in 50 mM potassium phosphate buffer, pH 6.4. (Top) Image of undiluted fibrils taken directly at the end of the reaction (for the SSNMR experiments the fibrils were further concentrated by centrifugation, washed with 50 mM potassium phosphate pH 6.4 buffer, and transferred into a Varian 3.2 mm limited-speed 36 µl zirconia rotor). (Bottom) Image taken following a 10-fold dilution of the starting fibril suspension. The scale bars correspond to 1 µm.
Chapter 3

Conformational Flexibility of Y145Stop Human Prion Protein Amyloid Fibrils Probed by Solid-State Nuclear Magnetic Resonance Spectroscopy


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3.1 Introduction

The structural rearrangement of a largely $\alpha$-helical cellular brain protein, dubbed prion protein or PrP$^C$, to highly organized, $\beta$-sheet-rich amyloid-like aggregates, PrP$^Sc$, underlies a class of fatal neurological disorders in mammals, referred to as transmissible spongiform encephalopathies or prion diseases.\(^1\,3\,99\,-\,101\) According to the ‘protein-only’ hypothesis,\(^102\,103\) the misfolded PrP$^Sc$ isoform itself acts as the infectious agent that self-propagates by a mechanism involving binding to PrP$^C$ and templating its conformational conversion to PrP$^Sc$ state. The finding that infectious PrP$^Sc$ particles can be generated \textit{in vitro},\(^68\,-\,70\) combined with discoveries of prion-like protein conformation-based inheritance phenomena in yeast and other fungi,\(^71\,72\,104\) have provided important data in support of the notion that proteins alone can function as self-replicating infectious agents. While most recent evidence points to the ability of PrP$^Sc$ aggregates to adopt a spectrum of
molecular conformations as being a key determinant of prion propagation, the molecular mechanisms of prion and amyloid conformational inheritance and infectivity remain generally poorly understood at the atomic level.\textsuperscript{105-107}

Previous studies by us and others have demonstrated that certain features of mammalian prion conversion to the amyloid state can be conveniently modeled \textit{in vitro}, using a C-truncated Y145Stop human PrP variant, huPrP23-144, linked to the pathogenesis of a hereditary cerebral amyloid angiopathy.\textsuperscript{27,108,109} Recombinant PrP23-144 is readily converted under physiologically relevant buffer conditions from soluble monomeric form to amyloid fibrils, and this conversion reaction occurs in a nucleation-dependent, autocatalytic manner.\textsuperscript{4,5} Although PrP23-144 fibrils have not been shown to be infectious \textit{in vivo}, this simple system provides a convenient model \textit{in vitro} for studying some of the most fundamental aspects of mammalian prion propagation including the phenomena of prion strains and species barriers.\textsuperscript{6,7} While a systematic series of biochemical and biophysical studies carried out using several homologous mammalian PrP23-144 sequences\textsuperscript{6,7} established that seeding specificities of PrP23-144 amyloid are a consequence of the unique conformational properties of different amyloid strains, the methods used in this analysis were of inherently low resolution, precluding the full molecular-level understanding of these phenomena.

In order to elucidate the detailed structural basis of PrP23-144 strains and seeding specificities, we have recently commenced the characterization of PrP23-144 amyloid fibrils by using high-resolution multidimensional magic-angle spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) techniques. Surprisingly, our initial studies
revealed that human PrP23-144 amyloid contains a highly compact, relatively rigid and β-sheet-rich core region of only ~30 amino acids (aa) located near the C-terminus. Concurrently, the remaining residues, corresponding primarily to the large N-terminal domain (aa ~23-111), exhibit significant conformational flexibility within the fibril lattice. The latter conclusion was based on results of variable temperature SSNMR studies, which showed that signals for the vast majority of huPrP23-144 residues were ‘invisible’ in conventional cross-polarization (CP) based SSNMR spectra recorded above -20°C due to the effective motional averaging of nuclear magnetic dipole-dipole couplings, but could be detected at lower temperatures upon freezing the fibrils.

Here, we extend our SSNMR analysis of the conformational flexibility of huPrP23-144 amyloid by detecting directly, at ambient conditions, the highly dynamic huPrP23-144 segments using J-coupling based MAS SSNMR methods, and probing the molecular motions occurring on the NMR timescale within the relatively rigid amyloid core domain using site-resolved SSNMR measurements of backbone dipolar order parameters and transverse spin relaxation rate constants. Altogether, these studies reveal that huPrP23-144 fibrils share common features with other amyloid fibrils composed of intact protein molecules including α-synuclein,\textsuperscript{10} tau,\textsuperscript{12,110} and fungal prion protein HET-s,\textsuperscript{16-19} and highlight the structural and dynamic complexities inherent to protein assemblies of this type.
3.2 Experimental Section

3.2.1 Preparation of $^{13}$C, $^{15}$N-Labeled huPrP23-144 Amyloid Fibrils.

Uniformly $^{13}$C, $^{15}$N-labeled huPrP23-144 was overexpressed in Escherichia coli and purified as described previously. Lyophilized huPrP23-144 was dissolved in ultrapure water at a concentration of 5 mg/ml (~0.4 mM). Fibril formation was initiated by addition of pH 6.4 potassium phosphate buffer to a final concentration of 50 mM, and allowed to proceed to completion overnight at 25°C. Fibrils were routinely characterized by atomic force microscopy to confirm sample homogeneity, pelleted by low-speed centrifugation, washed with several aliquots of 50 mM pH 6.4 potassium phosphate buffer, and centrifuged directly into a 3.2 mm limited-speed (36 μl) Varian (Palo Alto, CA) zirconia rotor. The rotor was sealed using custom-made spacers (Revolution NMR, Fort Collins, CO) to prevent sample dehydration during experiments. The final SSNMR sample contained ~12 mg of $^{13}$C, $^{15}$N-labeled huPrP23-144 fibrils.

3.2.2 Solid-State NMR Spectroscopy.

NMR experiments were performed at 11.7 T (499.8 MHz for $^1$H, 125.7 MHz for $^{13}$C, and 50.6 MHz for $^{15}$N), using a three-channel Varian spectrometer equipped with 3.2 mm triple-resonance T3 and BioMAS™ probes in $^1$H-$^{13}$C-$^{15}$N configuration. All experiments employed MAS rates, $\omega_r/2\pi$, in the ~9-11 kHz regime, and temperatures in the 0-30°C range, as indicated in the text and figure captions. Sample spinning rates were actively regulated to ca. ± 3 Hz using a Varian MAS control unit, and sample
temperatures were controlled by a stream of dry compressed air, delivered to the sample using a variable-temperature (VT) stack at a flow rate of 30 L/min. Note that all temperatures listed in the paper consistently refer to the VT gas temperature at the sample—the actual average sample temperatures were ~5°C higher due to frictional heating as determined by lead nitrate calibration.\textsuperscript{112}

The pulse sequence diagrams for all experiments used in this study are shown in Figures 3.1, 3.2 and 3.3 and the experimental parameters are summarized below. In all experiments typical $^1$H, $^{13}$C, and $^{15}$N 90° pulse lengths were 2.5, 5.0, and 5.0 µs, respectively, and ~70 kHz two pulse phase modulated (TPPM) proton decoupling\textsuperscript{42} was applied during the chemical shift evolution periods unless indicated otherwise. For all pulse sequence figures the 90° and 180° pulses are represented by thin and thick black rectangles, respectively, and have phase $\chi$ unless indicated otherwise.

2D refocused INEPT experiments\textsuperscript{13, 113} (Figure 3.1A) recorded at 11.111 kHz MAS rate were used to obtain $^1$H-$^{13}$C and $^1$H-$^{15}$N correlation spectra containing signals from the most flexible segments of huPrP23-144 fibrils. The total durations of $^1$H-$^{13}$C and $^1$H-$^{15}$N magnetization transfer periods were 4.4 ms and 8.0 ms, respectively. $^1$H chemical shift evolution is achieved by incrementing $t_{1a}$ with $t_{1b}$ held at zero until $t_{1a}$=$2\delta$, at which point $t_{1b}$ is incremented. The delay $\delta$ was set to 1.1 ms for $^{13}$C and 2.0 ms for $^{15}$N.

Refocused INEPT spectra for Figure 3.5 were recorded as follows: (A) 360* ($t_1$, $^1$H) × 2000* ($t_2$, $^{13}$C) data matrix with acquisition times of 18 ms ($t_1$) and 40 ms ($t_2$) and a total measurement time of 24 h; (B) 360* ($t_1$, $^1$H) × 2000* ($t_2$, $^{15}$N) data matrix with acquisition times of 18 ms ($t_1$) and 40 ms ($t_2$) and a total measurement time of 72 h.
Cross-peaks are drawn with the lowest contour at 20 times the RMS noise level.

Tentative assignments based on average chemical shift values in the BioMagResBank\textsuperscript{114} are indicated in the spectra (see Table 3.1).

The CTUC-COSY scheme,\textsuperscript{115} preceded by either $^1$H-$^{13}$C cross-polarization (Figure 3.1B) or refocused INEPT (Figure 3.1C), was used at 11.111 kHz MAS rate to record 2D $^{13}$C-$^{13}$C’ spectra with correlations arising exclusively from the rigid amyloid core residues or the flexible segments of huPrP23-144 fibrils, respectively. The total duration of the $^{13}$C-$^{13}$C mixing period was 18 ms, during which ~100 kHz TPPM $^1$H decoupling was applied. The delays were set to: $T = 4.5$ ms, $\Delta = 16$ ms, $\delta = 1.1$ ms.

CTUC-COSY spectra for Figure 3.6 were recorded as follows: (A) 100* ($t_1$, $^{13}$C) $\times$ 1000* ($t_2$, $^{13}$C) data matrix with acquisition times of 9 ms ($t_1$) and 20 ms ($t_2$) and a total measurement time of 32 h; (B) 100* ($t_1$, $^{13}$C) $\times$ 1300* ($t_2$, $^{13}$C) data matrix with acquisition times of 9 ms ($t_1$) and 26 ms ($t_2$) and a total measurement time of 12 h. Cross-peaks are drawn with the lowest contour at 10 times the RMS noise level. Assignments in Figure 3.6A are taken from Helmus et al.\textsuperscript{23} Spinning sidebands are indicated by asterisks. Tentative assignments in Figure 3.6B are based on average chemical shift values in the BioMagResBank.\textsuperscript{114}
Figure 3.1 (A) Semi-constant time refocused INEPT pulse scheme\textsuperscript{113, 116} used to record the 2D $^1$H-$^1$H and $^1$H-$^15$N correlation spectra in Figure 3.5. $^1$H chemical shift evolution is achieved by incrementing $t_{1a}$ with $t_{1b}$ held at zero until $t_{1a}=2\delta$, at which point $t_{1b}$ is incremented. The delay $\delta$ was set to 1.1 ms for $^{13}$C and 2.0 ms for $^{15}$N. (B,C) CTUC-COSY pulse schemes\textsuperscript{115} used to record the 2D $^{13}$C-$^{13}$C correlation spectra in Figure 3.6. The schemes in (B) and (C), respectively, were used to selectively observe residues found in the most rigid and flexible regions of huPrP23-144 fibrils. The delays were set to: $T = 4.5$ ms, $\Delta = 16$ ms, $\delta = 1.1$ ms. For all sequences the 90° and 180° pulses are represented by thin and thick black rectangles, respectively, and have phase $x$ unless indicated otherwise.
The effective $^{1}H^{N}-^{15}N$ and $^{1}H^{\alpha}-^{13}C^{\alpha}$ one-bond dipolar couplings for residues in the amyloid core region of huPrP23-144 fibrils were probed at a MAS frequency of 9.470 kHz using 3D dipolar-chemical shift experiments, which employed SPECIFIC CP to generate $^{15}N-^{13}C^{\alpha}$ correlations and the T-MREV pulse scheme (highlighted by gray rectangles) to achieve simultaneous $^{1}H-^{13}C$ or $^{1}H-^{15}N$ recoupling and $^{1}H-^{1}H$ decoupling (Figure 3.2A). The T-MREV $^{1}H$ radiofrequency (RF) field strength was set to 113.6 kHz (corresponding to a 90° $^{1}H$ pulse length of 2.2 μs) and four T-MREV elements were incorporated into each rotor cycle. The $^{15}N$ and $^{13}C$ chemical shifts are encoded during $t_1$ and $t_2$, respectively, and the dipolar coupling evolution takes place during $\tau_{\text{mix}}$.

The 3D T-MREV spectrum for Figure 3.8A was recorded in an interleaved fashion for $\tau_{\text{mix}}$ as a 152* ($t_1, ^{15}N$) × 1400* ($t_2, ^{13}C$) × 16 ($\tau_{\text{mix}}, ^{1}H^{N}-^{15}N$ dipolar coupling) data matrix with acquisition times of 16 ms ($t_1$), 28 ms ($t_2$) and 792 μs ($\tau_{\text{mix}}$) and a total measurement time of 108 h.

The effective $^{15}N-^{13}C^{\alpha}$ and $^{15}N-^{13}C'$ one-bond dipolar couplings were determined at 11.111 kHz MAS rate using a 3D band-selective TEDOR scheme (Figure 3.2B). The $^{13}C^{\alpha}-^{13}C'$ J-decoupling during the $^{15}N-^{13}C^{\alpha}$ or $^{15}N-^{13}C'$ recoupling periods was achieved using frequency selective $^{13}C$ r-SNOB pulses with duration 360 μs, applied in the $^{13}C^{\alpha}$ (≈54 ppm) or $^{13}C'$ spectral region (≈174 ppm), respectively. The short delays $\kappa$ ensure that the total delay between the first and second REDOR periods is equal to an integer number of rotor cycles. The 180° $^{15}N$ recoupling pulse train was applied at a field strength of ≈28 kHz and phase cycled according to the $xy-4$ scheme. TPPM $^{1}H$ decoupling at a RF field strength of ≈100 kHz was applied throughout the $^{15}N$-
$^{13}\text{C}$ dipolar mixing and $^{15}\text{N}$ chemical shift evolution periods. The $^{15}\text{N}$ and $^{13}\text{C}$ chemical shifts are encoded during $t_1$ and $t_2$, respectively, and the dipolar coupling evolution takes place during $\tau_{\text{mix}}$.

The 3D spectrum for Figure 3.8B was recorded in an interleaved fashion for $\tau_{\text{mix}}$ as a $70^* (t_1, ^{15}\text{N}) \times 1400^* (t_2, ^{13}\text{C}) \times 12 (\tau_{\text{mix}}, ^{15}\text{N}-^{13}\text{C}'$ dipolar coupling) data matrix with acquisition times of 12.6 ms ($t_1$), 28 ms ($t_2$) and 4.32 ms ($\tau_{\text{mix}}$) and a total measurement time of 94 h. Cross-peaks in Figure 3.8A and B are drawn with the lowest contour at 7 times the RMS noise level. Resonance assignments are based on Helmus et al.\textsuperscript{23} and additional experimental details are provided in the Experimental Section.
Figure 3.2 3D dipolar-chemical shift pulse schemes used to measure the $^{1}\text{H}^{\text{N}}$-$^{15}\text{N}$, $^{1}\text{H}^{\alpha}$-$^{13}\text{C}^{\alpha}$, $^{15}\text{N}$-$^{13}\text{C}^{\alpha}$ and $^{15}\text{N}$-$^{13}\text{C}'$ one-bond dipolar couplings in huPrP23-144 fibrils (see Figures 3.9-3.14). The $^{15}\text{N}$ and $^{13}\text{C}$ chemical shifts are encoded during $t_1$ and $t_2$, respectively, and the dipolar coupling evolution takes place during $\tau_{\text{mix}}$. (A) The $^{1}\text{H}^{\text{N}}$-$^{15}\text{N}$ and $^{1}\text{H}^{\alpha}$-$^{13}\text{C}^{\alpha}$ couplings were measured using the T-MREV-4 sequence (highlighted by gray rectangles) applied to reintroduce either $^{15}\text{N}$-$^{1}\text{H}$ or $^{13}\text{C}$-$^{1}\text{H}$ couplings with simultaneous $^{1}\text{H}$-$^{1}\text{H}$ decoupling. (B) The $^{15}\text{N}$-$^{13}\text{C}^{\alpha}$ and $^{15}\text{N}$-$^{13}\text{C}'$ couplings were measured using 3D band-selective TEDOR. The selective $^{13}\text{C}$ pulses were of the r-SNOB type with durations of 360 $\mu$s, and were applied in either the $^{13}\text{C}^{\alpha}$ region ($\sim$54 ppm) or the $^{13}\text{C}'$ region ($\sim$174 ppm). The short delays $\kappa$ ensure that the total delay between the first and second REDOR periods is equal to an integer number of rotor cycles. For all sequences, the 90° and 180° pulses are represented by thin and thick black rectangles, respectively, and have phase $x$. The 180° degree REDOR pulses on the $^{15}\text{N}$ channel (gray rectangles) were phase cycled according to the $xy$-4 scheme.
Measurements of selected $^1$H, $^{13}$C and $^{15}$N transverse relaxation rate constants, $R_{1\rho}$ and $R_2$, were performed at 11.111 kHz MAS by combining the 2D $^{15}$N-$^{13}$C$^{\alpha}$ SPECIFIC CP scheme with an evolution period, during which appropriate NMR coherences were allowed to decay (Figure 3.3). For each experiment a set of 2D $^{15}$N-$^{13}$C$^{\alpha}$ correlation spectra was recorded as a function of one of the relaxation delays, $\tau$, highlighted by gray rectangles in the figure.

For $^1$H$^N$ $R_{1\rho}$ measurements, a $\sim$90 kHz spin-lock field was applied on the $^1$H channel prior to $^1$H-$^{15}$N CP. For the $^{15}$N and $^{13}$C$^{\alpha}$ $R_{1\rho}$ measurements $\sim$28 kHz spin-lock fields (satisfying the condition $\omega_{RF} \approx 2.5\omega_r$) were applied on $^{15}$N and $^{13}$C channels, respectively, with concurrent $\sim$100 kHz $^1$H continuous wave (CW) decoupling. These RF field settings were optimized to avoid $^{15}$N and $^{13}$C dipolar and chemical shift rotary resonance conditions$^{128}$ and $^{15}$N/$^{13}$C-$^1$H dipolar recoupling.$^{129}$ For the $^{15}$N $R_2$ measurements a spin-echo period consisting of a single 180$^0$ $^{15}$N pulse with concurrent $\sim$70 kHz TPPM $^1$H decoupling was inserted immediately prior to $^{15}$N chemical shift evolution.

To minimize potential long-term fluctuations of peak intensities during extended 3D SSNMR experiments designed to measure dipolar or relaxation trajectories, the corresponding pulse sequences were implemented with: (i) a constant duty factor by appending additional RF pulses on the appropriate channels following signal acquisition, and (ii) sampling the dipolar and relaxation dimension increments in an interleaved fashion, as the inner-most loops of the 3D datasets.
Figure 3.3 3D SSNMR pulse schemes used to measure the $^{1}\text{H}^{N}$, $^{13}\text{C}^{\alpha}$ and $^{15}\text{N} R_{1p}$ and $^{15}\text{N} R_{2}$ relaxation rates in huPrP23-144 fibrils in residue specific fashion (see Figures 3.15-3.20). For each experiment a set of 2D $^{15}\text{N}$-$^{13}\text{C}^{\alpha}$ correlation spectra was recorded as a function of one of the relaxation delays, $\tau$, highlighted by gray rectangles. For the $^{15}\text{N}$ and $^{13}\text{C} R_{1p}$ measurements the $^{15}\text{N}$ and $^{13}\text{C}$ spin-lock and $^{1}\text{H}$ decoupling field strengths were optimized to avoid the $^{15}\text{N}$ and $^{13}\text{C}$ dipolar and chemical shift rotary resonance conditions$^{128}$ and $^{15}\text{N}/^{13}\text{C}-^{1}\text{H}$ dipolar recoupling.$^{129}$ In practice this was achieved by setting the $^{15}\text{N}$ and $^{13}\text{C}$ spin-lock fields to $\sim28$ kHz ($\omega_{\text{RF}} \approx 2.5\omega_{r}$) and the $^{1}\text{H}$ decoupling field to $\sim100$ kHz. The $90^\circ$ and $180^\circ$ pulses are represented by thin and thick black rectangles, respectively, and have phase $x$. 

64
3.2.3 Data Processing and Simulations.

All NMR spectra were processed and analyzed using the NMRPipe/NMRDraw software package, where a typical 3D NMR experiment designed to measure dipolar couplings or relaxation rates in site-resolved fashion consists of a series of 2D $^{15}$N-$^{13}$C correlation spectra recorded for different values of a dipolar evolution time, $\tau_{\text{mix}}$, or relaxation delay, $\tau$ (see pulse schemes in Figures 3.2 and 3.3). For each 2D $^{15}$N-$^{13}$C spectrum in the series, the residue-specific cross-peak volumes were obtained using in-house Python scripts by summing up spectral intensities within integration boxes with dimensions corresponding to approximately the full peak width at half maximum along the $^{15}$N and $^{13}$C frequency axes. Note that for each cross-peak, the integration box dimensions were defined using the 2D spectrum in the series having the highest overall intensity, and subsequently used to integrate each spectrum in the series. This procedure yielded the cross-peak volumes and their uncertainties, $\sigma$, according to: $\sigma = \sqrt{N} \sigma_{\text{noise}}$, where $N$ is the number of spectral intensities present within the box limits and summed up to obtain the cross-peak volume, and $\sigma_{\text{noise}}$ is the estimated experimental noise that corresponds effectively to the root-mean-squared (RMS) noise obtained within NMRDraw by examining noise distributions in empty regions of the spectra.

Simulations of the $^1H-^{15}$N and $^1H-^{13}$C T-MREV dipolar trajectories were carried out in the time-domain using an average Liouvillian approach, described in detail by Hohwy et al. and Rienstra et al. and implemented in FORTRAN. The adjustable fit parameters included: (1) the magnitude of the scaled one-bond dipolar coupling between the heavy atom, $^{13}$C or $^{15}$N, and the directly bonded $^1H$, (2) the average proton coherence
decay rate, $\Gamma_2$, and (3) an overall amplitude scaling factor. Moreover, in order to obtain
the most accurate estimate of the one-bond dipolar couplings of interest, the simulation
model also took into account two additional, weaker dipolar couplings between the heavy
atom and distant protons, which were not varied during the simulation.\textsuperscript{119, 120} No fitting
was done for proline ($^1\text{H}^\text{N} - ^1\text{H}^\text{N}$) and glycine ($^1\text{H}^\alpha - ^1\text{H}^\alpha$) residues as well as weak or
overlapped correlations.

Simulations of the $^{15}\text{N} - ^1\text{H}^\alpha$ and $^{15}\text{N} - ^1\text{H}^\alpha$ band-selective TEDOR dipolar trajectories
were carried out in the time-domain using an average Hamiltonian approach described
previously,\textsuperscript{121, 124} implemented in FORTRAN. The adjustable fit parameters included: (1)
the magnitude of the scaled one-bond $^{15}\text{N} - ^1\text{H}^\alpha$ dipolar coupling, (2) the $^1\text{H}^\alpha$ transverse
relaxation rate, modeled as a single exponential decay, and (3) an overall amplitude
scaling factor. In addition, the simulation model included: the passive dipolar coupling
between the $^1\text{H}^\alpha$ spin and the next nearest $^{15}\text{N}$ nucleus, the effects of finite $^{15}\text{N}$ pulses
during TEDOR mixing,\textsuperscript{130} and for the $^{15}\text{N} - ^1\text{H}^\alpha$ experiment for all residues except
glycine, the ~35 Hz $^{13}\text{C}^\alpha - ^1\text{H}^\alpha$ J-coupling not refocused by the shaped pulse. No fitting
was done for weak or overlapped correlations.

For each residue and coupling type the order parameter is calculated as
\begin{equation*}
\langle S \rangle = \frac{d_{IS}^{exp}}{d_{IS}^{rigid}}
\end{equation*}
where $d_{IS}^{exp}$ is the experimentally determined scaled dipolar coupling
and $d_{IS}^{rigid}$ is the corresponding dipolar coupling in the rigid-lattice limit. Bond lengths of
1.04 Å ($^1\text{H}^\text{N} - ^1\text{H}^\text{N}$), 1.12 Å ($^1\text{H}^\alpha - ^1\text{H}^\alpha$), 1.46 Å ($^{15}\text{N} - ^1\text{H}^\alpha$) and 1.33 Å ($^{15}\text{N} - ^1\text{H}^\alpha$), and pulse
sequence dipolar scaling factors of 0.485 (T-MREV-4)\textsuperscript{119} and 0.434 (TEDOR)\textsuperscript{130} yield
d$\text{IS}^{rigid}$ magnitudes of 5.25 kHz ($^1\text{H}^\text{N} - ^1\text{H}^\text{N}$), 10.4 kHz ($^1\text{H}^\alpha - ^1\text{H}^\alpha$), 427 Hz ($^{15}\text{N} - ^1\text{H}^\alpha$) and
565 Hz (15N-13C'). Note that the 1H\textsuperscript{N}-15N and 1H\textsuperscript{\alpha}-13C\textsuperscript{\alpha} bond lengths used to calculate d\textsubscript{IS\textsuperscript{rigid}} have been compensated for the effects of fast vibrational and librational averaging,\textsuperscript{117, 118, 131-133} while the 15N-13C\textsuperscript{\alpha} and 15N-13C' bond lengths correspond to the standard crystallographic values.\textsuperscript{134, 135} See Table 3.2 for a summary of these calculations.

The 1H, 13C and 15N transverse relaxation rate constants were extracted by fitting cross-peak trajectories as a function of the relaxation delay to single exponential decays, with R\textsubscript{1D} or R\textsubscript{2} being the only adjustable fit parameter.

### 3.2.4 Dipolar Coupling Error Estimation.

A Monte Carlo approach, analogous to that described by Rienstra et al.,\textsuperscript{120} was used to obtain the best estimates and uncertainties of the one-bond 1H\textsuperscript{N}-15N, 1H\textsuperscript{\alpha}-13C\textsuperscript{\alpha}, 15N-13C\textsuperscript{\alpha} and 15N-13C' dipolar couplings. Briefly, the simulated dipolar trajectory which gave the best fit to the corresponding experimental trajectory was used to generate a set of 5,000 synthetic dipolar trajectories. This was accomplished by adding to each time point of the best-fit simulation a random amount of noise drawn from a Gaussian distribution with the standard deviation, \( \sigma \), given by the noise level obtained from NMR spectra as described above. Each synthetic trajectory was subsequently re-fit with the same simulation model yielding a set of 5,000 dipolar couplings used to form a distribution (see Figures 3.10-3.13 for examples of representative distributions). The best estimate and uncertainty of the dipolar coupling corresponded to the average value and standard deviation of this distribution, respectively. To minimize the computational time associated with the Monte Carlo procedure, extensive grids of simulated dipolar
trajectories covering large ranges of the fitting parameters were constructed and repeatedly used to fit all synthetic trajectories. The error bars in Figure 3.14 correspond to $\pm 2\sigma$.

3.3 Results and Discussion

3.3.1 Direct Detection of Highly Flexible Segments of huPrP23-144 Amyloid.

Our initial solid-state NMR analysis of huPrP23-144 fibrils identified the presence of a relatively rigid and highly ordered amyloid core region encompassing ~30 residues near the C-terminus (Figure 3.4). Residues in the relatively rigid amyloid core region are shown in black font, with predicted $\beta$-strand segments highlighted by gray rectangles. Residues displaying significant conformational flexibility are shown in red font. The NMR chemical shifts indicate that the majority of these core residues adopt a $\beta$-strand conformation. Remarkably, most of the huPrP23-144 sequence, corresponding primarily to N-terminal amino acids 23-111, was ‘invisible’ in conventional SSNMR experiments performed at temperatures higher than -20°C due to the motional averaging of magnetic dipole-dipole couplings required for efficient magnetization transfer via cross-polarization. On the other hand, these highly flexible residues could be detected by CP-based SSNMR methods at ca. -30°C on frozen samples, where the major protein backbone motions in huPrP23-144 fibrils appear to be effectively quenched. Here we extend our analysis of the mobile huPrP23-144 amyloid segments by using solution-state NMR type (i.e., J-coupling based) techniques applied under MAS conditions. Unlike dipolar couplings, J-coupling interactions have a non-zero isotropic component and can
drive efficient homo- and heteronuclear magnetization transfers in solids even in the presence of significant conformational dynamics. This permits the direct observation of highly flexible domains in immobilized proteins, provided that the relaxation of NMR coherences is sufficiently slow.\(^{10, 12, 13, 16, 110}\)

Figure 3.5 shows \(^1\)H-\(^{13}\)C and \(^1\)H-\(^{15}\)N chemical shift correlation spectra of huPrP23-144 fibrils recorded at ~11 kHz MAS using a 2D refocused INEPT scheme\(^{13, 113}\) (Figure 3.1A). These spectra were recorded at 30°C, where the relaxation of \(^1\)H, \(^{13}\)C and \(^{15}\)N coherences is attenuated relative to 0°C, resulting in a higher INEPT transfer efficiency. Note that our previous studies confirm that the huPrP23-144 amyloid core remains highly ordered at all temperatures between -30°C and 30°C.\(^{23}\) The \(^1\)H-\(^{13}\)C correlation spectrum (Figure 3.5A) contains a multitude of intense resonances in the aliphatic and aromatic regions, most of which exhibit pronounced \(^{13}\)C-\(^{13}\)C J-splittings in the \(^{13}\)C dimension, indicating the effective motional averaging of \(^{13}\)C-\(^1\)H and \(^1\)H-\(^1\)H dipolar couplings. Most important, a number of characteristic correlations that can be attributed only to residues present outside the amyloid core region (e.g., Thr and Trp) are present in this \(^1\)H-\(^{13}\)C spectrum. On the other hand, notably suppressed are correlations involving methyl groups of residues found exclusively in the amyloid core region (Ala, Ile, Leu and Val)—this is primarily due to the rapid relaxation of proton coherences for these relatively rigid residues during the first half of the refocused INEPT sequence.

The \(^1\)H-\(^{15}\)N spectrum (Figure 3.5B) reveals resonance frequencies of approximately 8.1 ± 0.3 ppm for all detected backbone \(^1\)H\(^N\) atoms. Tentative assignments of Gly, Ser and Thr residues (as well as Asn, Gln and Trp side-chain correlations) could be made based
on their characteristic $^{15}$N shifts. Amide proton frequencies in this range are typical of unstructured proteins,\textsuperscript{30} indicating that the highly flexible N-terminal domain in huPrP23-144 fibrils resembles an ensemble of largely disordered states with random-coil-like conformations.\textsuperscript{136} This is in accord with our previous observations of pronounced inhomogeneous linebroadening for this region of huPrP23-144 in low-temperature CP-based SSNMR spectra.\textsuperscript{23} Additional evidence for the dynamic disorder in the N-terminal region of huPrP23-144 is provided by the relatively limited dispersion of resonances in the $^1$H-$^{13}$C spectrum. Indeed, by assuming a random-coil protein conformation we were able to tentatively assign most resonances in this spectrum by simply mapping the observed chemical shift values onto the average residue- and site-specific chemical shifts listed in the BioMagResBank database (BMRB, http://www.bmrb.wisc.edu).\textsuperscript{114} This analysis, summarized in Table 3.1, yielded the average shift deviations, $\langle \delta_{obs} - \delta_{BMRB} \rangle$, of -0.02 ± 0.10 ppm for $\text{H}^\alpha$ and -0.2 ± 0.7 ppm for $\text{C}^\alpha$/C$^\beta$, and allowed us to readily identify correlations corresponding to all amino acid types (Arg, Asn, Asp, Gln, Gly, His, Lys, Met, Pro, Ser, Thr, Trp, and Tyr) present outside the core region of huPrP23-144 amyloid. The chemical shifts (in ppm) in Table 3.1 are given relative to DSS. Average chemical shift values obtained from the BioMagResBank (http://www.bmrb.wisc.edu) are given in parentheses.
Figure 3.4 Amino acid sequence of huPrP23-144. Residues in the relatively rigid amyloid core region are shown in black font, with predicted β-strand segments highlighted by gray rectangles. Residues displaying significant conformational flexibility are shown in red font. A four-residue (Gly-Ser-Asp-Pro) N-terminal extension remaining after thrombin cleavage has been omitted for clarity.
Figure 3.5 Two-dimensional $^1$H-$^13$C (A) and $^1$H-$^15$N (B) correlation spectra of huPrP23-144 fibrils recorded at 30°C and 11.111 kHz MAS rate. Refocused INEPT$^{113}$ with a total duration of 4.4 or 8.0 ms was used to transfer magnetization from $^1$H to $^13$C or $^15$N, respectively. $^1$H chemical shift evolution was achieved in a semi-constant time manner$^{116}$ (Figure 3.1A). Spectra were recorded as follows: (A) 360° ($t_1$, $^1$H) × 2000° ($t_2$, $^{13}$C) data matrix with acquisition times of 18 ms ($t_1$) and 40 ms ($t_2$) and a total measurement time of 24 h; (B) 360° ($t_1$, $^1$H) × 2000° ($t_2$, $^{15}$N) data matrix with acquisition times of 18 ms ($t_1$) and 40 ms ($t_2$) and a total measurement time of 72 h. Cross-peaks are drawn with the lowest contour at 20 times the RMS noise level. Tentative assignments based on average chemical shift values in the BioMagResBank$^{114}$ are indicated in the spectra (see Table 3.1).
Table 3.1 Tentative $^1$H and $^{13}$C resonance assignments for the flexible residues in huPrP23-144 fibril. Assignments were obtained from 2D $^1$H-$^{13}$C and $^1$H-$^{15}$N correlation spectra in Figure 3.3. The chemical shifts (in ppm) are given relative to DSS. Average chemical shift values obtained from the BioMagResBank (http://www.bmrb.wisc.edu) are given in parentheses.
To further probe the most flexible segments of huPrP23-144 fibrils we recorded 2D $^{13}\text{C}^-^{13}\text{C}'$ correlation spectra (Figure 3.6), where the initial $^{13}\text{C}$ magnetization was prepared using either $^1\text{H}^{-^{13}\text{C}}$ CP or refocused INEPT and the magnetization transfer between coupled $^{13}\text{C}$ nuclei was mediated by one-bond $^{13}\text{C}^{-^{13}\text{C}}$ J-couplings. The spectrum in Figure 3.6A was acquired at 0°C using the CTUC-COSY pulse scheme (Figure 3.1B), with the $^{13}\text{C}^{-^{13}\text{C}}$ magnetization transfer preceded by $^1\text{H}^{-^{13}\text{C}}$ CP. Given that substantial $^1\text{H}^{-^{13}\text{C}}$ dipolar couplings are required for efficient CP, this scheme effectively suppresses signals arising from the most flexible regions of huPrP23-144 amyloid. Indeed, this ‘CP-COSY’ $^{13}\text{C}^{-^{13}\text{C}}$ spectrum contains backbone $^{13}\text{C}$ signals from only the amyloid core residues and is in good agreement with the previously reported 2D $^{13}\text{C}^{-^{13}\text{C}}$ RF-assisted proton driven spin diffusion spectrum. Figure 3.6B shows a 2D $^{13}\text{C}^-^{13}\text{C}'$ spectrum also acquired at 30°C using a CTUC-COSY-type scheme, but where the initial $^{13}\text{C}$ magnetization was prepared by refocused INEPT instead of CP (Figure 3.1C). In analogy to the $^1\text{H}^{-^{13}\text{C}}$ and $^1\text{H}^{-^{15}\text{N}}$ spectra in Figure 3.5, this ‘INEPT-COSY’ experiment filters out signals from the amyloid core region and highlights resonances arising exclusively from the most flexible residues of huPrP23-144 (e.g., Asn, Asp, Gln, and Thr). The chemical shift values observed in this $^{13}\text{C}^{-^{13}\text{C}}$ spectrum combined with the relatively low spectral resolution further substantiate the notion that the N-terminal domain of huPrP23-144 fibrils is most accurately described as a dynamic, random-coil-like ensemble of protein chains.
Figure 3.6 2D $^{13}$C-$^{13}$C' CTUC-COSY $^{115}$ spectra of huPrP23-144 fibrils recorded at 11.111 kHz MAS rate. (A) Spectrum at 0°C, with initial $^{13}$C magnetization generated using $^{1}$H-$^{13}$C CP (Figure 3.1B). (B) Spectrum at 30°C, with initial $^{13}$C magnetization generated using $^{1}$H-$^{13}$C refocused INEPT (Figure 3.1C). (C) Overlay of CP (blue) and refocused INEPT (red) spectra shown in (A) and (B). Spectra were recorded as follows: (A) 100* ($t_1$, $^{13}$C) × 1000* ($t_2$, $^{13}$C) data matrix with acquisition times of 9 ms ($t_1$) and 20 ms ($t_2$) and a total measurement time of 32 h; (B) 100* ($t_1$, $^{13}$C) × 1300* ($t_2$, $^{13}$C) data matrix with acquisition times of 9 ms ($t_1$) and 26 ms ($t_2$) and a total measurement time of 12 h. Cross-peaks are drawn with the lowest contour at 10 times the RMS noise level. Assignments in (A) are taken from Helmus et al. $^{23}$ Spinning sidebands are indicated by asterisks. Tentative assignments in (B) are based on average chemical shift values in the BioMagResBank.
3.3.2 Probing the Sub-Microsecond Timescale Backbone Dynamics in the Core Region of huPrP23-144 Amyloid.

In contrast to the highly flexible random-coil-like N-terminated domain, the core region of huPrP23-144 amyloid fibrils appears to be generally quite rigid. This relative rigidity permits signals from the core residues to be detected in CP-based solid-state NMR spectra. Nevertheless, the considerable variation of cross-peak intensities in such spectra, as discussed below, suggests that motional phenomena may be present within this core region as well. Here we describe our analysis of the protein backbone dynamics for the huPrP23-144 core residues.

The fingerprint 2D chemical shift correlation spectra recorded using conventional SSNMR methods (see Figures 3.5A and 3.8 for representative $^{13}\text{C}-^{13}\text{C}$ and $^{15}\text{N}-^{13}\text{C}$ spectra, respectively) reveal that signals arising from residues 112-113 and 140-141 present at the periphery of the amyloid core are visibly attenuated relative to most other cross-peaks. Moreover, missing completely are cross-peaks for residues Y128 and M129 within the core region, while resonances from adjacent residues (e.g., G127, L130 and G131) display notably reduced intensities—similar prior observations formed the basis for the hypothesis that residues 128 and 129 are found in a partially flexible loop within the huPrP23-144 amyloid core. A detailed analysis of peak intensities in analogous 3D SSNMR spectra corroborates these findings and also offers new insights that are not readily apparent from the 2D datasets. In Figure 3.7 we show representative strips from a 3D $^{13}\text{C}'-^{15}\text{N}-^{13}\text{C}^\alpha$ (CONCA) chemical shift correlation spectrum as well as a plot of cross-peak volume as a function of residue number. Strips are labeled by residue number.
according to the $^{15}$N frequency, and the $^{13}$C$'$ (F$_2$) frequency is given inside each strip. Peaks from neighboring spectral planes are marked by asterisks. Peak volumes were obtained using NMRPipe/NMRDraw$^{98}$ and are given relative to the volume of the most intense correlation (A116).

These data illustrate that the most rigid part of the huPrP23-144 fibril core, (i.e., that characterized by highest cross-peak intensities) corresponds to aa $\sim$115-120—interestingly, this region spans parts of strands $\beta$1 and $\beta$2 and contains the short intervening non-$\beta$ bend. The remaining huPrP23-144 core residues exhibit the following intensity pattern: (i) cross-peaks for residues 121-127 are seen to decrease gradually toward nearly zero intensity, (ii) no cross-peaks are observed for residues 128-129 and very weak signals are seen for residues 130-131 as noted above, and (iii) cross-peaks for residues 132-140 (except for P137, which has no amide proton) have intensities that are on average $\sim$40 ± 10% of those obtained for residues found in the most rigid part of the amyloid core. These findings are consistent with the presence of molecular motions of differing degrees throughout the entire core region of huPrP23-144 fibrils.
Figure 3.7 Cross-peak intensities for residues in huPrP23-144 amyloid core. (A) Selected strips from a 3D CONCA spectrum (see Helmus et al. for details of data acquisition). Strips are labeled by residue number according to the $^{15}$N frequency, and the $^{13}$C′ ($F_2$) frequency is given inside each strip. Peaks from neighboring spectral planes are marked by asterisks. (B) Relative peak volumes in the 3D CONCA spectrum at 0°C as a function of residue number. Peak volumes were obtained using NMRPipe/NMRDraw and are given relative to the volume of the most intense correlation (A116).
In order to probe for the presence of relatively fast, sub-μs timescale protein backbone motions, we have conducted residue-specific measurements of the magnitudes of one-bond dipolar couplings by using 3D dipolar chemical shift SSNMR methods. Such SSNMR measurements have been previously applied to microcrystalline and membrane-associated proteins to yield information about backbone dynamics in the form of dipolar order parameters, \( \langle S \rangle = \frac{d_{IS}^{exp}}{d_{IS}^{rigid}} \), where \( d_{IS}^{exp} \) is the experimentally-determined dipolar coupling between nuclear spins I and S, and \( d_{IS}^{rigid} \) is the corresponding dipolar coupling in the rigid-lattice limit. These order parameters effectively report on the extent of internuclear bond vector reorientation, where in the absence of dynamics we have \( d_{IS}^{exp}=d_{IS}^{rigid} \) and \( \langle S \rangle=1 \), while for fully isotropic motion both \( d_{IS}^{exp} \) and \( \langle S \rangle \) vanish.

Specifically, for each residue in the huPrP23-144 amyloid core we have measured up to four one-bond dipolar couplings including \( ^1H^N-^{15}N \), \( ^1H^\alpha-^{13}C^\alpha \), \( ^{15}N-^{13}C^\alpha \) and \( ^{15}N-^{13}C' \). Each type of coupling was measured using a separate 3D SSNMR experiment that consisted of a series of 2D \( ^{15}N-^{13}C \) correlation spectra recorded as a function of a variable mixing period, during which the appropriate dipolar coupling is reintroduced under MAS while other nuclear spin interactions are largely suppressed (see pulse schemes in Figure 3.2). Thus, for each residue, these 3D SSNMR experiments yield characteristic cross-peak dephasing (\( ^1H^N-^{15}N \) and \( ^1H^\alpha-^{13}C^\alpha \) couplings) or build-up (\( ^{15}N-^{13}C^\alpha \) and \( ^{15}N-^{13}C' \) couplings) trajectories, the exact profile of which is determined primarily by the magnitude of the large one-bond dipolar coupling of interest. In Figure 3.8 we show representative 2D \( ^{15}N-^{13}C \) planes from experiments designed to measure the \( ^1H^N-^{15}N \)
(panel A) and $^{15}\text{N}-^{13}\text{C}'$ (panel B) dipolar couplings to illustrate the resolution and sensitivity of these experiments for huPrP23-144 amyloid fibrils. Figure 3.8A is a 2D $^{15}\text{N}-^{13}\text{C}_\alpha$ plane from a 3D $^{1}\text{H}-^{15}\text{N}$ T-MREV experiment$^{119,120}$ recorded at 0°C and 9.470 kHz MAS with the dipolar evolution period. Figure 3.8B is a 2D $^{15}\text{N}-^{13}\text{C}'$ plane from a 3D band-selective TEDOR experiment$^{121}$ recorded at 0°C and 11.111 kHz MAS with the dipolar evolution period, $\tau_{\text{mix}}$, set to 1.44 ms. In Figure 3.9 we show representative dipolar trajectories (circles) and best fit simulation (solid line) for residues A117 and S132, located in the non-$\beta$ bend region between strands $\beta$1 and $\beta$2 and in the $\beta$3-strand, respectively. Additional dipolar trajectories are shown in Figures 3.10-3.3.13 and the experimental dipolar coupling values extracted from these trajectories are summarized in Table 3.2.

The most remarkable feature of the data in Figure 3.9 is that, in spite of being associated with huPrP23-144 core residues that exhibit markedly different cross-peak intensities in CP-based SSNMR spectra (see Figure 3.7), the respective dipolar trajectories and, hence, dipolar order parameters for residues A117 and S132 appear to be nearly identical. This suggests the lack of major differences in sub-μs timescale motions for these two sites. In fact, a complete analysis of the dipolar trajectories has enabled us to extend this conclusion to all huPrP23-144 amyloid core residues. In Figure 3.14 we show plots of the $^{1}\text{H}-^{15}\text{N}$, $^{1}\text{H}_\alpha-^{13}\text{C}_\alpha$, $^{15}\text{N}-^{13}\text{C}_\alpha$ and $^{15}\text{N}-^{13}\text{C}'$ dipolar order parameters as a function of residue number. These order parameters, which on average range from ~0.92 ($^{15}\text{N}-^{13}\text{C}'$ couplings) to ~0.98 ($^{1}\text{H}-^{15}\text{N}$ and $^{15}\text{N}-^{13}\text{C}_\alpha$ couplings) (see Table 3.2), indicate that not only are there no major differences in sub-μs timescale dynamics for residues
located in different parts of the amyloid core but also that most residues are near the rigid-lattice limit on this timescale. Indeed, dipolar order parameters in this regime are reminiscent of well-structured regions of microcrystalline proteins,\textsuperscript{142, 143, 145, 146} and, within a motional model that describes dipolar vector dynamics as a restricted diffusion in a cone characterized by a semi-angle $\alpha_0$,\textsuperscript{137, 147} the observed $<S>$ values correspond to motions with amplitudes of $\alpha_0 = \sim 10-20^\circ$. 
Figure 3.8 Representative 2D $^{15}$N-$^{13}$C planes from 3D dipolar chemical shift SSNMR experiments on huPrP23-144 amyloid fibrils. (A) 2D $^{15}$N-$^{13}$C$\alpha$ plane from a 3D $^1$H$^{15}$N-$^{15}$N T-MREV experiment$^{119, 120}$ recorded at 0°C and 9.470 kHz MAS with the dipolar evolution period, $\tau_{\text{mix}}$, set to 0 (Figure 3.2A). The 3D spectrum was recorded in an interleaved fashion for $\tau_{\text{mix}}$ as a 152* ($t_1$, $^{15}$N) $\times$ 1400* ($t_2$, $^{13}$C) $\times$ 16 ($\tau_{\text{mix}}$, $^1$H-$^{15}$N dipolar coupling) data matrix with acquisition times of 16 ms ($t_1$), 28 ms ($t_2$) and 792 $\mu$s ($\tau_{\text{mix}}$) and a total measurement time of 108 h. (B) 2D $^{15}$N-$^{13}$C$'$ plane from a 3D band-selective TEDOR experiment$^{121}$ recorded at 0°C and 11.111 kHz MAS with the dipolar evolution period, $\tau_{\text{mix}}$, set to 1.44 ms (Figure S2B). The 3D spectrum was recorded in an interleaved fashion for $\tau_{\text{mix}}$ as a 70* ($t_1$, $^{15}$N) $\times$ 1400* ($t_2$, $^{13}$C) $\times$ 12 ($\tau_{\text{mix}}$, $^{15}$N-$^{13}$C$'$ dipolar coupling) data matrix with acquisition times of 12.6 ms ($t_1$), 28 ms ($t_2$) and 4.32 ms ($\tau_{\text{mix}}$) and a total measurement time of 94 h. Cross-peaks in (A) and (B) are drawn with the lowest contour at 7 times the RMS noise level. Resonance assignments are based on Helmus et al.$^{23}$ and additional experimental details are provided in the Experimental Section.
Figure 3.9 Backbone dipolar couplings in the amyloid core region of huPrP23-144 fibrils. Representative measurements of $^1$H$^N$-$^{15}$N (A), $^1$H$^\alpha$-$^{13}$C$^\alpha$ (B), $^{15}$N-$^{13}$C$^\alpha$ (C) and $^{15}$N-$^{13}$C′ (D) one-bond dipolar couplings are shown for residues A117 and S132 (for $^{15}$N-$^{13}$C′ couplings, the $^{13}$C′ nucleus corresponds to the preceding residue). The dipolar trajectories were recorded using 3D dipolar-chemical shift correlation SSNMR experiments, as described in the text. For each trajectory the experimental cross-peak intensity in a 2D $^{15}$N-$^{13}$C$^\alpha$ (A-C) or $^{15}$N-$^{13}$C′ (D) correlation spectrum is shown as a function of the dipolar evolution time (circles), and the best-fit simulation is shown as a solid line. Additional trajectories are shown in Figures 3.10-3.13. See the Experimental Section for a detailed description of the simulations.
Figure 3.10 (Left) Experimental $^1$H-$^{15}$N dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D T-MREV dipolar-chemical shift correlation scheme (Figure 3.2A). Best-fit simulations of the experimental trajectories are shown as solid lines. (Right) Representative distributions from Monte Carlo simulations used to obtain the best estimate of the dipolar coupling including the uncertainty for each residue (the coupling and uncertainty are equal to the average and standard deviation of the corresponding distribution; see Table 3.2 for a summary of these data). Each Monte Carlo simulation involved: (i) generating 5,000 ‘synthetic dipolar trajectories’ by adding to each time point of the best-fit simulation a random amount of noise drawn from a Gaussian distribution with $\sigma$ given by the experimental noise level, and (ii) fitting each synthetic trajectory to obtain the set of 5,000 dipolar couplings used to form the distribution. See Experimental Section of the main text for additional details.
Figure 3.11 Experimental $^1\text{H} \alpha-^{13}\text{C} \alpha$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D T-MREV dipolar-chemical shift correlation scheme (Figure 3.2A). Best-fit simulations of the experimental trajectories are shown as solid lines. (Right) Representative distributions from Monte Carlo simulations used to obtain the best estimate of the dipolar coupling including the uncertainty for each residue (see Table 3.2 for a summary of these data). Additional details of the Monte Carlo simulations are given in Figure 3.10 caption.
Figure 3.12 Experimental $^{15}\text{N}-^{13}\text{C}^\alpha$ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D band-selective TEDOR scheme (Figure 3.2B). Best-fit simulations of the experimental trajectories are shown as solid lines. (Right) Representative distributions from Monte Carlo simulations used to obtain the best estimate of the dipolar coupling including the uncertainty for each residue (see Table 3.2 for a summary of these data). Additional details of the Monte Carlo simulations are given in Figure 3.10 caption.
Figure 3.13 Experimental $^{15}$N-$^{13}$C′ dipolar trajectories (circles) for representative residues in the amyloid core region of huPrP23-144 fibrils, recorded using a 3D band-selective TEDOR scheme (Figure 3.2B). Best-fit simulations of the experimental trajectories are shown as solid lines. (Right) Representative distributions from Monte Carlo simulations used to obtain the best estimate of the dipolar coupling including the uncertainty for each residue (see Table 3.2 for a summary of these data). Additional details of the Monte Carlo simulations are given in Figure 3.10 caption.
Figure 3.14 Backbone order parameters in the amyloid core region of huPrP23-144 fibrils derived from measurements of $^{1}H^{15}N$ (A), $^{1}H^{\alpha}{^{13}C^{\alpha}}$ (B), $^{15}N^{13}C^{\alpha}$ (C) and $^{15}N^{13}C^{'}$ (D) one-bond dipolar couplings. The error bars correspond to ±2σ. Data points missing in the plots correspond to proline (A) and glycine (B) residues, and weak or overlapped correlations in 2D $^{15}N^{13}C$ spectra. For each residue and coupling type the order parameter is calculated as $\langle S \rangle = d_{IS}^{exp} / d_{IS}^{rigid}$, where $d_{IS}^{exp}$ is the experimentally determined scaled dipolar coupling and $d_{IS}^{rigid}$ is the corresponding dipolar coupling in the rigid-lattice limit. Bond lengths of 1.04 Å ($^{1}H^{15}N$), 1.12 Å ($^{1}H^{\alpha}{^{13}C^{\alpha}}$), 1.46 Å ($^{15}N^{13}C^{\alpha}$) and 1.33 Å ($^{15}N^{13}C^{'}$), and pulse sequence dipolar scaling factors of 0.485 (TMREV-4) and 0.434 (TEDOR) yield $d_{IS}^{rigid}$ magnitudes of 5.25 kHz ($^{1}H^{15}N$), 10.4 kHz ($^{1}H^{\alpha}{^{13}C^{\alpha}}$), 427 Hz ($^{15}N^{13}C^{\alpha}$) and 565 Hz ($^{15}N^{13}C^{'}$). Note that the $^{1}H^{15}N$ and $^{1}H^{\alpha}{^{13}C^{\alpha}}$ bond lengths used to calculate $d_{IS}^{rigid}$ have been compensated for the effects of fast vibrational and librational averaging, while the $^{15}N^{13}C^{\alpha}$ and $^{15}N^{13}C^{'}$ bond lengths correspond to the standard crystallographic values. See Table 3.2 for a summary of these calculations.
Table 3.2 Scaled backbone dipolar couplings and order parameters for residues in the amyloid core region of huPrP23-144 fibrils. Order parameters are calculated as \( \langle S \rangle = \frac{d_{\text{exp}}}{d_{\text{rigid}}} \), where \( d_{\text{exp}} \) is the experimental scaled dipolar coupling and \( d_{\text{rigid}} \) is the corresponding scaled dipolar coupling expected in the rigid-lattice limit. The \( d_{\text{rigid}} \) values used in the calculations are 5.25 kHz \( \left( ^1\text{H}^\alpha^\text{N} \right) \), 10.4 kHz \( \left( ^1\text{H}^\alpha^\text{C}^\text{a} \right) \), 427 Hz \( \left( ^{15}\text{N}^\text{C}^\text{a} \right) \) and 565 Hz \( \left( ^{15}\text{N}^\text{C}^\text{a} \right) \). See Figure 3.14 caption of the main text for additional details.
3.3.3 Transverse Nuclear Relaxation Rates of huPrP23-144 Amyloid Core Residues.

Measurements of the backbone dipolar order parameters discussed above show that huPrP23-144 residues in the amyloid core region undergo rather limited and relatively uniform motions on the sub-μs timescale. Such restricted dynamics do not lead to significant attenuation of the one-bond nuclear dipolar couplings and are therefore not able to account for the considerable variation of cross-peak intensities observed in CP-based SSNMR correlation spectra. Therefore, in order to further probe this issue we have performed measurements of selected transverse relaxation rates for the backbone nuclei in the huPrP23-144 amyloid core region.

These measurements, which include rotating frame relaxation rates, $R_{1p}$, for $^1$H$^N$, $^{13}$C and $^{15}$N nuclei, and the $^{15}$N spin-spin relaxation rate, $R_2$, reveal considerable differences in magnitude for the various types of relaxation rate constants among the huPrP23-144 amyloid core residues. As an example, in Figure 3.15 we show $^1$H$^N$ $R_{1p}$ and $^{15}$N $R_2$ trajectories for residues A117, L130 and S132, which illustrate the sizeable variations in relaxation behavior that can be observed on a site-specific basis. For this small subset of residues, the relaxation data are highly correlated with the relative cross-peak intensities obtained in CP-based SSNMR experiments (see Figure 3.7). Specifically, for A117, which exhibits relatively intense cross-peaks in multidimensional correlation spectra, the $^1$H$^N$ and $^{15}$N nuclei are seen to relax relatively slowly. On the other hand, the same spins in L130 and S132 are associated with both increased transverse relaxation rates and significantly reduced cross-peak intensities relative to A117. A complete set of the residue- and site-specific transverse relaxation trajectories is presented in Figures
3.16-3.19, and plots of the transverse relaxation rate constants as a function of residue number obtained by fitting the experimental relaxation trajectories to single exponential decays are shown in Figure 3.20. While considerable site-to-site fluctuations are evident for different types of relaxation rate constants for the huPrP23-144 amyloid core residues, with the exception of $^{15}$N $R_{1p}$ rates which are $\sim$50-60 s$^{-1}$ and essentially invariant throughout the core region under the experimental conditions employed here, residues $\sim$115-120 consistently display relatively small relaxation rates, while larger $R_{1p}/R_2$ values (by as much as $\sim$50-100 s$^{-1}$) are observed, on the whole, for the C-terminal amino acids $\sim$130-141. These findings extend the conclusion made above for residues A117, L130 and S132 to the entire amyloid core region—namely, that the significant dispersion in relaxation rates of nuclear spin coherences is responsible for the large variation of cross-peak intensities in CP-based multidimensional SSNMR spectra of huPrP23-144 fibrils.
Figure 3.15 Selected site-resolved measurements of transverse relaxation rate constants, $R_{1p}$ and $R_2$, for residues in the amyloid core region of huPrP23-144 fibrils. $^1$H N $R_{1p}$ (A) and $^{15}$N $R_2$ (B) trajectories, recorded using pulse schemes in Figure 3.3, are shown for residues A117, L130 and S132. Best-fit simulations to decaying single exponential curves are shown as solid lines. Additional $^1$H N $R_{1p}$, $^{13}$C $R_{1p}$, $^{15}$N $R_{1p}$ and $^{15}$N $R_2$ trajectories are shown in Figures 3.16-3.19.
Figure 3.16 Experimental $^1$H $R_{1p}$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. Trajectories were recorded using the pulse scheme in Figure 3.3. Best-fit simulations to decaying single exponentials are shown as solid lines.
Figure 3.17 Experimental $^{13}$C$\alpha$ $R_{1\rho}$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. Trajectories were recorded using the pulse scheme in Figure 3.3. Best-fit simulations to decaying single exponentials are shown as solid lines.
**Figure 3.18** Experimental $^{15}$N R$_{1p}$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. Trajectories were recorded using the pulse scheme in Figure 3.3. Best-fit simulations to decaying single exponentials are shown as solid lines.
Figure 3.19 Experimental $^{15}\text{N}$ $R_2$ relaxation trajectories (circles) for residues in the amyloid core region of huPrP23-144 fibrils. Trajectories were recorded using the pulse scheme in Figure 3.3. Best-fit simulations to decaying single exponentials are shown as solid lines.
Figure 3.20 Site-specific $^1$H $R_{1\rho}$ (A), $^{13}$C$\alpha$ $R_{1\rho}$ (B), $^{15}$N $R_{1\rho}$ (C) and $^{15}$N $R_2$ (D) relaxation rate constants plotted as a function of residue number for the huPrP23-144 amyloid core region.
It is interesting to speculate here about the molecular level basis for the relatively large variations in certain types of transverse spin relaxation rates observed for the huPrP23-144 amyloid core residues. One possibility is that these variations are associated with different local densities of proton spins. This, however, appears to be rather unlikely, given that no consistent trends in relaxation rates are evident as a function of residue type or identities of the nearest neighbor residues. For example, a nearly two-fold difference in $^{15}\text{N} R_2$ is observed for glycines 114 and 119, both of which are flanked by alanine residues on either side. Another explanation relates to the presence of relatively slow, chemical exchange type phenomena, that occur in parts of the huPrP23-144 amyloid core region on the $\mu$s-ns timescale\textsuperscript{137, 138} and are thus undetectable via the dipolar order parameter measurements. Such processes, which would lead to the time-dependent modulation of chemical shifts and dipolar couplings, can potentially interfere with MAS and the applied RF fields leading to increased nuclear spin relaxation. While a definitive confirmation and quantitative description of motional phenomena of this type would require an extended series of SSNMR relaxation experiments\textsuperscript{137, 138} to be carried out on different types of isotopically-labeled huPrP23-144 fibril samples and is beyond the scope of the current study, to provide a rough order-of-magnitude estimate of the timescale of processes that are consistent with the experimentally observed relaxation rate variations we have performed calculations of $^{15}\text{N} R_2$ values using the theoretical spin-spin relaxation model presented by Rothwell and Waugh for the slow MAS regime\textsuperscript{148} and subsequently extended to arbitrary MAS rates by Griffin and co-workers.\textsuperscript{149} This model provides $R_2$ estimates for a simple system consisting of an isolated low-$\gamma$ nucleus S (e.g., $^{13}\text{C}$ or $^{15}\text{N}$) coupled to a single I-spin ($^1\text{H}$) and subject to
MAS, $^1$H continuous wave RF field and random rotational diffusion of the I-S dipole-dipole interaction with a correlation time $\tau_c$ as follows:

$$R_2 = \frac{\omega_{IS}^2}{30} \left[ 2f(\omega_{RF} + \omega_r) + 2f(\omega_{RF} - \omega_r) + f(\omega_{RF} + 2\omega_r) + f(\omega_{RF} - 2\omega_r) \right]$$

where $\omega_{IS}$ is the $^1$H-$^{15}$N dipolar coupling, $\omega_r$ is the MAS rate, $\omega_{RF}$ is the amplitude of the $^1$H RF field (all in rad·s$^{-1}$), and $f(\omega)$ is the spectral density function:

$$f(\omega) = \frac{\tau_c}{1 + \omega^2\tau_c^2}$$

Interestingly, these highly approximate calculations indicate that under the experimental conditions of this study molecular dynamics-related contributions to $^{15}$N $R_2$ of ~50-100 s$^{-1}$, that would be consistent with our experimental relaxation data (see Figure 3.20D), correspond to $\tau_c$ values in the ~0.1-1 ms regime. Thus, the occurrence of non-uniform, $\mu$s-ms timescale chemical exchange phenomena within the huPrP23-144 amyloid core region, while far from being a direct proof, offers at least a plausible explanation for the experimentally-observed disparity in transverse nuclear spin relaxation rates.

3.4 Conclusions

In the present study, we have probed the conformational flexibility of the Y145Stop mutant of human prion protein in the amyloid state by using several types of magic-angle spinning solid-state NMR techniques. Two-dimensional solid-state NMR spectra based on J-coupling-driven magnetization transfers have permitted the direct observation, at ambient conditions, of N-terminal amino acid residues in the region ~23-
111, which are undetectable under the same conditions via conventional cross-polarization-based methods. Combined with the analysis of $^1H$, $^{13}C$ and $^{15}N$ isotropic chemical shifts for these residues, our results strongly suggest that the large N-terminal domain exists in huPrP23-144 fibrils as a highly dynamic ensemble of protein chains with random-coil-like conformations.

Remarkably, the core region of huPrP23-144 amyloid, although on the whole quite rigid in comparison to the N-terminal domain, appears to experience non-negligible molecular motions on the NMR timescale as well, implied by the considerable variation of signal intensities in CP-based solid-state NMR spectra. Our measurements of the backbone $^1H^N$-$^{15}N$, $^1H^\alpha$-$^{13}C^\alpha$, $^{15}N$-$^{13}C^\alpha$, and $^{15}N$-$^{13}C'$ dipolar order parameters for the core amino acids, that are observable in such spectra, reveal that these residues are reminiscent of well-structured regions of microcrystalline proteins and undergo only highly restricted and relatively uniform molecular motions on the sub-$\mu$s timescale. On the other hand, significant dispersion in the relaxation rates of $^1H$, $^{13}C$ and $^{15}N$ nuclear spin coherences observed for the same huPrP23-144 core residues appears to be responsible for the large variation of spectral intensities. Although still somewhat speculative, the molecular basis of this finding is consistent with the presence of relatively slow, chemical exchange type phenomena taking place in this region of the protein on the $\mu$s-ms timescale. Interestingly, the results of our previous variable temperature NMR studies provide additional support for this chemical exchange hypothesis. Specifically, these experiments show that the most rigid amyloid core residues (aa 115-120) exhibit nearly identical resonance widths in 2D $^{15}N$-$^{13}C^\alpha$ correlation spectra recorded at 0°C and upon freezing.
the sample at -30°C, which indicates that these residues exist in huPrP23-144 fibrils in a single predominant conformation. In contrast, several core residues that are found in the C-terminal β3-strand and give rise to relatively well isolated signals in both 0°C and -30°C spectra, including S132, I138 and I139, display pronounced inhomogeneous linebroadening upon freezing the fibrils at -30°C, consistent with the presence of several similar, but not identical, conformers for this region of the protein.

While for amyloid fibrils composed of short polypeptides the rigid amyloid core region typically encompasses the entire backbone and side-chains, within a steric-zipper type motif, analogous amyloid assemblies formed by intact protein molecules are, perhaps not surprisingly, far more complex. Indeed, the finding that Y145Stop human PrP amyloid fibrils contain both highly flexible large segments as well as a relatively rigid, well-structured core domain that overall exhibits more restricted motions on different timescales appears to be general in nature, as similar observations have been made for several other protein-based amyloid structures that have been investigated to date in atomic-level detail. Interestingly, the concurrent presence of such rigid and flexible protein domains is quite consistent with the initial molecular models of amyloid fibrils, where the packing of individual protein monomers into an amyloid scaffold was presumed to be generally associated with substantial rearrangements of the native protein fold to yield a compact β-sheet core flanked by other types of structural motifs. We note, however, that the existence of amyloid architectures consisting of protein molecules in a native-like conformation has also been recently reported, further underlining the structural and dynamic complexities intrinsic to these types of biomacromolecular assemblies. The fundamental
insights into the dynamic properties of human PrP23-144 amyloid obtained in this study should facilitate future efforts aimed at the full understanding of the molecular basis of amino acid sequence- and strain-dependent conformational inheritance in this biologically-relevant in vitro model.6,7 Questions in this regard are of central importance in prion and amyloid research.
Chapter 4

Intermolecular Alignment of Y145Stop Human Prion Protein Amyloid Fibrils Probed by Solid-State Nuclear Magnetic Resonance Spectroscopy

4.1 Introduction

With a complete resonance assignment of the amyloid core of huPrP23-144 as presented in Chapter 2, a number of advanced solid-state NMR experiments can be performed to elucidate the structure of the fibril. Other research groups have successfully used MAS SSNMR experimental techniques to study a number of proteins in their amyloid fibril state, including Aβ(1-40)\(^8\), \(^9\), α-synuclein\(^10\), \(^11\), tau\(^12\), \(^13\), β2m\(^14\), curli\(^15\), and the fungal prion proteins: HET-s\(^16\)-\(^20\), Sup35p\(^21\) and Ure2p\(^22\). In this chapter, we investigate the intermolecular alignment and distances present in the tertiary structure of huPrP23-144 amyloid fibrils using a series of ZF-TEDOR (\(z\)-filtered transferred echo double resonance) and BS-TEDOR (band selective transferred echo double resonance)\(^121\) \(^15\)N-\(^13\)C correlation experiments. A similar study of the β2m amyloid fibrils was recently published by Debelouchina et al.\(^156\).
4.2 Results and Discussion

4.2.1 2D ZF-TEDOR Experiments

For the ZF-TEDOR and BS-TEDOR experiments in this chapter, a total of six huPrP23-144 fibril samples were prepared with different isotopic labeling schemes in the same manner as described in sections 2.4.1 and 3.2.1. Uniformly labeled $^{13}\text{C}$ and $^{15}\text{N}$ samples made with [1,3-$^{13}\text{C}$]-glycerol or [2-$^{13}\text{C}$]-glycerol as the sole carbon source, referred to as “uniform 1,3-glycerol huPrP23-144” and “uniform 2-glycerol huPrP23-144”, served as control samples with $^{15}\text{N}$-$^{13}\text{C}$ correlation experiments performed on these samples showing contribution from both intermolecular and intramolecular $^{15}\text{N}$-$^{13}\text{C}$ contacts. In addition, a dilution of independently synthesized uniformly labeled $^{15}\text{N}$ and [1,3-$^{13}\text{C}$]-glycerol or [2-$^{13}\text{C}$]-glycerol samples were diluted in a 1:3 ratio with natural abundance huPrP23-144. Correlation spectra of these samples, referred to as “diluted 1,3-glycerol huPrP23-144” and “diluted 2-glycerol huPrP23-144”, show peaks from predominately intramolecular contacts. Finally, samples consisting of uniformly $^{15}\text{N}$ labeled huPrP23-144 mixed in a 1:1 ratio with either [1,3-$^{13}\text{C}$]-glycerol or [2-$^{13}\text{C}$]-glycerol huPrP23-144, referred to as “mixed 1,3-glycerol huPrP23-144” and “mixed 2-glycerol huPrP23-144” were used to detect intermolecular $^{15}\text{N}$-$^{13}\text{C}$ contacts and to measure the approximate distance between neighboring molecules.

To determine the intermolecular alignment between neighboring huPrP23-144 fibrils, a number of 2D ZF-TEDOR spectra were collected on all six samples to obtain site-specific information on the $^{15}\text{N}$-$^{13}\text{C}$ contacts present. The NCO region of the ZF-TEDOR spectrum of the uniform 1,3-glycerol huPrP23-144 is shown in Figure 4.1A and
the spectrum of dilute 1,3-glycerol huPrP23-144 samples shown in Figure 4.1B. Both of these spectra were collected at a short mixing time (1.8 ms), overlap extensively with each other and show a number of one bond $^{15}N_{i+1}^{13}CO_i$ cross peaks, which can be assigned from earlier cross-polarization based correlation experiments. Although the uniform 1,3-glycerol huPrP23-144 sample could, in nature, contain intermolecular contacts it is unlikely that at such a short mixing time such contacts would have sufficient intensity for detection. When a longer $^{15}N$-$^{13}C$ mixing time is used, additional peaks from $^{15}N_i^{13}C'_i$ contacts are also seen (data not shown) which may come from intramolecular or intermolecular contacts, the uniform labeling of the sample does not allow us to differentiate between these two possibilities. The same NCO region of the ZF-TEDOR spectrum of the mixed 1,3-glycerol huPrP23-144 sample collected with a 18.0 ms $^{15}N$-$^{13}C$ mixing time, shown in Figure 4.1C, contains a number of peaks, the majority of which could be assigned using the known chemical shift assignments of huPrP23-144 fibrils. These $^{15}N_{i+1}^{13}CO_i$, $^{15}N_i^{13}CO_i$ as well as two $^{15}N_{i+2}^{13}CO_i$ peaks (A116N-G114CO and G119N-A117CO) arise exclusively from intermolecular contacts and suggest that the huPrP23-144 fibrils align in a parallel in-register configuration. This parallel in-register configuration is further supported by examining the NCα region of the uniform, dilute and mixed 2-glycerol ZF-TEDOR spectra shown in Figure 4.2. The uniform and dilute spectra, collected with a 1.44 ms $^{15}N$-$^{13}C$ mixing time, largely overlap and contain only one bond $^{15}N_i^{13}Ca_i$ peaks. Whereas the mixed spectrum, with a longer $^{15}N$-$^{13}C$ mixing time of 15.84 ms, contains peaks from $^{15}N_{i-1}^{13}Ca_i$, $^{15}N_i^{13}Ca_i$, and $^{15}N_{i+1}$-
$^{13}\text{C}_\alpha_i$ intermolecular contacts consistent with a parallel in-register alignment of neighboring molecules.

The interpretation of the NCx region of the uniform, dilute and mixed 1,3-glycerol huPrP23-144 ZF-TEDOR spectra in Figure 4.3 requires additional care. This region contains peaks from contacts between $^{15}\text{N}$ nuclei on the protein backbone and $^{13}\text{C}$ nuclei in the side chains which, when in the same residue, are separated by $\sim2-4$ Å and therefore require a relatively long $^{15}\text{N}-^{13}\text{C}$ mixing time for detection. During this long mixing period which is required for the intensity of the side-chain-to-backbone intramolecular contacts to build up, side-chain-to-$^{15}\text{N}$ nuclei on neighboring fibrils may also develop significant intensity to contribute to the spectrum. Therefore, the uniformly 1,3-glycerol huPrP23-144 NCx spectrum in Figure 4.3A contains peaks which arise from both intramolecular $^{15}\text{N}-^{13}\text{C}$ contacts as well as intermolecular $^{15}\text{N}-^{13}\text{C}$ contacts. From this spectrum alone, it cannot be determined if a given peak results from an intramolecular contact, an intermolecular contact, or as a result of both types of contacts. This spectrum contains a number of interesting long-range restraints which can be broken down into contacts arising from three distinct structural folds. First the contacts to $\text{C}_\delta1$ and $\text{C}_\delta2$ of the L125 side chain: A115N-L125CD1, A115N-L125CD2, and G114N-L125CD1. Second, the contact to I139$\gamma2$: A115N-I139CG2, A116N-I139CG2, and A117N-I139CG2. And third, the contacts to A116$\beta$: S132N-A116CB and G131N-A116CB. Determining if these peaks are the result of intramolecular or intermolecular contacts would provide significant restraints to the structure of both a single huPrP23-144 fibril as well as additional information on the intermolecular alignment and packing of
neighboring fibrils. The NCx spectrum of the diluted 1,3-glycerol huPrP23-144 and mixed 1,3-glycerol huPrP23-144 provide just such information. The majority of the peaks in the NCx spectrum of the diluted 1,3-glycerol huPrP23-144 in Figure 4.3B are a result of intramolecular $^{15}N_i$-$^{13}Cx_{i+1}$, $^{15}N_{i+1}$-$^{13}C_{Xi}$, or $^{15}N_{i+1}$-$^{13}Cx_{i}$ contacts which do not provide significant restraints on the fibril structure. The strong S132N-A116CB peak, which is notably missing from the spectrum of mixed 1,3-glycerol huPrP23-144, indicated that this contact is intramolecular in nature and provides a restraint on the global fold of a single fibril. The small A117N-I139CG2 peak, especially in light of the strength of this peak as well as the presence of the A117N-I139CG2 peak in the mixed 1,3-glycerol huPrP23-144 spectrum suggests that the I139Cγ2 contacts should be interpreted as intermolecular restraints. The NCx spectra of the mixed 1,3-glycerol huPrP23-144 in Figure 4.3C contains a number of intermolecular $^{15}N_i$-$^{13}C_{Xi}$, $^{15}N_{i+1}$-$^{13}C_{Xi}$, and $^{15}N_{i+1}$-$^{13}Cx_{i}$ contacts consistent with the in-register parallel alignment of fibrils determined earlier. In addition to the two I139Cγ2 peaks, the presence of all three of the L125Cδ contacts in the spectrum indicate that these two structural folds should be interpreted as intermolecular restraints on the fibril’s tertiary structure.

In summary, the 2D ZF-TEDOR spectra collected on the various huPrP23-144 samples indicate that huPrP23-144 fibrils align in-register and parallel to each other, with the various contacts consistent with the alignment indicated in Figure 4.4. Here, residues with in-register parallel contacts are underlined with the corresponding spectrum in which they are observed, red being those present in the NCO spectrum, blue the NCα spectrum and green the NCx spectrum. As observed from this figure, contacts arising
from the in-register parallel alignment are seen throughout the amyloid core indicative of a regular repeating packing of this entire region of the fibril. In addition, the NCx 2D ZF-TEDOR spectrum allows us to separate the three long-range structural folds into intra- and intermolecular restraints. The S132N and G131N to A116Cβ contacts are intramolecular in nature, whereas the L125Cδ to A115N and G114N contacts are intermolecular in nature. The three contacts to I139Cγ2 from A115N, A116N and A117N appear to be predominately intermolecular in nature although may have some intramolecular contribution.
Figure 4.1 NCO region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 1,3-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. The 2D spectra of uniform 1,3-glycerol huPrP23-144 fibrils (A) and dilute 1,3-glycerol huPrP23-144 fibrils (B) were collected with 1.8 ms $^{13}$C-$^{15}$N TEDOR mixing time and recorded as a 74* ($t_1$, $^{15}$N) $\times$ 1400* ($t_2$, $^{13}$C) data matrix with acquisition times of 13.3 ms ($t_1$) and 28 ms ($t_2$), and a measurement time of 13.5 h and 27 h respectively. The 2D spectrum of mixed 1,3-glycerol huPrP23-144 fibrils (C) was collected with 18.0 ms $^{13}$C-$^{15}$N TEDOR mixing time and recorded as a 78* ($t_1$, $^{15}$N) $\times$ 1250* ($t_2$, $^{13}$C) data matrix with acquisition times of 14.0 ms ($t_1$) and 25 ms ($t_2$), and a measurement time of 212 h. Assignments are taken from Helmus et al. 23.
Figure 4.2 NCα region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 2-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. The 2D spectra of uniform 2-glycerol huPrP23-144 fibrils (A) and dilute 2-glycerol huPrP23-144 fibrils (B) were collected with 1.44 ms $^{13}$C-$^{15}$N TEDOR mixing time and recorded as a 74* ($t_1$, $^{15}$N) $\times$ 1200* ($t_2$, $^{13}$C) data matrix with acquisition times of 13.3 ms ($t_1$) and 24 ms ($t_2$), and a measurement time of 7 h and 27 h respectively. The 2D spectrum of mixed 2-glycerol huPrP23-144 fibrils (C) was collected with 15.84 ms $^{13}$C-$^{15}$N TEDOR mixing time and recorded as a 78* ($t_1$, $^{15}$N) $\times$ 1200* ($t_2$, $^{13}$C) data matrix with acquisition times of 13.3 ms ($t_1$) and 24 ms ($t_2$), and a measurement time of 216 h. Assignments are taken from Helmus et al.23
Figure 4.3 NCx region of a 2D ZF-TEDOR experiment on uniform, dilute and mixed 1,3-glycerol huPrP23-144 fibrils, acquired at 11.1 kHz MAS rate and 0°C. The 2D spectra of uniform 1,3-glycerol huPrP23-144 fibrils (A), dilute 1,3-glycerol huPrP23-144 fibrils (B) and mixed 1,3-glycerol huPrP23-144 fibrils (C) were collected with 1.8 ms $^{13}$C-$^{15}$N TEDOR mixing time and recorded as a $74^*$ ($t_1$, $^{15}$N) $\times$ $1200^*$ ($t_2$, $^{13}$C) data matrix with acquisition times of 13.3 ms ($t_1$) and 24 ms ($t_2$), and a measurement time of 66 h, 108 h and 212 h respectively.
Figure 4.4 Amino acid sequence of the amyloid core of huPrP23-144 fibrils with contacts consistent with an in-register parallel intermolecular alignment underlined as seen in the NCO (red), NCx (green) and NCα (blue) ZF-TEDOR spectra.
4.2.2 Time Resolved 1D BS-TEDOR and ZF-TEDOR experiments

In order to further probe the tertiary structure of huPrP23-144, a time resolved 1D BS-TEDOR\textsuperscript{121} experiment was performed on the mixed 1,3-glycerol huPrP23-144 samples in order to determine the approximate distances between neighboring fibrils. In this BS-TEDOR experiment, a shaped $^{13}\text{C}$ pulse was applied during the middle of the two REDOR blocks of the pulse sequence to remove any residual $^{13}\text{CO}-^{13}\text{C}\alpha$ J-coupling present in the sparsely labeled sample. In the mixed 2-glycerol huPrP23-144 sample, the glycerol labeling scheme removes nearly all $^{13}\text{CO}-^{13}\text{C}\alpha$ J-coupling so a time-resolved 1D ZF-TEDOR\textsuperscript{121} experiment was performed on this sample to provide a duplicate measurement of the distances between neighboring molecules. As described in section 4.3.2, simulations of these experiments provide an approximate distance between the amyloid core regions of neighboring huPrP23-144 molecules of 4.4 Å. A summary of the experimental data and quality of the fit are shown in Figure 4.5. Here the experimental and simulated curves corresponding to a 4.0 Å, 4.4 Å and 4.8 Å inter-fibril distance are shown with the experimentally determined relaxation rate for the BS-TEDOR experiment on a mixed 1,3-glycerol huPrP23-144 in panel A and for the ZF-TEDOR experiment on mixed 2-glycerol huPrP23-144 in panel C. Both trajectories show excellent agreement with the simulated trajectory at 4.4 Å indicating that this is a good approximation of the distance between neighboring fibrils. The atoms from the GNNQQNY fibril model\textsuperscript{158} used to simulate the trajectories are shown in panels B and D of Figure 4.6.
Figure 4.5 Trajectories and fibril models for inter-fibril distance measurements. Experimental trajectories and simulated curves for BS-TEDOR experiment on mixed 1,3-glycerol huPrP23-144 (A) and ZF-TEDOR experiment on mixed 2-glycerol huPrP23-144 (C). The model fibril system, based on the GNNQQNY fibril\textsuperscript{158}, with the atoms included in the simulated spin system indicated as spheres for the 1,3-glycerol huPrP23-144 simulation (B) and 2-glycerol huPrP23-144 simulation (D).
4.3 Materials and Methods

4.3.1 Solid-State NMR Spectroscopy.

NMR experiments were performed at 11.7 T (499.8 MHz for \(^1\)H, 125.7 MHz for \(^{13}\)C, and 50.6 MHz for \(^{15}\)N) using a three-channel Varian spectrometer equipped with 3.2 mm triple-resonance T3 and BioMAS™ probes\(^{111}\) in an \(^1\)H-\(^{13}\)C-\(^{15}\)N configuration. All experiments employed MAS rates, \(\omega_r/2\pi\), of 11.1 kHz actively regulated to ca. ± 3 Hz using a Varian MAS control unit, and a sample temperature of 0°C controlled by a stream of dry compressed air, delivered to the sample using a variable-temperature (VT) stack at a flow rate of 30 L/min. This temperature refers to the VT gas temperature at the sample—the actual average sample temperatures were ~5°C higher due to frictional heating as determined by lead nitrate calibration.\(^{112}\) The pulse sequences used in this study are described in detail elsewhere\(^{121}\) and the key experimental parameters are summarized below. Typical \(^1\)H, \(^{13}\)C, and \(^{15}\)N 90° pulse lengths were 2.5, 5.0, and 6.0 \(\mu\)s, respectively, and ~70 kHz two pulse phase modulated (TPPM) proton decoupling\(^{42}\) was applied during the chemical shift evolution periods and detection. \(^{15}\)N 180° pulses of length 18 \(\mu\)s were used to recouple the \(^{15}\)N-\(^{13}\)C dipole-dipole interaction during the REDOR periods of the ZF-TEDOR and BS-TEDOR experiments with simultaneous application of 100 kHz TPPM\(^{42}\) proton decoupling. A 5.4 ms z-filter was used to suppress anti-phase and multiple-quantum spin coherences in the ZF-TEDOR experiment.

The 2D ZF-TEDOR spectra of the NCO region presented in Figure 4.1 were collected in ~13.5, ~27, and ~212 hours for the uniform, diluted and mixed 1,3-glycerol
samples, respectively. The 2D ZF-TEDOR spectra of the NCα region presented in Figure 4.2 were collected in ~7, 27, and ~216 hours for the uniform, diluted and mixed 2-glycerol samples, respectively. The 2D ZF-TEDOR spectra of the NCx region presented in Figure 4.3 were collected in ~66, ~108, and ~212 hours for the uniform, diluted and mixed 1,3-glycerol samples, respectively.

4.3.2 Data Processing and Simulations.

Distances between huPrP fibrils were measured using a BS-TEDOR experiment on the mixed 1,3-glycerol huPrP23-144 sample and a ZF-TEDOR experiment on the mixed 2-glycerol huPrP23-144 sample. The pulse sequences used for these experiments are given in Figure 4.6. For the BS-TEDOR experiment on 1,3-glycerol huPrP23-144, 30 experimental TEDOR $^{13}$C-$^{15}$N mixing times were collected linearly from 0.72 ms to 21.6 ms in steps of 0.72 ms using a BS-TEDOR pulse sequence containing a 360 µs r-SNOB shaped pulse centered in the carbonyl region of the spectrum to refocus any residual $^{13}$CO-$^{13}$Cα J-couplings. Each 1D BS-TEDOR spectrum was collected in ~1.4 hours resulting in a total experimental time to collect the BS-TEDOR trajectory of ~41 hours (1920 scans per FID, 2.5 sec recycle delay).

The relaxation rate during the TEDOR mixing was established using a spin-echo experiment with a shaped refocusing pulse identical to that used in the BS-TEDOR experiment. Twenty experimental points were collected with relaxation times varying linearly from 0.18 ms to 20.7 ms in steps of 1.08 ms. Each 1D spectrum was collected in ~5.5 minutes resulting in a total experimental time of 109 minutes to collect the
relaxation trajectory (128 scans per FID, 2.5 sec recycle delay). The experimental trajectories were fit to a single decaying exponential:

\[ A_t = A_0 \cdot \exp\left(\frac{-R}{t}\right) \]

using a least-squares approach. The relaxation rate for the mixed 1,3-glycerol sample was found to be 81.67 sec\(^{-1}\) with an excellent fit to experimental data.

The BS-TEDOR efficiency was established by scaling the intensities of the BS-TEDOR trajectory by the intensities of the CO region of a 1D \(^{13}\)C CP spectrum collected with an identical number of scans as was used to collect the BS-TEDOR trajectory, this 1D took \(\sim\)82 minutes to collect.

Similarly for the ZF-TEDOR experiment on 2-glycerol huPrP23-144, twelve experimental TEDOR \(^{13}\)C-\(^{15}\)N mixing times were collected linearly from 1.8 ms to 21.6 ms in steps of 1.8 ms. Each 1D ZF-TEDOR spectrum was collected in \(\sim\)5.7 hours resulting in a total experimental time to collect the ZS-TEDOR trajectory of \(\sim\)68 hours (8192 scans per FID, 2.5 sec recycle delay).

The relaxation rate during the TEDOR mixing was established in the same manner as in the BS-TEDOR experiment with each 1D spectrum taking \(\sim\)42 minutes to collect resulting in a total experimental time of \(\sim\)14.3 hours. (1024 scans per FID, 2.5 sec recycle delay). The relaxation rate for the mixed 2-glycerol sample was found to be 136.4 sec\(^{-1}\) with an excellent fit to experimental data.

The ZF-TEDOR efficiency was established by scaling the intensities of the ZF-TEDOR trajectory by the intensities of the C\(\alpha\) region of a 1D \(^{13}\)C CP spectrum collected
with an identical number of scans as was used to collect the ZF-TEDOR trajectory. This 1D took ~5.7 hours to collect.

Trajectories for the relaxation, the BS-TEDOR and ZF-TEDOR experiments as well as the amplitude of the 1D CP reference spectra were determined by a summation of the CO or Cα region of the spectrum after processing the data using NMRPipe\textsuperscript{98}. TEDOR and CP spectra were processed with 500 Hz of exponential line broadening. Relaxation spectra were processed with a squared sine-bell apodization.

The best fit distance between the fibrils was determined by simulating a grid of distances, with a 0.1 Å spread, of a model fibril system and calculating the RMSD between these simulations and the experimentally determined trajectory. An intermolecular distance of 4.4 Å was found to give the lowest RMSD to the experimental data. These simulated BS-TEDOR and ZF-TEDOR trajectories were calculated using SPINEVOLUTION\textsuperscript{159} on a model system based upon the GNNQQNY fibrils\textsuperscript{158}. Three separate trajectories were calculated for the spin systems representing the three possible \textsuperscript{13}C/\textsuperscript{15}N labeling arrangements of the fibrils; the three spin system containing the ASN-6 CO or Cα atom on the central fibril and the two closest \textsuperscript{15}N atoms on the +1 fibril, the three spin system containing the ASN-6 CO or Cα atom on the central fibril and the two closest \textsuperscript{15}N atoms on the -1 fibril, and a five spin system containing the ASN-6 CO or Cα atom on the central fibril and the two closest \textsuperscript{15}N atoms on both the +1 and -1 fibril. These three simulations were averaged in a 1:1:1 ratio representing the ratio in a well-mixed sample of 50% \textsuperscript{13}C labeled and 50% \textsuperscript{15}N labeled fibrils.
Figure 4.6 ZF-TEDOR and BS-TEDOR pulse sequences. (A) 2D/3D z-filtered TEDOR pulse sequence. Narrow and wide solid rectangles represent $\pi/2$ and $\pi$ pulses, respectively. The two REDOR periods in which two $\pi$ pulses are applied to the $^{15}$N channel are phase cycled according to the $xy-4$ scheme$^{79}$. Multiple quantum and anti-phase coherences are removed by the two z-filter periods, $\Delta$. The short delay, $\tau$, is applied so that the total period between the REDOR periods is equal to an integer number of rotor cycles. The phase cycles are as follows: $\varphi_1 = 01$, $\varphi_2 = 0022$, $\varphi_3 = 4\times(0)\ 4\times(1)$, $\varphi_4 = 8\times(1)\ 8\times(3)$, $\varphi_5 = 16\times(0)\ 16\times(2)\ 16\times(1)\ 16\times(3)$, and the receiver cycled as $1331331\ 3131331\ 3131331\ 3131331\ 3131331\ 3131331\ 20020202\ 02202002\ 02202002\ 20020220$ where $0=x$, $1=y$, $2=-x$, $3=-y$. All remaining pulses have phase $x$ unless otherwise noted. Hypercomplex data were acquired by shifting $\varphi_2$ according to Ruben and co-workers$^{160}$. (B) 1D band-selective TEDOR pulse sequences in which a band-selective $^{13}$C r-SNOB $\pi$ pulse refocuses $^{13}$C-$^{13}$C J-couplings outside the pulse bandwidth. The small delays $\delta$ are applied so that the total period between the REDOR periods is equal to an integer number of rotor cycles. The phase cycles are as follows $\varphi_1 = 4\times(0)\ 4\times(2)$, $\varphi_2 = 0022$, $\varphi_3 = 02$ and the receiver 02202002. All remaining pulses have phase $x$ unless otherwise noted. Additional details on the ZF-TEDOR and BS-TEDOR pulse sequences can be found in the article by Jaroniec et. al.$^{121}$.
Chapter 5
Torsion Angle Measurement

5.1 Introduction

In Chapter 2, magic-angle spinning solid state NMR was used to examine the Y145Stop variant of the human prion protein (huPrP23-144). Using 2D and 3D $^{15}$N and $^{13}$C correlation spectroscopy, it was found that huPrP23-144 fibrils consist of a rigid, ~30 amino acid amyloid-core from residue 112 to 141 consisting of three $\beta$-sheets with the remainder of the protein being conformationally flexible. With the complete resonance assignment of the amyloid core, site-specific structural restraints can be measured such as inter-nuclear distances and torsion angles, which are required for determining an atomic-resolution structure of the human prion protein. This chapter will examine three dipolar chemical shift correlation experiments performed on huPrP23-144 fibrils to restrain the backbone torsion angles, $\psi$ and $\varphi$. The following chapter, Chapter 6, will examine experiments which report on carbon-nitrogen and carbon-carbon distances in the fibril, focusing on the long-range restraints these experiments provide.

5.2 Projection Angle Measurements

As noted in Chapter 1, the dipole-dipole interaction between nuclei has an angular dependence, that is to say, the interaction is anisotropic. Although magic-angle spinning
removes the dipole-dipole interaction, well designed radio-frequency pulses can reintroduce this interaction, which can then be exploited to provide a number of useful structural restraints, including the relative orientation of two nuclear dipole tensors, which is called a projection angle. These projection angles can be related to the backbone and side-chain torsion angles, which determine the structure of a protein. Figure 5.1 provides a graphical representation of the location of the three backbone torsion angles in proteins, $\phi$, $\psi$, and $\omega$, as well as the names of the various atoms discussed in this chapter. Of these three torsion angles, only $\phi$ and $\psi$ are required to predict the structure of a protein as the peptide bond restricts $\omega$ to 180°. A number of magic-angle spinning solid state NMR experiments have been designed\textsuperscript{120, 161-171} to measure projection angles in protein samples. In this chapter, we examine three such experiments, a $^1$H-i-$^{15}$N$_i$-^{13}C$^\alpha$-^{1}H$^\alpha_i$ experiment, a $^1$H$_{i+1}$-$^{15}$N$_{i+1}$-^{13}C$^\alpha$-^{1}H$^\alpha_i$ experiment\textsuperscript{120} and a $^{15}$N$_{i+1}$-^{13}C$^\alpha$-^{13}C$^\alpha$-^{15}N$_i$ experiment\textsuperscript{170} as applied to a uniformly $^{13}$C and $^{15}$N labeled sample of huPrP23-144 fibrils.

To explain how these dipole-tensor correlation experiments work, consider a generic $^1$H-$^{15}$N-$^{13}$C-$^1$H experiment. In this experiment, $^{15}$N transverse magnetization is generated and, in a time-resolved manner, dephased by reintroducing the $^1$H-$^{15}$N dipole-dipole interaction. The dephased $^{15}$N magnetization is frequency labeled and transferred to $^{13}$C transverse magnetization. This $^{13}$C magnetization is also dephased by reintroducing the $^1$H-$^{13}$C dipole-dipole interaction in a time-resolved manner and then detected along a second dimension. The result is a series of 2D $^{15}$N-$^{13}$C correlation
spectra in which each cross peak is modulated by the relative orientation of the $^{1}\text{H}-^{15}\text{N}$ and $^{1}\text{H}-^{13}\text{C}$ vectors. The intensity of a given $^{15}\text{N}-^{13}\text{C}$ cross peak is given by:

$$A(\tau_N, \tau_C, t_{\text{mix}}) = D_N(\tau_N) \times T(t_{\text{mix}}) \times D_C(\tau_C)$$

Here $D_N$ is the dephasing of the $^{15}\text{N}$ transverse coherence due to the reintroduction of the $^{1}\text{H}-^{15}\text{N}$ dipole-dipole interaction, $D_C$ is the dephasing of the $^{13}\text{C}$ transverse coherence due to the reintroduction of the $^{1}\text{H}-^{13}\text{C}$ dipole-dipole interaction, and $T(t_{\text{mix}})$ refers to the relative polarization transfer amplitude resulting from the $^{15}\text{N}$ to $^{13}\text{C}$ transfer. Each of these functions depends on the relative orientation with respect to the applied magnetic field of the molecular segment being considered. Therefore, in powdered samples, an average over all crystallite orientations, either analytically or more often numerically for a represented set of orientations, must be taken in order to describe the observed cross peak modulation. The functional forms of $D_N$ and $D_C$ are:

$$D(t) = \cos (\omega \cdot \tau)$$

where $\omega$ is the orientation-dependent dipolar coupling being reintroduced ($^{1}\text{H}-^{15}\text{N}$ or $^{1}\text{H}-^{13}\text{C}$) and $\tau$ is the dipolar evolution time. Formally, $T(t_{\text{mix}})$ also has an angular dependence, but in many cases$^{120}$, including the three experiments described in this chapter, an isotropic function can be assumed (i.e. $T(t_{\text{mix}})=1$).

The cross peaks of the generic $^{1}\text{H}-^{15}\text{N}-^{13}\text{C}-^{1}\text{H}$ experiment can therefore be approximated by:

$$A(t_1, t_2) = \cos (\omega_{HN} \tau_N) \times \cos (\omega_{HC} \tau_C)$$

where $\omega_{HN}$ and $\omega_{HC}$ are the orientation-dependent dipolar couplings reintroduced for times $\tau_N$ and $\tau_C$, and the overbar indicates an average over all crystallites. The two
dephasing periods, $\tau_N$ and $\tau_C$, could be varied independently resulting in an effective 4D experiment, but the time requirements for this experiment would be prohibitively long. In practice, the two dephasing periods are adjusted together so that a 3D experiment is collected. When the arguments of the cosine functions have approximately the same magnitude ($\omega_{HN}\tau_N \approx \omega_{HC}\tau_C$) for each crystallite, which occurs when the dipole vectors under study are parallel or anti-parallel, this equation displays very distinctive features$^{167}$. Therefore, for molecular topologies in which the two dipole vectors being correlated are nearly parallel, a projection angle of 0°, or anti-parallel, 180°, these projection angle experiments will have optimum sensitivity and angular resolution. Typically, the region of highest sensitivity is taken to be within 30° of one of these co-linear geometries. In addition, because the cosine function is even, $\cos(x) = \cos(-x)$, and anti-symmetric around 90°, $\cos(x) = -\cos(180°-x)$, the projection angle will also have these symmetries. Therefore the projection angle can be restricted between 0° and 90° knowing that for a given projection angle $\theta$, there exists three additional degenerate solutions, $\theta$, 180° - $\theta$ and -180° + $\theta$. 
Figure 5.1 Model of the peptide backbone. Backbone torsion angles $\phi$, $\psi$, and $\omega$ indicated. Of these three, only $\phi$ and $\psi$ are needed to determine the protein structure as $\omega$ is restricted to $180^\circ$ by the peptide bond.
5.3 $^1H_i-^{15}N_t-^{13}C_{\alpha_i}-^1H_{\alpha_i}$ Experiment

The $^1H_i-^{15}N_t-^{13}C_{\alpha_i}-^1H_{\alpha_i}$ experiment for the measurement of the $\varphi$ torsion angle has been described in detail elsewhere$^{120}$. Here we will provide a brief description of the experiment, discuss the experimental and simulation parameters, and show the results from the measurement of $\varphi$ and its corresponding projection angle in huPrP23-144. Additional experimental and simulation parameter are discussed in the Materials and Methods section. The pulse sequence used for the $^1H_i-^{15}N_t-^{13}C_{\alpha_i}-^1H_{\alpha_i}$ experiment is shown in Figure 5.2. In this experiment, initial magnetization from the proton spins is transferred using cross polarization to $^{15}N$. A constant-time transverse MREV (T-MREV) element$^{119}$ reintroduces the $^1H-^{15}N$ dipole-dipole coupling while simultaneously decoupling the $^1H-^1H$ dipole-dipole interactions for time $2\tau$. After $^{15}N$ frequency labeling in $t_1$, the magnetization is transferred to the neighboring $^{13}C_{\alpha}$ using a SPECIFIC CP$^{77}$. A second constant-time T-MREV element reintroduces the $^1H-^{13}C$ dipole-dipole interaction for time $\tau$ before detection of the $^{13}C$ signal in $t_2$. The resulting 3D experiment is comprised of a collection of 2D $^{15}N-^{13}C_{\alpha}$ correlation spectra (not shown) similar to the NC$\alpha$ spectrum presented in Figure 2.1. The two T-MREV dephasing periods are adjusted together in the third dimension with the $^{15}N$ channel dephasing period, $2\tau$, being twice as long as the $^{13}C$ dephasing period, $\tau$. Typical $^1H-^{15}N$ dipole coupling constants are roughly half those of $^1H-^{13}C_{\alpha}$ constants, so doubling the $^{15}N$ dephasing time results in the two dipolar arguments being approximately equal leading to the greatest possible interference effects as well as optimal sensitivity between the $^1H-^{15}N$ and $^1H-^{13}C_{\alpha}$ dephasing curves.
The relationship between the molecular torsion angle $\varphi$ and the measured projection angle between the $^1\text{H}_i^N\text{-}^{15}\text{N}_i$ and $^1\text{H}_i^\alpha\text{-}^{13}\text{C}_i^\alpha$ dipole vectors is displayed in Figure 5.3 with the region of highest sensitivity highlighted in gray. As indicated by the Ramachandran diagram in Figure 5.4, this experiment is most sensitive in the gray region, $-150^\circ < \varphi < -90^\circ$, which includes the $\beta$-sheet region of the Ramachandran space. Simulated dephasing curves, shown in Figure 5.5, indicate the high sensitivity for molecular geometries in which the $^1\text{H}_i^N\text{-}^{15}\text{N}_i$ and $^1\text{H}_i^\alpha\text{-}^{13}\text{C}_i^\alpha$ dipole vectors are nearly co-linear (red lines) as opposed to regions with low-sensitivity far away from this co-linear geometry (black lines).

Representative, experimental dephasing curves and fits to simulation of the $^1\text{H}_i\text{-}^{15}\text{N}_i\text{-}^{13}\text{C}_i^\alpha\text{-}^1\text{H}_i^\alpha$ experiment on huPrP23-144 are shown in Figure 5.6. This figure indicates that the projection angles (expressed here as $180^\circ\Theta$) for the A115, V121, A133 and I139 residues, labeled in blue, are in the sensitive region ($\Theta < 30^\circ$) which is consistent with a $\varphi$ torsion angle in the $\beta$-sheet region of the Ramachandran space. Residues A116 and A117 have projection angles far away from this region and therefore are predicted not to be in a $\beta$-sheet conformation. A summary of the projection angles measured in this experiment are listed in the second column of Table 5.1.
Figure 5.2 $^{1}H_i$-$^{15}N_i$-13C$\alpha$-$^{1}H^\alpha_i$ pulse sequence. This sequence was used to record a 3D experiment on huPrP23-144 to measure the projection angle between $^{1}H_i$-$^{15}N_i$ and $^{1}H^\alpha_i$-13C$\alpha$ dipole vectors in the sample. This projection angle can be related to the $\varphi$ backbone torsion angle. In the 3D experiment a series of 2D $^{15}N$-13C correlation spectra were collected with $^{15}N$ and 13C chemical shift evolution in $t_1$ and $t_2$ respectively. The third dimension, $\tau$, represents the amount of time for which the $^{1}H^N_i$-$^{15}N_i$ and $^{1}H^\alpha_i$-13C$\alpha$ couplings are dephased using the T-MREV-4$^{119,120}$ sequence which reintroduces either the $^{15}N$-$^{1}H$ or 13C-$^{1}H$ couplings while simultaneous decoupling $^{1}H$-$^{15}N$. The $^{1}H^N_i$-$^{15}N_i$ and $^{1}H^\alpha_i$-13C$\alpha$ dipole couplings are dephased in a 2:1 ratio; the $^{1}H^N_i$-$^{15}N_i$ dipole coupling for 2$\tau$ and the $^{1}H^\alpha_i$-13C$\alpha$ for $\tau$. 

![Diagram of pulse sequence](image-url)
Figure 5.3  Dependence of the projection angle, $\Theta$, measured in the $^1{\text{H}}_r$-$^{15}$N$_r$-$^{13}$C$_r$-$^{1}H_i$ experiment on the molecular torsion angle $\phi$. Regions of high angular resolution and sensitivity are highlighted in grey where the projection angle is within 30° of the collinear geometry.
Figure 5.4 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^1$H$_r$-$^{15}$N$_r$-$^{13}$C$_r$-$^1$H$_r$ experiment.
Figure 5.5 Simulated $^1\text{H}_i$-$^{15}\text{N}_i$-$^{13}\text{C}^\alpha_i$-$^1\text{H}^\alpha_i$ dephasing curves. Curves were calculated using an in-house FORTRAN program for the projections angles indicated by the labels. Red curves are for projections angles in the sensitive region of the experiment where the $^1\text{H}_i$-$^{15}\text{N}_i$ and $^1\text{H}^\alpha_i$-$^{13}\text{C}^\alpha_i$ dipole vectors are nearly co-linear, black curves are those far away from the sensitive region.
Figure 5.6 Sample experimental and best-fit simulated $^1H_i$-$^{15}N_i$-$^{13}C_{\alpha_i}$-$^1H_{\alpha_i}$ dephasing curves. The experimental curves were recorded using the 3D $^1H_i$-$^{15}N_i$-$^{13}C_{\alpha_i}$-$^1H_{\alpha_i}$ dipolar correlation experiment described in the text. For each trajectory the experimental cross-peak intensity in a 2D $^{15}N$-$^{13}C_{\alpha}$ correlation spectrum is shown as a function of the $^1H$-$^{13}C$ dephasing time $\tau$ (circles), and the best-fit simulation is shown as a solid line. Curves were fit using a grid of simulation using a collection of in-house Python and FORTRAN programs, see the Materials and Methods section for details. The projections angles for residues A115, V121, A133 and I139, labeled in blue, are in the sensitive region, which is consistent with a $\beta$-sheet conformation. Residues A116 and A117, labeled in green, do not have projection angles in the sensitive region and are predicted not to adopt a $\beta$-sheet conformation.
5.4 $^1H_{i+1}-^{15}N_{i+1}-{^{13}C_i}r^-^1H^a_i$ Experiment

Altering the $^1H_{i+1}-^{15}N_{i+1}-{^{13}C_i}r^-^1H^a_i$ experiment to transfer the $^{15}N$ magnetization to $^{13}CO$ and introducing a homonuclear $^{13}C-{^{13}C}$ recoupling sequence to transfer magnetization from the $^{13}CO$ to the neighboring $^{13}C\alpha$, one can measure the projection angle between the $^1H_{i+1}-^{15}N_{i+1}$ and $^{13}C\alpha^-^1H^a_i$ dipole vectors which can be related to the $\psi$ torsion angle. The pulse sequence for this $^1H_{i+1}-^{15}N_{i+1}-{^{13}C_i}r^-^1H^a_i$ experiment is shown in Figure 5.7 where RFDR$^{172}$ is used as the homonuclear $^{13}C-{^{13}C}$ recoupling sequence to transfer $^{13}CO$ polarization to $^{13}C\alpha$. The resulting 3D experiment is comprised of a collection of 2D $^{15}N-{^{13}C}\alpha$ correlation spectra (not shown) similar to the 2D NCOCX spectra collected in Chapter 2. The T-MREV dephasing periods are adjusted together in the same manner as the $^1H_{i+1}-^{15}N_{i+1}-{^{13}C_i}r^-^1H^a_i$ experiment.

The relationship between the molecular torsion angle $\psi$ and the measured projection angle between the $^1H_{i+1}-^{15}N_{i+1}$ and $^{13}C\alpha^-^1H^a_i$ dipole vectors is displayed in Figure 5.8 with the region of highest sensitivity highlighted in gray. As indicated by the Ramachandran diagram in Figure 5.9, this experiment is most sensitive in the gray region, $90^\circ < \psi < 150^\circ$, which includes the $\beta$-sheet region of the Ramachandran space.

Representative experimental dephasing curves and fits to simulation of the $^1H_{i+1}-^{15}N_{i+1}-{^{13}C_i}r^-^1H^a_i$ experiment on huPrP23-144 are shown in Figure 5.10. This figure indicates that the projection angles for the V121, P137 and I138 residues, labeled in blue, are in the sensitive region ($\Theta < 30^\circ$) which is consistent with a $\psi$ torsion angle in the $\beta$-sheet region of the Ramachandran space. Residues A115, A118 and V122 have projection angles far away from this region and therefore are predicted not to be in a $\beta$-
sheet conformation. A summary of the projection angles measured in this experiment are listed in the fourth column of Table 5.1
Figure 5.7 $^{1}H_{i+1}$-$^{15}N_{i+1}$-$^{13}C_{\alpha}$-$^{1}H_{\alpha}$ pulse sequence. This sequence was used to record a 3D experiment on huPrP23-144 to measure the projection angle between $^{1}H_{i+1}$-$^{15}N_{i+1}$ and $^{1}H_{\alpha}$-$^{13}C_{\alpha}$ dipole vectors in the sample. This projection angle can be related to the $\psi$ backbone torsion angle. In the 3D experiment a series of 2D $^{15}N$-$^{13}C$ correlation spectra were collected with $^{15}N$ and $^{13}C$ chemical shift evolution in $t_1$ and $t_2$ respectively. The third dimension, $\tau$, represents the amount of time for which the $^{1}H^{N,15}N$ and $^{1}H^{\alpha,13}C_{\alpha}$ couplings are dephased using the T-MREV-4$^{119,120}$ sequence which reintroduces either the $^{15}N$-$^{1}H$ or $^{13}C$-$^{1}H$ couplings while simultaneous decoupling $^{1}H$-$^{1}H$. The $^{1}H^{N,15}N$ and $^{1}H^{\alpha,13}C_{\alpha}$ dipole couplings are dephased in a 2:1 ratio; the $^{1}H^{N,15}N$ dipole coupling for $2\tau$ and the $^{1}H^{\alpha,13}C_{\alpha}$ for $\tau$. A 1.48 ms RFDR mixing period was used to transfer polarization from the $^{13}CO$ nuclei to the neighboring $^{13}C_{\alpha}$ nuclei after the $^{15}N$-$^{13}C$ CP.
Figure 5.8 Dependence of the projection angle, \( \Theta \), measured in the \( ^1\text{H}_i-i^+\text{N}_i-i^+\text{C}_i^\alpha-i^+\text{H}_i^\alpha \) experiment on the molecular torsion angle \( \psi \). Regions of high angular resolution and sensitivity are highlighted in grey where the projection angle is within 30° of the collinear geometry.
Figure 5.9 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^1H_{i+1}$-$^{15}N_{i+1}$-$^{13}C^\alpha$-$^1H^\alpha_i$ experiment.
Figure 5.10 Sample experimental and best-fit simulated $^1H_{i+1} - ^{15}N_{i+1} - ^{13}C_{\alpha i} - ^1H_{\alpha i+1}$ dephasing curves. The experimental curves were recorded using the 3D $^1H_{i+1} - ^{15}N_{i+1} - ^{13}C_{\alpha i} - ^1H_{\alpha i}$ dipolar correlation experiment described in the text. For each trajectory the experimental cross-peak intensity in a 2D $^{15}N_{-}^{13}C_{\alpha}$ correlation spectrum is shown as a function of the $^1H_{-}^{13}C$ dephasing time, $\tau$, (circles), and the best-fit simulation is shown as a solid line. Curves were fit using a grid of simulation using a collection of in-house Python and FORTRAN programs, see the Materials and Methods section for details. The projections angles for residues V121, P137 and I138, labeled in blue, are in the sensitive region, which is consistent with a $\beta$-sheet conformation. Residues A115, A118 and V122, labeled in green, do not have projection angles in the sensitive region and are predicted not to adopt a $\beta$-sheet conformation.
5.5 $^{15}\text{N}_{i+1} - ^{13}\text{C}^\prime - ^{13}\text{C}^\alpha - ^{15}\text{N}_i$ Experiment

The $^{15}\text{N}_{i+1} - ^{13}\text{C}^\prime - ^{13}\text{C}^\alpha - ^{15}\text{N}_i$ experiment for the measurement of the $\psi$ torsion angle has been described in detail elsewhere$^{167, 170, 173}$. In this section, we will provide a brief description of the experiment, discuss the experimental and simulation parameters, and show the results from the measurement of $\psi$ and its corresponding projection angle in huPrP23-144. Additional experimental parameter and simulation methods are discussed in the Materials and Methods section. The pulse sequence used for the $^{15}\text{N}_{i+1} - ^{13}\text{C}^\prime - ^{13}\text{C}^\alpha - ^{15}\text{N}_i$ experiment, referred to as the NCOCA NCCN experiment by Ladizhansky et al.$^{170}$, is shown in Figure 5.11. In this experiment, initial magnetization from the proton spins is transferred using cross polarization to $^{15}\text{N}$, frequency labeled in $t_1$, and then transferred to the neighboring $^{13}\text{CO}$ using a SPECIFIC CP$^{77}$. A time resolved REDOR pulse train is then used to reintroduce the $^{13}\text{C} - ^{15}\text{N}$ dipole-dipole coupling resulting in a dephasing of the $^{13}\text{CO}$ magnetization. From here, RFDR$^{172}$ is used to transfer $^{13}\text{CO}$ polarization to the neighboring $^{13}\text{C}^\alpha$ which is again dephased using a time resolved REDOR pulse train before detection. The resulting 3D experiment is comprised of a collection of 2D $^{15}\text{N}$-$^{13}\text{C}^\alpha$ correlation spectra (not shown) similar to the 2D NCOCX spectrum collected in Chapter 2. The REDOR dephasing periods are applied for identical times.

The relationship between the molecular torsion angle $\psi$ and the measured projection angle between the $^{15}\text{N}_{i+1} - ^{13}\text{C}^\prime$ and $^{13}\text{C}^\alpha - ^{15}\text{N}_i$ dipole vectors is displayed in Figure 5.12 with the region of highest sensitivity highlighted in gray. As indicated by the Ramachandran diagram in Figure 5.13, this experiment is most sensitive in the gray region, $150^\circ < |\psi| < 180^\circ$. Simulated dephasing curves are shown in Figure 5.13 for this
experiment showing high sensitivity for molecular geometries in which the $^{15}\text{N}_{i+1}-^{13}\text{C}'_i$ and $^{13}\text{C}'_i-^{15}\text{N}_i$ dipole vectors are nearly co-linear (red lines) as opposed to regions with low-sensitivity far away from this co-linear geometry (black lines).

Representative experimental dephasing curves and fits to simulation of the $^{15}\text{N}_{i+1}-^{13}\text{C}'_i-^{13}\text{C}'_i-^{15}\text{N}_i$ experiment on huPrP23-144 are shown in Figure 5.15. As can be seen in this figure, the experiment, which is most sensitive when $\psi$ is close to its trans conformation, did not find any projection angles in the sensitive region. This is not surprising as the trans conformation of $\psi$ is not common in proteins. Therefore, all the trajectories in Figure 5.15, as well as nearly all the other experimental trajectories measured, are not in the sensitive region of the experiment and provide only limited structural restraints. A summary of the projections angles measured in this experiment are listed in the sixth column of Table 5.1
This sequence was used to record a 3D experiment on huPrP23-144 to measure the projection angle between $^{15}\text{N}_{i+1}-^{13}\text{C}'_i$ and $^{13}\text{C}_\alpha_i$ dipole vectors in the sample. This projection angle can be related to the $\psi$ backbone torsion angle. In the 3D experiment a series of 2D $^{15}\text{N}-^{13}\text{C}$ correlation spectra were collected with $^{15}\text{N}$ and $^{13}\text{C}$ chemical shift evolution in $t_1$ and $t_2$ respectively. The third dimension, $\tau_{CN}$, represents the amount of time for which the $^{15}\text{N}_{i+1}-^{13}\text{C}'_i$ and $^{13}\text{C}_\alpha_i$-$^{15}\text{N}_i$ couplings are dephased using a REDOR sequence to reintroduces the $^{15}\text{N}_{i+1}-^{13}\text{C}'_i$ and $^{13}\text{C}_\alpha_i$-$^{15}\text{N}_i$ dipole couplings.
Figure 5.12 Dependence of the projection angle $\Theta$ measured in the $^{15}N_{i+1}-{^{13}C_i'}-{^{13}C_i}{^{15}N_i}$ experiment on the molecular torsion angle $\psi$. Regions of high angular resolution and sensitivity are highlighted in grey where the projection angle is within $30^\circ$ of the collinear geometry.
Figure 5.13 Ramachandran diagram with the shaded region indicating the region of high resolution and sensitivity of the $^{15}\text{N}_{i+1}^{13}\text{C}^{-}\text{C}^{\alpha}_{r-1}^{15}\text{N}_{i}$ experiment.
Figure 5.14 Simulated $^{15}\text{N}_{i+1} - ^{13}\text{C}_i - ^{13}\text{C}_i - ^{15}\text{N}_i$ dephasing curves. Curves were calculated using an in-house FORTRAN program for the projections angles indicated by the labeled. Red curves are for projections angles in the sensitive region of the experiment where the $^{15}\text{N}_{i+1} - ^{13}\text{C}_i$ and $^{13}\text{C}_i - ^{15}\text{N}_i$ dipole vectors are nearly co-linear, black curves are those far away from the sensitive region.
Figure 5.15 Sample experimental and best-fit simulated \(^{15}\text{N}_{i} \leftrightarrow^{13}\text{C}' \leftrightarrow^{13}\text{C}' \leftrightarrow^{15}\text{N}_{i}\) dephasing curves. The experimental curves were recorded using the 3D \(^{15}\text{N}_{i} \leftrightarrow^{13}\text{C}' \leftrightarrow^{13}\text{C}' \leftrightarrow^{13}\text{C}' \leftrightarrow^{15}\text{N}_{i}\) dipolar correlation experiment described in the text. For each trajectory the experimental cross-peak intensity in a 2D \(^{15}\text{N} \leftrightarrow^{13}\text{C}\) correlation spectrum is shown as a function of the \(^{15}\text{N} \leftrightarrow^{13}\text{C}\) dephasing time, \(\tau_{\text{CN}}\), (circles), and the best-fit simulation is shown as a solid line. Curves were fit using a grid of simulation using a collection of in-house Python and FORTRAN programs, see the Materials and Methods section for details.
5.6 Comparison to TALOS Prediction

The TALOS+\textsuperscript{174, 175} program predicts the backbone torsion angles for a protein, when provided a table of chemical shift assignments, by mining a large database of proteins with known structures and chemical shift assignments. From these torsion angles, the corresponding projection angles measured in the three dipole correlation experiments described above can be calculated. The correlation between these two methods is examined in Figure 5.16 and shows reasonable agreement between the two methods. In the region where the dipolar correlation experiments are most sensitive, which is highlighted in yellow, the correlation between these two methods is high for both residues predicted by TALOS to be in β-sheets, indicated by black dots, and in strands, indicated by red dots. A summary of the projections angles calculated from TALOS+ are listed in the odd column of Table 5.1
Figure 5.16 Correlation plots between the experimental measured projections angles versus those predicted by TALOS. Regions in which the projection angle experiment has high angular sensitivity and resolution are highlighted in yellow.
<table>
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<th>Residue</th>
<th>$^{1}$H$<em>{\text{i}}$$^{15}$N$</em>{\text{i+1}}$$^{13}$C$<em>{\text{q,i}}$$^{1}$H$</em>{\text{q}}$</th>
<th>$^{1}$H$<em>{\text{i+1}}$$^{15}$N$</em>{\text{i+1}}$$^{13}$C$<em>{\text{q,i}}$$^{1}$H$</em>{\text{q}}$</th>
<th>$^{15}$N$<em>{\text{i+1}}$$^{13}$C$</em>{\text{q,i}}$$^{13}$C$<em>{\text{q,i}}$$^{15}$N$</em>{\text{i}}$</th>
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<td></td>
<td>Experiment</td>
<td>TALOS</td>
<td>Experiment</td>
</tr>
<tr>
<td>A113</td>
<td>34.5±1.0 / 75.5±2.0</td>
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<td>16.8</td>
</tr>
<tr>
<td>G114</td>
<td>10.0</td>
<td>26.1</td>
<td>43.0±8.0 / 70.0±11.0</td>
</tr>
<tr>
<td>A115</td>
<td>17.5±0.5</td>
<td>11.1</td>
<td>41.0±3.0 / 64.5±5.0</td>
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<tr>
<td>A116</td>
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<td>47.7</td>
<td>32.0±2.0 / 77.5±4.0</td>
</tr>
<tr>
<td>A117</td>
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<td>26.2</td>
<td>31.5±2.0 / 80.5±5.0</td>
</tr>
<tr>
<td>A118</td>
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<tr>
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<td>82.1</td>
<td>43.0±8.0 / 70.0±11.0</td>
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<td>42.5±7.0 / 61.5±5.0</td>
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<tr>
<td>M129</td>
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<td></td>
</tr>
<tr>
<td>L130</td>
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<td>21.5</td>
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<tr>
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<td>19.0±5.0</td>
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<tr>
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<td>22.0</td>
<td>39.5±4.0 / 68.5±5.0</td>
</tr>
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<td>13.4</td>
<td>17.2</td>
</tr>
<tr>
<td>M134</td>
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<td>15.2</td>
<td>24.5±4.0</td>
</tr>
<tr>
<td>S135</td>
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<td>10.0</td>
<td>20.5±3.0</td>
</tr>
<tr>
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<td>27.5</td>
<td>32.3</td>
</tr>
<tr>
<td>P137</td>
<td>17.3</td>
<td>23.5±3.0</td>
<td>16.4</td>
</tr>
<tr>
<td>I138</td>
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<td>18.7</td>
<td>24.5±3.0</td>
</tr>
<tr>
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<td>15.8</td>
<td>18.0±3.0</td>
</tr>
<tr>
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<td>57.9</td>
<td>89.8</td>
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Table 5.1 Summary of huPrP23-144 experimentally measured projection angles and the corresponding projections angles predicted from TALOS. Errors for the projection angles were determined using a Monte Carlo analysis as described by Rienstra et al.\textsuperscript{120}
5.7 Materials and Methods

All torsion angle experiments described in this chapter were performed at 11.7 T (499.8 MHz for $^1$H, 125.7 MHz for $^{13}$C, and 50.6 MHz for $^{15}$N), using a three-channel Varian spectrometer equipped with 3.2 mm BioMAS™ probes in the $^1$H-$^{13}$C-$^{15}$N configuration. All experiments employed MAS rates, $\omega_r/2\pi$, in the ~9-11 kHz regime, and a temperature of 0° C. Sample spinning rates were actively regulated to ca. ± 3 Hz using a Varian MAS control unit, and sample temperatures were controlled by a stream of dry compressed air, delivered to the sample using a variable-temperature (VT) stack at a flow rate of 30 L/min. The 0° C operating temperature refers to the VT gas temperature at the sample—the actual average sample temperatures were ~5° C higher due to frictional heating as determined by lead nitrate calibration. All experiments were performed on a uniformly $^{13}$C,$^{15}$N labeled sample of huPrP23-144 fibrils. Details on the expression, purification, and preparation of this sample can be found in sections 2.4 and 3.2.1.

A $^1$H$_i$-$^{15}$N$_i$-$^{13}$C$^\alpha_i$-$^{1}$H$^\alpha_i$ experiment was performed on huPrP23-144 in order to measure the φ torsion angles. A spinning speed of 9469.7 Hz (corresponding to a 105.6 μs rotor period) was used to accommodate a 113.6 kHz T-MREV-4 element with four C elements per rotor period (2.2 μs $^1$H 90°). Twelve experimental 2D $^{15}$N-$^{13}$C correlation spectra were collected with $^{13}$C T-MREV dephasing times varying linearly from 0 μs to 290.4 μs in steps of 26.4 μs and correspondingly doubled $^{15}$N T-MREV dephasing times (0 μs to 580.8 μs in steps of 52.8 μs). Other experimental parameters were similar to those used in SSNMR experiments described earlier; see sections 2.4.3, 3.2.2, and 4.3.1. Experimental trajectories were extracted from the 3D experiments, in
the manner described in section 3.2.3, and fit using a grid of simulated dephasing curves generated from an in-house collection of Python and FORTRAN programs. The simulations were performed in a similar manner to those described in detail elsewhere. Briefly, an isotropic $^{15}$N-$^{13}$C polarization transfer was assumed, the two closest $^1$H’s to the $^{15}$N or $^{13}$C nuclei were included, and the differential relaxation effects of the T-MREV elements were considered. The fixed and free parameters for simulation were as follows: $^1$H-$^{15}$N, $^1$H-$^{15}$N, $^1$H-$^{13}$C and $^1$H-$^{13}$C distances were fixed to 1.04 Å, 2.12 Å, 2.18 Å, 1.12 Å, respectively. The T-MREV scaling factor, $\kappa$, for the $^1$H-$^{15}$N and $^1$H-$^{13}$C dephasing were fixed at the values found for the relevant coupling in the $^1$H-$^{13}$C and $^1$H-$^{15}$N order-parameter experiments described in section 3.3.2. The torsion angle, $\Theta$, was allowed to vary from 0° to 90° in steps of 0.5°, and the effective $^{15}$N and $^{13}$C relaxation constants were allowed to vary within ±100 s$^{-1}$ of the rates found for relevant coupling in the order-parameter experiments in section 3.3.2. A set of 3,722 Euler angles generated using the ZCW algorithm was used to perform the powder averaging. The estimated errors in the projection angles were determined using a Monte Carlo analysis as described by Rienstra et al. A $^1$H$_{i+1}$-$^{15}$N$_{i+1}$-$^{13}$C$_i$-$^1$H$_i$ experiment was performed on huPrP23-144 in order to measure the $\psi$ torsion angles with parameters similar to the $^1$H$_i$-$^{15}$N$_i$-$^{13}$C$_i$-$^1$H$_i$ experiment. A 1.48 ms RFDR mixing period, 14 rotor periods, was used to transfer polarization from the $^{13}$CO nuclei to neighboring $^{13}$C nuclei. Experimental trajectories were extracted and fit in the same manner as described for the $^1$H$_i$-$^{15}$N$_i$-$^{13}$C$_i$-$^1$H$_i$ experiment.
A $^{15}\text{N}_{i+1}-^{13}\text{C}'_i-^{13}\text{C}^\alpha_i-^{15}\text{N}_i$ experiment was performed on huPrP23-144 in order to measure the $\psi$ torsion angles. Experimental NMR parameters were similar to those for the ZF-TEDOR experiments described in Chapter 4 and to the other dipolar correlation experiments discussed in this Chapter. Eleven experimental 2D $^{15}\text{N}-^{13}\text{C}$ correlation spectra were collected with REDOR mixing times for both dephasing periods varying linearly from 0 ms to 1.8 ms in steps of 180 $\mu$s. Experimental trajectories were extracted from the 3D experiment in the manner described in section 3.2.3 and fit using a grid of simulated dephasing curves generated from an in-house collection of Python and FORTRAN programs. Simulations were performed in a similar manner to those described in detail elsewhere$^{170}$. An isotropic $^{15}\text{N}-^{13}\text{C}$ and $^{13}\text{C}-^{13}\text{C}$ polarization transfer was assumed and the closest $^{15}\text{N}$ to the $^{13}\text{C}$ nuclei being dephased were included in the simulation. The fixed and free parameters for the simulations were as follows: $^{15}\text{N}_{i+1}-^{13}\text{C}'_i$ and $^{13}\text{C}^\alpha_i-^{15}\text{N}_i$ distances were fixed to 1.33 Å and 1.45 Å, respectively. A scaling factor, $k$, of 0.95 was used to account for the REDOR finite pulses effects$^{130}$ and vibrational effects$^{132}$. The torsion angle, $\Theta$, was allowed to vary from 0° to 90° in steps of 0.25°, and exponential relaxation was applied to the resulting trajectory with rates varying from 0 to 1000 s$^{-1}$. A set of 3,722 Euler angles generated using the ZCW algorithm$^{176-178}$ was used to perform the powder averaging. The estimated errors in the projection angles were determined using a Monte Carlo analysis as described by Rienstra et al.$^{120}$
Chapter 6

Distance Measurements

6.1 Introduction

In addition to the restraints on the torsion angles provided by the dipole correlation experiments discussed in the last Chapter, a number of solid state NMR experiments have been designed to provide distance restraints between nuclei in solid protein samples\(^61, 62, 121, 123, 124, 179-183\). In this chapter, a ZF-TEDOR experiment\(^{121}\) to measure \(^{15}\)N-\(^{13}\)C distances will be discussed followed by a discussion of DARR\(^61, 62\) experiments which measure \(^{13}\)C-\(^{13}\)C distances.

6.2 TEDOR Measurements

In Chapter 4, magic-angle solid state NMR TEDOR experiments were used to determine the intermolecular alignment of huPrP23-144 fibrils by examining \(^{15}\)N-\(^{13}\)C contacts in 2D ZF-TEDOR spectra. If multiple 2D ZF-TEDOR spectra are collected with different \(^{15}\)N-\(^{13}\)C mixing times, the resulting cross peaks have intensities modulated by the \(^{15}\)N-\(^{13}\)C dipole-dipole couplings contributing to the peak. Fitting these cross peak trajectories allows a quantitative determination of the dipole-dipole coupling constant and therefore the \(^{15}\)N-\(^{13}\)C distance\(^{121, 124}\). In a protein sample, this technique can be used to measure \(^{15}\)N-\(^{13}\)C distances up to \(\sim 6\) Å. In this section, the results of a 3D ZF-TEDOR
experiment on a diluted 1,3-glycerol huPrP23-144 sample is discussed. A similar experiment was performed on a diluted 2-glycerol huPrP23-144 sample, but no structurally interesting distances could be determined from this data.

A 3D ZF-TEDOR experiment was performed on a sample of diluted 1,3-glycerol huPrP23-144 fibrils to measure $^{15}$N-$^{13}$C distances, experimental details are provided in the Materials and Methods section. A small region of the 2D spectrum from this 3D experiment is shown in Figure 6.1 at four different TEDOR mixing times. In the shortest $^{15}$N-$^{13}$C mixing time of 3.6 ms, panel A, the short (~2.5 Å) $^{15}$N$_{i}$-$^{13}$C$_{\beta_i}$ cross peaks are the only peaks with significant intensity as the other contacts have not had sufficient time to develop. By 9.0 ms of mixing, panel B, the valine and isoleucine $^{15}$N$_{i}$-$^{13}$C$_{\gamma_i}$ contacts begin to appear as well as a few inter-residue $^{15}$N$_{i+1}$-$^{13}$C$_{x_i}$ contacts: G119N-A118CB, A117N-A116CB, A118N-A117CB, G123N-V122CG1, V122N-V121CG1. These and similar inter-residue contacts contribute significantly to the spectrum with 14.4 ms of $^{15}$N-$^{13}$C mixing, panel C, and a long range S132N-A116CB cross peak also appears. This peak has even greater intensity at 19.8 ms of mixing; whereas many of the other peaks are beginning to succumb to the T$_2$ relaxation during the long mixing period.

By fitting the cross peak trajectories from this experiment a quantitative measurement of the $^{15}$N-$^{13}$C distance represented by the cross peak can be determined. Details on this fitting are given in the Materials and Methods section. Many of the observed cross peaks in this experiment were from one or two $^{15}$N-$^{13}$C bond contacts. Distances for such contacts do not depend on backbone or side-chain torsion angles and therefore, the $^{13}$C-$^{15}$N distances determined from fitting these trajectories do not provide
meaningful constraints on the protein structure and will not be discussed. Sample cross peak trajectories with fits are shown in Figure 6.2. In this figure, the two S132CO trajectories in panel A are examples of one bond, A133N-S132CO, and two bond, S132N-S132CO, contacts which do not provide meaningful restraints on the protein structure but can be fit quite well. Similarly the two bond $^{15}\text{N}_i-^{13}\text{C}\beta_i$ trajectories, A117N-A117CB and A118N-A118CB, in panels B and C, do not provide significant structural restraints, but the three bond $^{15}\text{N}_{i+1}-^{13}\text{C}\beta_i$ distances, A118N-A117CB and G119N-A118CB, provide restraints on the backbone $\psi$ torsion angle. In panel D, the V122N-V122CG1 distances provide a significant restraint on the valine side chain conformation. A total of 19 structurally interesting intramolecular distances were determined using this experiment, which are listed in Table 6.1 along with the trivial distances that were fit simultaneously.
Figure 6.1 Sample 2D NCx regions of the 3D ZF-TEDOR experiment. Spectra were collected at 11.11 kHz MAS rate and 0° C with $^{13}\text{C}_{-^{15}}\text{N}$ TEDOR mixing times of 3.6 ms (A), 9.0 ms (B), 14.4 ms (C) and 19.8 ms (D) on a diluted 1,3-glycerol huPrP23-144 sample. The 3D experiment was recorded in an interleaved manner as a $74^* (t_1, ^{15}\text{N}) \times 1200^* (t_2, ^{13}\text{C}) \times 12 (\tau_{\text{mix}})$ data matrix with acquisition times of 13.2 ms ($t_1$), 24 ms ($t_2$) and 21.6 ms ($\tau_{\text{mix}}$), and a total measurement time of 328 hours. Assignments are taken from Helmus et al.\textsuperscript{23}
Figure 6.2 Representative ZF-TEDOR experimental trajectories and best-fit simulations. The ZF-TEDOR trajectories were recorded using a 3D ZT-TEDOR SSNMR experiments on diluted 1,3-glycerol huPrP23-144, as described in the text. For each trajectory the experimental cross-peak intensities for all $^{15}\text{N}$-$^{13}\text{C}$ cross peaks for a given $^{13}\text{C}$ nuclei are shown as a function of the $^{13}\text{C}$-$^{15}\text{N}$ TEDOR mixing time (circles), and the best-fit simulation is shown as a solid line. Additional trajectories are shown in Figures 6.3. Experimental trajectories were fit using a constrained least-squares optimization to the analytical expression given in the text, with all trajectories for a given $^{13}\text{C}$ nuclei fit simultaneously.
Figure 6.3 ZF-TEDOR trajectories and best-fit simulations for all non-trivial distances in the 3D ZF-TEDOR experiment. See Figure 6.2 caption for a description of the experimental trajectories and simulations.
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Table 6.1 ¹³C-¹⁵N distances measured by the 3D ZF-TEDOR experiment on diluted 1,3-glycerol huPrP23-144 fibrils. ¹⁵N atoms and ¹⁵N-¹³C distances are given for each ¹³C nuclei in which at least one distance was structurally interesting (i.e. not describing a one or two bond distance). Distances were determined from the dipole coupling constants of the best-fit simulation of the ZF-TEDOR trajectories presented in Figure 6.3.
6.3 RAD/DARR Measurements

In addition to the $^{15}$N-$^{13}$C distances discussed in the last section, a number of $^{13}$C-$^{13}$C distances were measured using a series of 2D $^{13}$C-$^{13}$C DARR (dipolar assisted rotational resonance)$^{61}$, also referred to as RAD (RF assisted diffusion)$^{62}$, experiments. In these experiments, a series of 2D $^{13}$C-$^{13}$C correlation spectra were collected with varying $^{13}$C-$^{13}$C DARR mixing times, experimental details are given in the Materials and Methods section. For each mixing time, empirically determined distance limits can be applied based on the qualitative intensity of each peak detected in the spectrum. For example, in a 500 ms DARR spectrum, a strong peak is expected to correspond to a $^{13}$C-$^{13}$C internuclear distance less than 5.8 Å, a medium peak to a distance no less than 6.7 Å, and a weak peak to a distance no less than 8.2 Å$^{184}$. In many cases, peaks in these DARR spectra exhibit large overlap, and an unambiguous assignment of such peaks is not possible. A number of structural modeling programs$^{185-193}$ take into account ambiguous restraints and these restraints will be reported here.

Representative regions from the $^{13}$CO-$^{13}$Cx and $^{13}$Cx-$^{13}$Cx regions of a 500 ms mixing time DARR experiment on a uniform 1,3-glycerol huPrP23-144 sample are shown in Figure 6.4 and 6.5, with a number of unambiguous long-range peaks labeled. A total of 10 unambiguous and 18 ambiguous long range constraints, which are listed in Table 6.2, were determined from four DARR experiments on a uniform 1,3-glycerol huPrP23-144 sample. In this table, as well as Table 6.3 which lists constraints from a series of 2D DARR spectra on a uniform 2-glycerol huPrP23-144 sample, unambiguous restraints from cross peaks where a single, unique assignment could be made are listed on
the left side as $^{13}\text{C}_A$ and $^{13}\text{C}_B$. As the collected DARR spectra are symmetric, peaks from both $^{13}\text{C}_A$-$^{13}\text{C}_B$ and $^{13}\text{C}_B$-$^{13}\text{C}_A$ may be detected, but will be listed as a single restraint in the table with $^{13}\text{C}_A$ having the lower residue number. Ambiguous restraints, where the peak may arise from two possible assignments or the overlap of two peaks, are listed in Table 6.2 and 6.3 as $^{13}\text{C}_A$, $^{13}\text{C}_{B1}$, and $^{13}\text{C}_{B2}$, where $^{13}\text{C}_A$ is the unambiguous assigned $^{13}\text{C}$ in the first dimension and $^{13}\text{C}_{B1}$ and $^{13}\text{C}_{B2}$ are the two possible $^{13}\text{C}$ assignments in the second dimension. Peaks where both assignments were ambiguous or more than two assignments are possible for one of the dimensions are not included in these tables. Qualitative classification of these peaks as strong, medium or weak for the various spectra was also performed but is not shown.

As in the discussion of the 2D ZF-TEDOR on uniformly labeled samples in Section 4.2.1, the cross peaks in these DARR spectra may arise from intermolecular or intramolecular contacts. The use of diluted samples may allow differentiation between these two types of contacts. The two, 2D DARR spectra collected on a diluted 1,3-glycerol huPrP23-144 sample with mixing times of 100 ms and 500 ms, despite a long signal averaging period or ~27 hours and ~108 hours, did not have sufficient sensitivity to classify the long range peaks in the uniform 1,3-glycerol huPrP23-144 DARR spectra as intermolecular or intramolecular. If these experiments were repeated at higher field, it may be possible to sort these restraints into those of intermolecular nature and those coming from intramolecular contacts.
The $^{13}\text{C}\alpha-^{13}\text{C}\alpha$ regions of a 500 ms mixing time DARR experiment on a uniform 2-glycerol huPrP23-144 sample are shown in Figure 6.6 with a number of unambiguous long-range peaks labeled. A total of 8 unambiguous and 2 ambiguous long-range constraints were determined from four DARR experiments on a uniform 2-glycerol huPrP23-144 fibril sample. These constraints are listed in Table 6.3. Two, 2D DARR spectra were collected on a diluted 2-glycerol huPrP23-144 sample with mixing times of 100 ms and 350 ms and experimental collection times of ~25 and ~120 hours, but as in the case of the diluted 1,3-glycerol huPrP23-144 spectra, these did not have sufficient sensitivity to differentiate between intramolecular and intermolecular restraints.
Figure 6.4 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 1,3-glycerol huPrP23-144 with 500 ms DARR mixing. The spectrum, collected at 11.11 kHz MAS rate and 0° C, was recorded as a 1080 (t$_1$, $^{13}$C) × 1250* (t$_2$, $^{13}$C) data matrix with acquisition times of 15.1 ms (t$_1$), and 25 ms (t$_2$) and a total measurement time of 87 hours. Boxed peaks represent long range $^{13}$C-$^{13}$C contacts.
Figure 6.5 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 1,3-glycerol huPrP23-144 with 375 ms DARR mixing. The spectrum, collected at 11.11 kHz MAS rate and 0° C, was recorded as a 1080 ($t_1$, $^{13}$C) × 1250* ($t_2$, $^{13}$C) data matrix with acquisition times of 15.1 ms ($t_1$), and 25 ms ($t_2$) and a total measurement time of 84 hours. Boxed peaks represent long range $^{13}$C-$^{13}$C contacts.
Figure 6.6 Region from a 2D $^{13}$C-$^{13}$C DARR spectrum of uniform 2-glycerol huPrP23-144 with 500 ms of DARR mixing. The spectrum, collected at 11.11 kHz MAS rate and 0° C, was recorded as a 1080 ($t_1$, $^{13}$C) × 1250* ($t_2$, $^{13}$C) data matrix with acquisition times of 15.1 ms ($t_1$), and 25 ms ($t_2$) and a total measurement time of 87 hours. Boxed peaks represent long range $^{13}$C-$^{13}$C contacts.
Table 6.2 Listing of $^{13}\text{C}$-$^{13}\text{C}$ cross peaks from long range contacts detected in the DARR spectrum of uniform 1,3-glycerol huPrP23-144. Unambiguous cross-peaks, where the assignments of both $^{13}\text{C}$ resonances was well determined, are listed on the left side of the table. In these symmetric DARR spectra, peaks from both $^{13}\text{C}_A$-$^{13}\text{C}_B$ and $^{13}\text{C}_B$-$^{13}\text{C}_A$ may be seen but the peak is only listed once in the table with the $^{13}\text{C}$ with lowest residue number assigned as $^{13}\text{C}_A$. Ambiguous cross-peaks, where one of the $^{13}\text{C}$ resonance was in good agreement with two or more assignments, are listed on the right side of the table. The unambiguous $^{13}\text{C}$ assignment is listed as $^{13}\text{C}_A$ with the two ambiguous assignments listed as $^{13}\text{C}_{B1}$ and $^{13}\text{C}_{B2}$, again with the assignment with lowest residue number listed first. Peaks where both assignments were ambiguous or more than two assignment where possible for a single resonance are not included in this table.

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Table 6.3 Listing of $^{13}$C-$^{13}$C cross peaks from long range contacts detected in the DARR spectrum of uniform 2-glycerol huPrP23-144. Unambiguous cross-peaks, where the assignments of both $^{13}$C resonances was well determined, are listed on the left side of the table. In these symmetric DARR spectra, peaks from both $^{13}$C$_{A}$-$^{13}$C$_{B}$ and $^{13}$C$_{B}$-$^{13}$C$_{A}$ may be seen but the peak is only listed once in the table with the $^{13}$C with lowest residue number assigned as $^{13}$C$_{A}$. Ambiguous cross-peaks, where one of the $^{13}$C resonance was in good agreement with two or more assignments, are listed on the right side of the table. The unambiguous $^{13}$C assignment is listed as $^{13}$C$_{A}$ with the two ambiguous assignments listed as $^{13}$C$_{B1}$ and $^{13}$C$_{B2}$, again with the assignment with lowest residue number listed first. Peaks where both assignments were ambiguous or more than two assignment where possible for a single resonance are not included in this table.

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6.4 Materials and Methods

NMR experiments were performed at 11.7 T using a three-channel Varian spectrometer equipped with 3.2 mm triple-resonance T3 and BioMAS™ probes in $^1$H-$^13$C-$^{15}$N configuration with a 11.11kHz MAS rate and 0° C temperature controlled as described earlier.

A 3D ZF-TEDOR experiment was performed on a sample of diluted 1,3-glycerol huPrP23-144. Experimental parameters were similar to those described in section 4.3.1 with the pulse sequence given in Figure 4.6A. In total, twelve 2D ZF-TEDOR spectra were collected with mixing times varying linearly from 1.8 ms to 21.6 ms. Each 2D ZF-TEDOR spectrum required ~27 hours to collect resulting in an experimental time of ~328 hours for the full 3D. Peak assignments were determined from earlier CP and TEDOR experiments. Experimental trajectories were extracted from the 3D experiments in the manner described in section 3.2.3. Data was fit using a constrained least-squared optimization to the analytical expression:

$$I(t) = A_0 e^{-R t} \left(1 - \left[J_0(\sqrt{2} d_1 t)\right]^2 \prod_{k=2}^{N} \left(1 + [J_0(\sqrt{2} d_k t)]^2\right) \prod_{l=2}^{M} \cos^2 \left(\frac{\pi J_l t}{2}\right)\right)$$

where $I(t)$ is the intensity for a peak at the $^{15}$N-$^{13}$C mixing time $t$, $A_0$ is an overall scaling factor, $R$ is the relaxation rate, $J_0$ is the Bessel function of zeroth order, $d_1$ is the active coupling, the dipole coupling constant for the peak being examined, $d_2, d_3, ... d_N$ are the passive couplings, dipole coupling constants for peaks with the same $^{13}$C nuclei as the active coupling but different $^{15}$N nuclei, and $J_l$ are the active $^{13}$C J-couplings (if any) for the $^{13}$C. In cases where only a single $^{15}$N cross peak was detected for a given $^{13}$C
resonance, the first product was omitted from the expression. Similarly, for $^{13}$C with no active $J$-couplings, the second product was omitted. Peak trajectories arising from the same $^{13}$C nuclei but different $^{15}$N nuclei were fit simultaneously to determine a set of global best fit parameters ($A_0$, $R$, $d_1$, $d_2$, $\ldots$, $d_N$) by selecting the relevant dipole coupling to act as the active coupling in each trajectory. This equation has been found to provide a molecular, orientation-independent approximation for TEDOR curves$^{194}$.

Four 2D $^{13}$C-$^{13}$C DARR spectra were collected on a uniform 1,3-glycerol huPrP23-144 fibril sample with mixing times of 100 ms, 250 ms, 375 ms and 500 ms, and experimental collection times of ~18 hours, ~81 hours, ~84 hours, and ~87 hours, respectively and other experimental parameters similar to those described earlier.

Four 2D $^{13}$C-$^{13}$C DARR spectra were collected on a uniform 2-glycerol $^{13}$C labeled sample of huPrP23-144 fibrils with mixing times of 100 ms, 250 ms, 375 ms and 500 ms, and experimental collection times of ~25 hours, ~108 hours, ~84 hours, and ~87 hours, respectively and other experimental parameters similar to those described earlier.
References


174


