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Intermolecular Alignment in Y145Stop Human Prion Protein Amyloid Fibrils Probed by Solid-State NMR Spectroscopy

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Abstract

The Y145Stop mutant of human prion protein, huPrP23-144, has been linked to PrP cerebral amyloid angiopathy, an inherited amyloid disease, and also serves as a valuable *in vitro* model for investigating the molecular basis of amyloid strains. Prior studies of huPrP23-144 amyloid by magic-angle spinning (MAS) solid-state NMR revealed a compact β -rich amyloid core region near the C-terminus and an unstructured N-terminal domain. Here, with the focus on understanding the higher order architecture of huPrP23-144 fibrils, we probe the intermolecular alignment of β -strands within the amyloid core using MAS NMR techniques and fibrils formed from equimolar mixtures of ^{15}N -labeled protein and ^{13}C -huPrP23-144 prepared with $[1,3\text{-}^{13}\text{C}]$ or $[2\text{-}^{13}\text{C}]\text{glycerol}$. Numerous intermolecular correlations involving backbone atoms observed in 2D ^{15}N - ^{13}C spectra unequivocally suggest an overall parallel in-register alignment of the β -sheet core. Additional experiments that report on intermolecular ^{15}N - ^{13}CO and ^{15}N - $^{13}\text{C}\alpha$ dipolar couplings yield an estimated strand spacing that is within ~10% of the ~4.7–4.8 Å distances typical for parallel β -sheets.

Conformational conversion of the prion protein (PrP) to ordered β -sheet-rich amyloid-like aggregates is associated with the pathogenesis of a group of fatal neurodegenerative diseases known as transmissible spongiform encephalopathies.¹ Although once controversial, the notion that prions can propagate via a “proteinonly” mechanism with the misfolded aggregates acting as self-replicating infectious agents is becoming widely accepted, particularly in light of recent findings that infectious particles can be generated *in vitro* from purified prion protein² and observations regarding conformation-based inheritance in fungi.³ While the ability of prions to propagate appears to be encoded in the three-dimensional structure of PrP aggregates, the molecular mechanisms underlying this phenomenon are currently not well understood with atomic-level detail.⁴

The C-terminally truncated Y145Stop variant of human PrP (huPrP23-144) is associated with a heritable cerebral amyloid angiopathy.⁵ Though this type of prionopathy appears to be noninfectious, previously we have shown that huPrP23-144 provides a valuable model *in vitro* for studying the molecular basis of amyloid formation as well as the phenomena of amyloid strains and seeding specificity.⁶ More recently, we have initiated multidimensional magic-angle spinning (MAS) solid-state nuclear magnetic resonance (NMR) studies of the

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ASSOCIATED CONTENT

Supporting Information. Experimental and data analysis sections, NMR and fiber X-ray diffraction data, and structural models. This material is available free of charge via the Internet at <http://pubs.acs.org>.

structure and conformational dynamics of amyloid fibrils prepared from recombinant ^{13}C , ^{15}N -enriched huPrP23-144.^{7,8} These studies revealed that huPrP23-144 fibrils contain a relatively rigid amyloid core region composed of only ~30 C-terminal amino acids (aa), with many residues in a β -conformation. In contrast, a large N-terminal domain comprising aa ~23–111 was found to be a highly flexible ensemble of random-coil-like conformers.

Here, with the emphasis on gaining insight into the higher order structure of huPrP23-144 fibrils, we probe the intermolecular alignment of β -strands in the amyloid core region by MAS solid-state NMR spectroscopy. Solid-state NMR methods are uniquely well-suited for this type of analysis and have previously been used to investigate the core architecture for numerous peptide-derived fibrils,⁹ such as β -amyloid¹⁰ and fragments of amylin,¹¹ transthyretin¹² and β_2 -microglobulin,¹³ as well as several larger proteins or protein domains⁹ including Sup35p(1–253),¹⁴ Ure2p,¹⁵ β_2 -microglobulin,¹⁶ HET-s(218–289)¹⁷ and HET-s.¹⁸ In this study we utilize transferred echo double resonance (TEDOR) NMR techniques^{19,20} and fibril samples prepared from equimolar mixtures of ^{15}N -huPrP23-144 and sparsely ^{13}C -labeled huPrP23-144 to record 2D ^{15}N - ^{13}C chemical shift correlation spectra containing a large number of intermolecular correlations involving the backbone ^{15}N , ^{13}CO and $^{13}\text{C}\alpha$ nuclei. We also evaluate the magnitudes of intermolecular ^{15}N - ^{13}CO and ^{15}N - $^{13}\text{C}\alpha$ dipolar couplings, which report on the strand spacing within the β -sheet core.

The huPrP23-144 amyloid fibril samples were grown in pH 6.4 phosphate buffer from physical mixtures of ^{15}N - and ^{13}C -labeled monomers in a 1:1 molar ratio, where the ^{13}C -labeled proteins were generated using [1,3- ^{13}C] or [2- ^{13}C]glycerol based expression media^{21,22} (see Supporting Information for details). This type of ^{13}C -enrichment, which removes many one-bond ^{13}C - ^{13}C dipolar and J-couplings and leads to improved spectral resolution and higher magnetization transfer efficiencies,^{22,23} is particularly beneficial in the context of ^{15}N - ^{13}C TEDOR experiments.^{16,24} In addition to the mixed 1,3- ^{13}C / ^{15}N and 2- ^{13}C / ^{15}N huPrP23-144 fibrils we also prepared control amyloid samples from pure 1,3- ^{13}C , ^{15}N - and 2- ^{13}C , ^{15}N -labeled huPrP23-144, as well as 1,3- ^{13}C , ^{15}N - and 2- ^{13}C , ^{15}N -huPrP23-144 diluted in natural abundance (^{12}C , ^{14}N) protein in a 1:3 molar ratio.

For the undiluted and diluted 1,3- ^{13}C , ^{15}N - and 2- ^{13}C , ^{15}N -labeled control amyloid samples application of the z -filtered TEDOR pulse sequence²⁰ with short mixing times ($\tau_{\text{TEDOR}} \approx 1.5\text{--}2\text{ ms}$) and without ^{15}N evolution yields spectra displaying $^{13}\text{CO}/^{13}\text{C}\alpha$ intensities that are ~20–25% of those obtained for the corresponding cross-polarization (CP) reference ^{13}C spectra, indicating magnetization transfer via strong ~1 kHz one-bond ^{15}N - ^{13}C dipolar couplings. In contrast, no appreciable intensity is obtained for mixed 1,3- ^{13}C / ^{15}N and 2- ^{13}C / ^{15}N fibrils under the same experimental conditions (see TEDOR trajectories in Figure 2), confirming that contributions to the spectra from one-bond ^{15}N - ^{13}C couplings involving the ~1% of natural abundance ^{13}C atoms in ^{15}N -huPrP23-144 are negligible, obviating the need for preparation of mixed ^{13}C / ^{15}N fibril samples containing specifically ^{13}C -depleted, ^{15}N -labeled protein. The use of longer TEDOR mixing times, on the order of ~15–20 ms, however, results in a clear build-up of $^{13}\text{CO}/^{13}\text{C}\alpha$ intensities, up to ~2–4% of those found in CP spectra. The latter finding is consistent with magnetization transfer via weak intermolecular ^{15}N - ^{13}C dipolar couplings corresponding to distances in the ~4–5 Å regime (see discussion below), associated with neighboring ^{15}N - and 1,3- ^{13}C - or 2- ^{13}C -labeled huPrP23-144 molecules in the fibril lattice.

In order to identify the specific protein sites responsible for the intermolecular backbone ^{15}N - ^{13}C contacts we recorded a series of 2D ^{15}N - ^{13}CO (NCO) and ^{15}N - $^{13}\text{C}\alpha$ (NCA) TEDOR chemical shift correlation spectra for different huPrP23-144 fibril samples

(Figure 1). The short mixing time reference NCO and NCA spectra for 1,3- ^{13}C , ^{15}N - and 2- ^{13}C , ^{15}N -labeled fibrils, respectively, contain exclusively intramolecular one-bond ^{15}N - ^{13}CO and ^{15}N - $^{13}\text{C}\alpha$ correlations, which could be readily identified based on previous assignments established for uniformly ^{13}C , ^{15}N -enriched huPrP23-144 amyloid.⁷ Moreover, the fact that pairs of spectra for corresponding undiluted/diluted fibrils are effectively identical, indicates the high reproducibility of the sample preparation protocols and experiments employed in this study.

The NCO and NCA spectra for mixed 1,3- $^{13}\text{C}/^{15}\text{N}$ and 2- $^{13}\text{C}/^{15}\text{N}$ huPrP23-144 fibrils (Figures 1D and 1G) were recorded with TEDOR mixing times of 18 ms and 15.84 ms, respectively. Together, these two spectra contain ~30 relatively intense correlations involving the backbone atoms for the amyloid core residues, with ^{15}N , ^{13}CO and $^{13}\text{C}\alpha$ frequencies closely matching those observed for the control 1,3- ^{13}C , ^{15}N - and 2- ^{13}C , ^{15}N -labeled samples. The NCO data set contains a significantly greater number of cross-peaks relative to NCA, most likely due to more favorable transverse relaxation properties for ^{13}CO vs. $^{13}\text{C}\alpha$ nuclei and larger magnitudes of ^{15}N - ^{13}C dipolar couplings. A careful inspection of these data reveals that the majority of resonances present in the NCO and NCA spectra result from intermolecular $^{15}\text{N}_i$ - $^{13}\text{CO}_{i-1}$ and $^{15}\text{N}_i$ - $^{13}\text{C}\alpha_i$ transfers, respectively, with most of the remaining cross-peaks corresponding to $^{15}\text{N}_i$ - $^{13}\text{CO}_i$ and $^{15}\text{N}_i$ - $^{13}\text{C}\alpha_{i-1}$ transfers, respectively. In addition, a set of ^{15}N - ^{13}CX (NCX) TEDOR spectra, containing correlations involving side-chain ^{13}C methyl groups, was recorded for fibrils grown from ^{13}C or ^{13}C , ^{15}N proteins prepared using the [1,3- ^{13}C]glycerol labeling scheme (Figure S1). The spectrum for mixed 1,3- $^{13}\text{C}/^{15}\text{N}$ fibrils displays 10 intermolecular $^{15}\text{N}_i$ - $^{13}\text{C}_i$ cross-peaks, which further support the correlations detected in the NCO and NCA spectra. A graphical summary of the correlations observed in NCO, NCA and NCX spectra (Figure 1A) shows the presence of intermolecular contacts for the vast majority of residues within the ~113–125 region (which constitutes the most rigid part of the amyloid core⁸) as well as multiple residues in the C-terminal β -strand (aa ~132–139).

As noted above, the long-mixing time TEDOR experiments for the mixed $^{13}\text{C}/^{15}\text{N}$ fibrils report exclusively on intermolecular contacts. Furthermore, any contributions to the spectra arising from intramolecular ^{15}N - ^{13}C couplings involving residual ^{13}C nuclei present at natural abundance in ^{15}N -huPrP23-144 are below the detection limit of the measurements, a notion further supported by the fact that a number of correlations—that should normally be observed if such natural abundance background effects were significant—are missing in 2D ^{15}N - ^{13}C spectra in Figures 1 and S1 (e.g., G124N-G123CO, S132N-G131CO, A133N-C α , A115N-C β , etc.). Thus, the present data strongly indicate that β -strands in the core region of huPrP23-144 amyloid fibrils are organized in a parallel, in-register fashion. Within this structural model the nearest-neighbor and next-nearest-neighbor backbone ^{15}N - $^{13}\text{CO}/^{13}\text{C}\alpha$ distances correspond to $^{15}\text{N}_i$ - $^{13}\text{CO}_{i-1}$ (~4.2 Å) and $^{15}\text{N}_i$ - $^{13}\text{CO}_i$ (~5.2–5.4 Å), and $^{15}\text{N}_i$ - $^{13}\text{C}\alpha_i$ (~4.7–4.8 Å) and $^{15}\text{N}_i$ - $^{13}\text{C}\alpha_{i-1}$ (~5.2–5.4 Å), respectively (Figure S2), consistent with the correlations observed in the NCO and NCA spectra.

Although limited sensitivity of TEDOR experiments for mixed $^{13}\text{C}/^{15}\text{N}$ fibrils precluded the determination of intermolecular ^{15}N - ^{13}C distances in residue-specific fashion, we were able to estimate the spacing between adjacent huPrP23-144 molecules in the β -sheet core by recording 1D TEDOR trajectories that simultaneously report on all ^{15}N - ^{13}CO or ^{15}N - $^{13}\text{C}\alpha$ dipolar couplings (with the main contributions to the trajectories arising from the strongest $^{15}\text{N}_i$ - $^{13}\text{CO}_{i-1}/^{15}\text{N}_i$ - $^{13}\text{C}\alpha_i$ cross-peaks in the 2D spectra in Figure 1). These trajectories, shown in Figure 2, were simulated as a function of intermolecular spacing as described in detail in the Supporting Information (by considering all ^{15}N - $^{13}\text{CO}/^{13}\text{C}\alpha$ distances < 6 Å within the parallel in-register model expected for the mixed $^{13}\text{C}/^{15}\text{N}$ fibril samples and assuming that these distances are the same for all residues). The

calculated ^{15}N - ^{13}C and ^{15}N - ^{13}Ca trajectories are in good agreement with their experimental counterparts, and both datasets yield a self-consistent estimate of $d = 4.4 \text{ \AA}$ for the β -strand spacing. Although this estimated value is somewhat below that expected considering typical hydrogen-bond topologies in parallel β -sheets, it falls within $\sim 10\%$ of the canonical $\sim 4.7\text{--}4.8 \text{ \AA}$ strand spacings typically observed for this motif. The interstrand spacing for huPrP23-144 amyloid fibrils as determined by fiber X-ray diffraction is $4.65 \text{ \AA}$ (Figure S3). Relatively small underestimation of this value by simulations of TEDOR trajectories is not entirely surprising given that, due to relatively small magnitudes of the ^{15}N - ^{13}C couplings, such simulations depend to some degree on additional parameters such as the effective transverse ^{13}C spin relaxation rate during TEDOR mixing and overall spectral amplitude scaling.²⁰ In this study, we made an attempt to eliminate the dependence of data modeling on these extra parameters by using reference experiments as described in the Supporting Information. However, it should be noted that, using values for the relaxation and scaling parameters that differed somewhat from those determined in the reference experiments, good agreement between experimental and simulated trajectories could also be obtained for intermolecular spacings in the range of $\sim 4.5\text{--}5 \text{ \AA}$.

In conclusion, the wealth of site-resolved solid-state NMR data presented here for fibrils prepared from highly ^{13}C - and ^{15}N -enriched protein molecules establish, rather unequivocally, that the huPrP23-144 β -sheet core displays an overall parallel in-register alignment. While given the ^{15}N - ^{13}C correlations observed in 2D TEDOR spectra it is possible that several residues at the edges of β -strands have geometry that locally deviates from an ideal parallel in-register β -sheet arrangement, our experiments clearly rule out the existence of other topologies such as antiparallel β -sheets and parallel β -sheet motifs displaying a uniform registry shift of one or more residues. A parallel, in register β -structure has been observed for many other amyloidogenic polypeptides.⁹ A similar arrangement has also been found for fibrils formed by full-length PrP and PrP fragment 90–231,^{28,29} even though the residues forming the β -core of the latter amyloids (aa $\sim 160\text{--}220$)²⁸ are different from those encompassing the β -strands in huPrP23-144. The fundamental insights into the intermolecular arrangement of β -strands in human PrP23-144 fibrils gained from the present study provide a stepping stone toward the construction of high-resolution structural models and full understanding of the molecular basis of PrP23-144 amyloid strains and their seeding specificities.

Supplementary Material

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Acknowledgments

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Figure 1.

(A) Partial amino acid sequence of huPrP23-144. Residues exhibiting significant conformational dynamics are shown in red font, and relatively rigid amyloid core residues are shown in black font^{7,8} with the three β -strand segments predicted using TALOS+²⁵ highlighted by gray rectangles. Also shown is a summary of the intermolecular ^{15}N - ^{13}C correlations observed in z -filtered TEDOR spectra recorded for the mixed 1,3- $^{13}\text{C}/^{15}\text{N}$ - and 2- $^{13}\text{C}/^{15}\text{N}$ huPrP23-144 fibril samples (panels (D) and (G) and panel (C) of Figure S1). Thick green rectangles under residue i indicate that an unambiguous intermolecular ^{15}N - ^{13}C correlation was observed between the amide ^{15}N of residue i and $^{13}\text{CO}_{i-1}$ and/or $^{13}\text{CO}_i$ (NCO), $^{13}\text{CO}_{i-1}$ and/or $^{13}\text{CO}_i$ (NCA), and side-chain ^{13}C of residue i (NCX). Narrow green

rectangles correspond to ambiguous intermolecular ^{15}N - ^{13}C correlations. (B–D) ^{15}N - ^{13}CO (NCO) correlation spectra of huPrP23-144 amyloid fibrils recorded at 500 MHz ^1H frequency, 11.111 kHz MAS rate and effective sample temperature of $\sim 5^\circ\text{C}$ using the z -filtered TEDOR pulse scheme²⁰ with ^{15}N - ^{13}C dipolar mixing times, τ_{TEDOR} , indicated in the spectra. The fibril samples were prepared from (B) 1,3- ^{13}C , ^{15}N -huPrP23-144, (C) a mixture of 1,3- ^{13}C , ^{15}N -huPrP23-144 and natural abundance (^{12}C , ^{14}N) huPrP23-144 in a 1:3 molar ratio, and (D) a mixture of 1,3- ^{13}C -huPrP23-144 and ^{15}N -huPrP23-144 in a 1:1 molar ratio. The experiment times for spectra in panels (B–D) were ~ 14 , ~ 27 and ~ 212 h, respectively. (E–G) Similar to panels (B–D), but for ^{15}N - $^{13}\text{C}\alpha$ (NCA) spectra and fibril samples grown from huPrP23-144 prepared using $[2\text{-}^{13}\text{C}]$ instead of $[1,3\text{-}^{13}\text{C}]$ glycerol. The experiment times were ~ 7 , ~ 27 and ~ 216 h, respectively.

Figure 2.

Experimental (points) and simulated (lines) 1D TEDOR trajectories that report simultaneously on all intermolecular (A) ^{15}N - ^{13}CO and (C) ^{15}N - $^{13}\text{C}\alpha$ distances in huPrP23-144 amyloid fibrils. Experimental trajectories in panels (A) and (C) were recorded for the mixed 1,3- $^{13}\text{C}/^{15}\text{N}$ and 2- $^{13}\text{C}/^{15}\text{N}$ fibril samples, respectively, as described in the Supporting Information and the ^{13}CO and $^{13}\text{C}\alpha$ intensities in the plots are scaled by corresponding intensities in CP ^{13}C reference spectra recorded under identical conditions. The total experiment times were ~ 41 h and ~ 68 h, respectively. (B, D) Model spin systems, based on the X-ray structure of amyloid-like microcrystals of the peptide GNNQQNY²⁶ displaying a parallel in-register alignment of the β -strands (PDB ID 2OMM), used to generate the simulated TEDOR trajectories corresponding to experimental data in panels (A) and (C), respectively, within the SPINEVOLUTION software,²⁷ with the atoms included in

the simulations indicated by spheres. The simulated trajectories were generated as described in detail in the Supporting Information, as a function of a single distance parameter, d , corresponding to the spacing (in Å) between adjacent β -strands as indicated in the structural models. The best-fit ^{15}N - $^{13}\text{C}\text{O}$ and ^{15}N - $^{13}\text{C}\alpha$ simulations, corresponding to $d = 4.4$ Å, are shown as solid lines and simulations for d values of $\pm 10\%$ from the best-fit are shown as dashed lines.