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Structured regions of α -synuclein fibrils include the early-onset Parkinson's disease mutation sites

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Abstract

α -Synuclein (AS) fibrils are the major component of Lewy bodies, the pathological hallmark of Parkinson's disease (PD). Here, we use results from an extensive investigation employing solid-state NMR to present a detailed structural characterization and conformational dynamics quantification of full-length AS fibrils. Our results show that the core extends with a repeated structural motif. This result disagrees with the previously proposed fold of AS fibrils obtained with limited solid-state NMR data. Additionally, our results demonstrate that the three single point mutations associated with early-onset PD—A30P, E46K and A53T—are located in structured regions. We find that E46K and A53T mutations, located in rigid β -strands of the wild-type fibrils, are associated with major and minor structural perturbations, respectively.

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Supplementary Data

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Keywords

Lewy bodies; magic-angle spinning; solid-state NMR; conformational dynamics; structural perturbations

Introduction

Parkinson's disease (PD) is the most common movement disorder, affecting approximately 1–2% of the population over age 65.¹ PD is characterized by the presence of Lewy bodies (LBs),² which are intracytoplasmic aggregates composed largely of fibrillar α -synuclein (AS).³ AS is a —natively unfolded protein in solution,⁴ whose function is not well understood. AS forms α -helices throughout the N-terminal domain when exposed to phospholipids, detergents or membrane vesicles⁵ and can convert to insoluble fibrils with β -sheet secondary structure *in vitro*;⁶ these fibrils have been shown to have a morphology similar to that found in LBs.^{6,7} Additionally, three single missense mutations (A30P, E46K, A53T) in the AS gene.^{8–10} and multiplication of the wild-type (WT) AS allele.^{11,12} have been identified in early-onset PD. More recent studies have demonstrated that exogenous fibrils can seed LB-like inclusions in cell culture.¹³ It was also recently proposed that AS pathology can be propagated by direct neuron-to-neuron transmission of AS aggregates *in vivo*.¹⁴ Together, these findings implicate AS misfolding in both sporadic and familial PD. However, why and how these structural changes occur in the protein is not yet understood.

Early structural studies of AS fibrils supported the existence of a central β -sheet core, but did not provide detailed information on the extent of the structured regions and the topology.^{6,15} High-resolution magic-angle spinning (MAS) solid-state nuclear magnetic resonance (SSNMR) spectroscopy provides site-specific structural information on amyloidogenic peptides and proteins.^{16–26} These molecules are inaccessible to other structural determination techniques, such as solution NMR, because they are not soluble, or X-ray diffraction, because they do not form crystals. Previous SSNMR studies of AS fibrils—from our laboratory and others—reported substantial numbers of resonance assignments, but were lacking key stretches of amino acid residues such as the region between residues 43 to 49 where the single point mutation E46K is located.^{27–31} Some of these previous studies attributed the missing signals to their potential localization within dynamic loops,^{27–31} and proposed a possible fold of AS fibrils on the basis of these assumptions. None reported the overall high-resolution fold of the protein, detailed side chain conformations, hydrogen bonding patterns or packing arrangements.

Here we show that state-of-the-art SSNMR experiments, combined with improved sample preparation and labeling schemes, have enabled us to detect many of the previously unobserved residues and to perform detailed analysis of the sidechains, as well as the backbone. Although some of these newly-assigned residues previously were identified, but not assigned, at low temperature with fully hydrated, frozen fibrils,²⁸ they were not detected in previous SSNMR studies at ambient conditions due to conformational dynamics of prior sample preparations. To understand the origin of the missing peaks in previous studies, here we present comprehensive studies, including high sensitivity dipolar correlation experiments, scalar-coupling mediated correlation spectroscopy, site-specific measurements of the dipolar order parameters and relaxation rates and a systematic comparison of signal intensities. These results reveal that many of these critical signals, which previously escaped detection, are located in β -sheet strands that impact the fibril quaternary structure, in disagreement with the previously proposed fold of the fibrils.³¹ Additionally, our results demonstrate that the three early-onset PD mutation sites are located in structured regions of the fibrils, with E46 and A53 located in rigid β -strands of the WT fibrils and A30 in a

random coil region. These results are in agreement with the major and minor perturbations observed upon E46K and A53T mutation, respectively, and the relative lack of perturbation for A30P.

Results

Comprehensive chemical shift assignments demonstrate secondary structure details of the AS fibrillar core

AS fibrils were prepared as previously described²⁹ and identical spectra (Figure 1) were obtained from several independent sample batches ($|\Delta\delta|_{\text{avg}} < 0.2$ ppm). Previous SSNMR studies²⁷ reported differences in the fibril morphology that were attributed to minor variations in the fibrillation conditions.³² Our results indicate high reproducibility of the sample preparation, with or without seeding with prior fibril generations (Figure 1c), an essential requirement for using different labeling schemes and performing structural comparisons between the WT and mutants. As previously demonstrated, regardless of sample hydration, portions of the core chemical shift assignments were conserved, with the exception of residues located in loops such as S87 and I88.²⁹ We subsequently determined the level of optimal hydration to maximize overall spectral sensitivity and resolution to 36% water by mass (Figure 1, see Materials and Methods).

The resulting two-dimensional (2D) spectra are well resolved (<0.2 ppm inhomogeneous broadening); however, due to the highly repetitive AS sequence, 3D spectra are essential to perform *de novo* chemical shift assignments. Thus, we collected extensive sets of spectra to assign the ^{13}C and ^{15}N chemical shifts unambiguously. Figure 2a illustrates a strip plot of the 3D NCACX, NCOX and CAN(CO)CX with 50 ms and CON(CA)CX with 10 ms dipolar assisted rotational resonance (DARR) mixing times on uniform (U)- ^{13}C , ^{15}N AS fibrils to demonstrate the sequential backbone connectivity for residues 89 to 95. A backbone walk with these 3D experiments was conducted for residues 44 to 96, leading to a high degree of assignment confidence for the majority of fibril core signals (residues 38 to 96). To complement the 3D spectra, where peak intensities varied by a factor of five or more among residue types, we performed higher sensitivity 2D experiments with longer mixing times (e.g., 250 ms) to confirm inter-residue correlations (Figures 2b and 2c). In addition to the U- ^{13}C , ^{15}N -labeled samples, sparse ^{13}C isotopic labeling patterns were also utilized to acquire higher quality spectra.^{33,34} The samples included: (a) U- ^{15}N AS fibrils with 1,3- ^{13}C glycerol and (b) U- ^{15}N AS fibrils with 2- ^{13}C glycerol. In these samples, most of the one-bond ^{13}C - ^{13}C J -couplings were removed, which increased the sensitivity and allowed the detection of additional residues. Data acquired on these samples enabled the detection of 15 additional residues using cross-polarization (CP)-dipolar experiments, such as residues 33 or 106, as shown by the ^{13}C - ^{13}C 2D correlation experiment with DARR mixing in Figure 2c, such as residues 33 or 106. Further 3D spectra, in which we employed SPC-5₃ mixing³⁵ for targeted polarization transfers with maximal sensitivity, confirmed the number of unique signal patterns by residue type for the U- ^{13}C , ^{15}N labeled samples (Figure 3a). 3D experiments were also acquired on the sparsely labeled samples as described in Figures 3b and 3c, as well as the ZF-TEDOR (Z-filtered transferred-echo double-resonance) experiment³⁶ to assist in the chemical shift assignments of Lys sidechain signals, Gly-Ala residue pairs and Thr N-CB correlations outside the core. Finally, spectra collected at 900 MHz ^1H frequency (Figure 2b) offered superior resolution³⁷ to confirm and complete the assignments. Together, this extensive set of NMR data facilitated assignments of 91% of ^{15}N and ^{13}C resonances (95% of the backbone and 86% of the side chains) of the detected residues between 38 and 96. Additionally, using dipolar-based experiments, we were also able to assign residues in small stretches outside the core, such as E105 to A107 or G111 to I112, and residues present only in the N- or the C-terminus, such as T or Y, using dipolar-based experiments. This suggests the presence of locally structured regions in both termini.

Highly confident resonance assignments directly yield substantial structural information. Empirical prediction of the backbone torsion angles Φ and Ψ from residues 30 to 110 of AS fibrils was obtained by using the ^{13}C (CA, CB and CO) and ^{15}N chemical shift assignments and the TALOS+ program.³⁸ Figure 4 shows a plot of (a) the dihedral angles and (b) the predicted order parameters (S^2) reported by TALOS+. TALOS+ provides a prediction of the secondary structure that demonstrates the presence of a repeated motif in the core of AS fibrils from residues 38 to 66 and 68 to 96 (Figure 4d). Taken together, these secondary chemical shift analysis results indicate a repeated motif including a long β -strand (of ~12 residues, L38 to V49) and two short β -strands (of ~4 residues each, V52–V55 and V63–V66) separated by a loop of several residues; the motif repeats with a 12-residue β -strands (G68–Q79) followed by two short β -strands (T81–G84 and A90–K96). These results demonstrate differences in the secondary structure that do not agree with a proposed fold of AS.³¹ Additionally, our results show that the three single point mutation sites, including A30 and E46, which had not been detected in previous NMR studies,²⁷ are located in structured regions of the fibrils.

Chemical shifts also report directly on sidechain conformation preferences, most notably for Val methyl groups.³⁹ Whereas previous studies²⁷ of AS did not report distinguishable differences among the Val methyl assignments, the present report demonstrates distinctive chemical shifts for many Val methyl groups. The likely conformations of V48, V49 and V82 is p ($\chi_1 \sim +60^\circ$), V55, V74 and V95 is t ($\chi_1 \sim +180^\circ$), and V71 and V77 is m ($\chi_1 \sim -60^\circ$). Although these are not high-resolution dihedral restraints, the chemical shifts are consistent with the presence of well-ordered side chains. In contrast, the observation of a single methyl frequency each for V40, V52 and V70 indicates a lack of strong conformational preference. We attribute these variations to sidechain packing (or lack thereof) in the fibril core.

Conformational dynamics of the fibril backbone

Giasson *et al.* demonstrated that residues 71 to 82 are necessary for AS fibril assembly,⁴⁰ and therefore it is not surprising that these rigid residues yield some of the strongest SSNMR signals. We confirmed this in a site-specific manner by performing a 3D CANCO experiment and by normalizing the cross peak intensities (Figure 4c) to the strongest signal (V77CA-V77N-A76C). The intensities in the CANCO report qualitatively on dynamic properties of the fibril, with the majority of cross peaks uniquely resolved. An advantage of the CANCO experiment, relative to NCACX or NCOCX experiments involving DARR mixing, is that peak intensities depend in the CANCO primarily upon the (T_2 and $T_{1\rho}$) relaxation rates of the spins on which polarization resides during the chemical shift evolution and CP periods. In NCACX (or NCOCX) experiments, the peak intensities depend to a greater extent on the residue type, due to large variations in numbers of sidechain ^{13}C sites and chemical shift patterns. All but a few residues from T64 to K96 have ~50% or greater intensity on this scale. The newly identified β -strand from L38 to V49 has a much weaker signature, and peripheral regions (residues less than 38 and greater than 96) are not observed in this experimentally demanding dipolar 3D experiment.

As previously demonstrated for the Y145Stop prion protein,⁴¹ such variations in intensity can arise despite a lack of large-scale backbone motions. For AS, we investigated this hypothesis further by measuring site-resolved ^1H - ^{15}N and ^1H - ^{13}C dipolar couplings, where the time-averaged coupling approximates the order parameter ($\langle S \rangle$), using the recoupling pulse sequence R18₁⁷ (Ref. 42). The ^1HN - ^{15}N (Figure 5a) and ^1HA - ^{13}C (Figure 5b) dipolar trajectories for representative residues provide direct measurements that elaborate upon, but agree well with, the TALOS+ predictions (Figure 4a). For example, S42, A89 and F94 all have near rigid-lattice limit dipolar couplings for both backbone sites ($\langle S \rangle$ greater than 0.94). T59 has only a very slight increase in motion ($\langle S \rangle \sim 0.92$ on average), despite being within a loop, and G67 likewise shows no significant averaging despite a

distinct backbone conformation. On the other hand, S87 displays an average $\langle S \rangle$ value of 0.88 ± 0.02 , consistent with the TALOS+ value for S^2 of ~ 0.75 .

Although these small variations in average coupling ($\sim 10\%$) are experimentally significant, they fail to account for the large differences in peak intensities in the 3D spectra, which can range up to a factor of 10 for individual cross peaks. Therefore we next considered the rotating frame relaxation rates as a potential source of signal intensity variation, by measuring site-resolved $^1\text{H}^{\text{N}}$ R1 ρ values (Figure 5c). Under the conditions examined, the majority of core residues have relaxation rates less than 90 s^{-1} , and the rates coincide well with the peak intensities observed in the CANCO spectrum; for example, V77 has a small measured relaxation rate (V77, $70 \pm 4 \text{ s}^{-1}$), similar to other residues that were not previously assigned such as Y39 or S42 (Y39, $63 \pm 10 \text{ s}^{-1}$; S42, $75 \pm 5 \text{ s}^{-1}$). In contrast, several residues with depressed signal intensities have higher rates (A53, $115 \pm 15 \text{ s}^{-1}$; T59, $122 \pm 10 \text{ s}^{-1}$; G73, $118 \pm 15 \text{ s}^{-1}$). Therefore, the dispersion in relaxation rates appears to be responsible for the large variations in spectral intensities previously reported for other systems.⁴¹ We postulate that these increased rates may be due to small amplitudes, microsecond-to-millisecond timescale motions.

For faster and/or larger amplitude motions, dipolar methods failed to detect the signals at all, in particular for residues in the terminal domains. Therefore we implemented J -based transfer experiments (Figures 6). Constant-time uniform-sign cross-peak (CTUC) correlation spectroscopy (COSY) and the CACO IPAP (in-phase anti-phase)^{43,44} experiments resolved regions of the spectra that were not resolved or observed at all in dipolar spectra (Figures 6a and 6b). We attempted to perform the COSY experiment with an initial INEPT ^1H - ^{13}C transfer⁴⁵ but found the sensitivity to be inadequate. Therefore, we modified the J -based experiments to include ^{13}C direct polarization (DP), instead of ^1H - ^{13}C cross polarization (CP), and low power ^1H SPINAL decoupling (60 kHz), which served as a ^{13}C T_2 filter to dephase the most rigid regions, resulting in much stronger relative peak intensities for the mobile residues (Figure 6c). These results are in agreement with the differences in intensity in the CP-dipolar experiments (Figure 4c), as evidenced by the presence of mobile residues such as T64, S87 and I88 (Figure 6). Additional residues not previously detected in the CP-dipolar based experiments, and therefore more dynamic, were also detected. These residues included several Ala with random coil chemical shifts, as well as Val, Glu/Gln, Asp/Asn, Gly and Lys, suggesting that both the C- and the N-termini have locally structured regions but are more dynamic than the core (Figure 6d).

Structural perturbations found in the fibrillar core upon mutation of two single point mutants located in the β -strand regions

In addition to the multiplication of the WT AS allele,^{11,12} three single point mutations (A30P, E46K and A53T) in the AS gene have been identified in familial early-onset PD.^{8–10} Our results demonstrate that residues E46 and A53 are both located in the middle of rigid β -strands in the WT fibrils, while A30 is located in a loop region (Figure 4d). To further support these results, we prepared fibrils of the three single-point mutants (A30P, E46K and A53T) following the same procedure of fibril preparation and hydration as for the WT fibrils and acquired ^{13}C - ^{13}C 2D spectra under identical conditions. Multidimensional experiments, equivalent to the subset acquired for the U- ^{13}C , ^{15}N WT at 600 MHz ^1H frequency, were also acquired to obtain *de novo* chemical shift assignments for the three mutants. These experiments indicate substantial alteration to the structure upon mutation, with major perturbations ($|\Delta\delta|_{\text{avg}} \sim 0.3\text{--}2.0 \text{ ppm}$) upon E46K mutation and minor perturbations ($|\Delta\delta|_{\text{avg}} \sim 0.1\text{--}0.9 \text{ ppm}$) upon A53T mutation, as shown in the CC2D expansion of the Thr, Ala or Gly regions (Figure 7). Almost no perturbation was detected for A30P ($|\Delta\delta|_{\text{avg}} < 0.2 \text{ ppm}$, Figure 7), which is located in a loop region. Regardless of these structural changes, a comparison of

electron micrographs of the WT versus the three AS mutant fibrils demonstrates no major changes to the fibril morphology upon mutation (Figure 8).

Discussion

Structured regions of AS fibrils include the early-onset PD mutation sites

Although it has been known for decades that LBs are the characteristic hallmark of PD,² it was not discovered until 1997 that the main protein component of LBs is fibrillar AS.³ AS fibrils have motivated numerous structural studies.^{6,7,15,27–31,46,47} Despite the considerable effort, the structural characterization of AS fibrils at atomic level is still incomplete. MAS SSNMR, a technique uniquely positioned to achieve information on amyloid fibrils at an atomic level, requires first and foremost the determination of confident chemical shift assignments. Previous SSNMR studies of AS fibrils made substantial progress in this regard.^{27–31} Here we have advanced these studies with new combinations of SSNMR experiments, improved sample preparation and sparse labeling schemes, permitting the detection and assignment of numerous residues not previously observed by SSNMR. Nearly identical chemical shifts were obtained for the regions previously detected with dried or fully hydrated fibrils,²⁹ with some local variations such as residues 86 to 88 (Ref. 29) or residues 64 to 67. Our results extend significantly upon previous studies, with the detection of a total of 106 residues versus 62 (Ref. 27) or 59 (Ref. 28). In addition, we have assigned 91% of the ¹⁵N and ¹³C resonances of the detected residues between 38 and 96 residues where others reported ~58% (Ref. 27) or ~48% (Ref. 28). These experiments demonstrate that the three early-onset PD mutation sites are structured, with a β -sheet core that extends from residues 38 to 96 and local random coil regions in the termini, a finding in agreement with previous electron paramagnetic resonance studies of the Langen group where they detected distance distributions of <15 Å between the nitroxide spin labels from residues ~34 to ~101 (ref. 46). Additionally, our results demonstrate that E46 is located in one of the two longest β -strands of the fibril. This disagrees with previous studies that assumed it was located in a loop region, because signals were not detected for E46 or neighboring residues.^{27,31} A53 is located in one of the four short beta-strands and A30 in a loop region near the N-terminus. Signals that correspond to residues 30 to 39 were not assigned by previous SSNMR studies.^{27,31} A comparison of our chemical shifts to those from by Heise *et al.*²⁷ strongly suggests a difference in morphology, possibly as a result of different fibrillation conditions.³²

Secondary structure of AS fibrils includes a repeated β -strand motif

Our results demonstrate the presence of additional structured regions in the fibrillar core of WT AS, which have led to significant differences in the secondary structure from those previously proposed.^{27,31} Vilar *et al.*³¹ proposed a possible structural model of AS fibrils based on electron microscopy (EM) experiments and relatively limited SSNMR data of a fragment of AS. On the basis of hydrogen/deuterium (H/D) exchange NMR experiments, the authors reported the presence of five protected regions that they presumed to be five distinct β -sheet strands. In general, amyloid fibrils present segments in the fibrils core that are well protected from H/D exchanges, regardless of local secondary structure. Other, less protected segments may be beta-strand, but less stable and more well exposed to solvent. Thus, H/D exchange cannot be used to draw definitive structural conclusions. Our results demonstrate the presence of differences in the secondary structure versus previous studies, based on a significantly more extensive investigation with solid-state NMR and complete chemical shift assignments. The secondary structure includes a repeated motif that is composed of a long β -strand followed by two short β -strands located from residues 38 to 66 and again from 68 to 96 (Figure 4d). The length of the long β -strand (~5 nm, 12 residues) is compatible with our

electron micrographs (Figure 1), with the fibril width reported by Vilar *et al.* and also directly observed by EM in LBs from PD patients.^{6,7,48}

Conformational dynamics measurements reveal the rigid regions of AS fibrils

The detection of a total of 106 residues in the sequence that accounts for the residues located in the core and residue types located only in the N-terminus (Thr, Val) or the C-terminus (Pro, Asp, Asn, Tyr) demonstrates the presence of partial structure in both the N- and the C-termini. Previous investigations of the mobile regions of AS fibrils²⁷ encountered problems caused by the presence of AS monomer that the authors identified by comparison to the assignments of AS monomer in solution. We have overcome this problem by washing the fibril samples. In our study, type-specific residue assignments, most notably for unambiguous cases such as Ala or Asp/Asn, demonstrate substantial differences (>0.5 ppm) from solution NMR of the unfolded state (BMRB 16300). Additionally, we detected the mobile regions using *J*-based experiments that agree with the residues located outside the core and the residues detected with the dipolar based experiments using samples with sparse labeling schemes and a 3D NCACB experiment with SPC5₃ mixing. Our investigations indicate the presence of conformational dynamics along the AS fibrillar core and demonstrate that the residues described by Giasson *et al.* to be necessary for AS fibril assembly⁴⁰ are among the strongest solid-state NMR signals and therefore are located in the more rigid regions of the fibril.

The early-onset PD mutants that fibrillate faster than the wild-type have distinctive fibrillar forms

Significant additional insight from our study includes the localization of the early onset mutation sites (A30P, E46K and A53T)^{8–10} to structured regions of WT fibrils. These results suggest a major effect on the fibril structure upon E46K or A53T mutation, both of which are located in β -sheet regions and therefore expected to cause more structural perturbation than A30P. The solution NMR structure of AS in mixed detergent micelles demonstrates that this region may serve as a key conformational switch.⁴⁹ Investigations of phospholipid binding revealed differences in the binding affinity of the mutants compared to the WT that could suggest increased vulnerability to self-aggregation.⁵⁰ Thus the mutation sites may be directly involved in dictating fibrillation. Additionally, our results demonstrate effects of E46 and A53 mutation on the final fibrillar state. In E46K, a negatively charged amino acid, which is located in the middle of a long β -strand, is mutated to a positively charged amino acid that creates a major perturbation of the structure of AS fibrils. In A53T, an Ala located in one of the short β -strands is mutated into a Thr, which generates a local perturbation to the final fibrillar structure. The A30P mutation does not have a significant impact on the fibril structure that we have attributed to A30 being located in a random coil. These results indicate that the two mutants that have faster fibrillation rates (E46K and A53T) compared to the WT protein also have distinctive final fibrillar states compared to WT, while A30P has a slower fibrillation rate but a fibril structure similar to that of the WT.

Materials and Methods

AS protein expression and purification

U-¹³C, ¹⁵N-labeled WT, A30P, E46K and A53T AS were expressed and purified as previously described.⁵¹ U-¹⁵N WT AS with 2-¹³C glycerol and U-¹⁵N WT AS with 1,3-¹³C glycerol AS were prepared with the same protocol, but ¹⁵N ammonium chloride was used as sole nitrogen source and either 2-¹³C-glycerol and calcium ¹³C-carbonate, or 1,3-¹³C-glycerol and natural abundance carbonate as the sole carbon sources, respectively.

Preparation of AS fibrils

Dry (WT, A30P, E46K and A53T) AS fibrils were prepared as previously described.²⁹ For washed samples, the pellet was washed with Milli-Q water, subjected to ultracentrifugation and dried. The dried pellets were packed into 3.2 mm MAS NMR rotors (Varian, Inc., Palo Alto and Walnut Creek, CA and Fort Collins, CO; now part of Agilent Technologies, Santa Clara, CA and Loveland, CO), rehydrated to 36% water by mass and kept hydrated by Kel-F and rubber spacers. Non-seeded AS fibrils were formed in the same conditions but incubated for a total of 4 weeks.

Sample hydration

Previous studies from our group have demonstrated an important effect of the hydration to the resolution and sensitivity of the solid-state NMR spectra of the full-length α -synuclein fibrils.²⁹ Here, we optimized the hydration level of α -synuclein fibrils to maximize the resolution and sensitivity of the solid-state NMR multidimensional experiment, which was essential to complete the chemical shift assignments. To optimize the hydration level, fibrils were dried by N_2 gas until no changes in mass were observed, and a 1H 1D and a ^{13}C - ^{13}C 2D spectra were acquired (Figure 9). The hydration of the sample was gradually increased and a 1H 1D and a ^{13}C - ^{13}C 2D spectra were acquired at each hydration level up to 50 % (mass of water/total mass), as shown in Figure 9.

As reported in Table 1, the pulse width for both for 1H and ^{13}C become longer upon hydration and the overall cross-polarization decreases its efficiency. However, we observe significant improvement in the ^{13}C linewidths and signal to noise of individual peaks (see table below) that we attributed to changes in the timescale dynamics of certain regions of the full-length protein. Average linewidths (LW) $\pm$ standard deviation and normalized signal to noise were obtained by measuring the LW and signal to noise for a total of 20 resolved peaks in ^{13}C - ^{13}C 2D experiments. Conditions marked in bold correspond to the optimal conditions selected for the multidimensional NMR experiments.

Solid-state NMR spectroscopy

MAS SSNMR experiments were performed using a 11.7 Tesla (500 MHz) Varian Infinity Plus spectrometer with a 3.2 mm Varian BalunTM probe, a 14.1 Tesla (600 MHz) Varian Infinity Plus spectrometer with a 3.2 mm T3 Varian 1H - ^{13}C - ^{15}N probe, a 17.6 Tesla (750 MHz) Varian Unity Inova spectrometer with a Varian 3.2 mm BalunTM 1H - ^{13}C - ^{15}N probe and a 21.1 Tesla (900 MHz) Varian Unity Inova spectrometer equipped with a 3.2 mm Varian BioMASTM 1H - ^{13}C - ^{15}N probe. All experiments (unless indicated) were performed with the variable-temperature (VT) gas adjusted to 10 °C, which corresponded to a sample temperature of ~12–17 °C determined by ethylene glycol calibration. All experiments (unless indicated) utilized tangent ramped CP,⁵² TPPM⁵³ or SPINAL 64 (Ref. 54,55) 1H decoupling was applied during acquisition and evolution periods with a field strength of ~80 kHz (unless indicated). For 3D ^{15}N - ^{13}C - ^{13}C and ^{13}C - ^{15}N - ^{13}C correlation experiments, band-selective SPECIFIC CP⁵⁶ was used for polarization transfer between ^{15}N and ^{13}C and DARR⁵⁷ or SPC5₃ (Ref. 35) mixing were used for ^{13}C - ^{13}C polarization transfer. Chemical shifts were referenced externally with adamantane (assuming the downfield peak 40.48 ppm).⁵⁸

Electron Microscopy

AS fibrils were applied to formvar carbon coated grids (300 mesh), fixated with Karnovsky's fixative, negatively stained with 2 % ammonium molybdate (W/v), and viewed in a Hitachi H600 Transmission Electron Microscope (TEM), operating at 75 kV.

Data processing and simulations

All spectra were processed with nmrPipe⁵⁹ with back linear prediction and polynomial baseline correction in the direct dimension. Zero filling and Lorentzian-to-Gaussian apodization and/or cosine bells were applied for each dimension before Fourier transformation. Peak picking, assignments and peak height measurements were performed in Sparky.⁶⁰ Extraction of the evolution curves (t_2) from the 3D MAS solid-state NMR experiments was conducted by using the autoFit.tcl package in nmrPipe,⁵⁹ after having picked the peaks in the first ^{15}N - ^{13}C (F1–F3) plane. Dipolar coupling trajectories were fit to exact spin simulations by using in-house FORTRAN-77 code that called both external MINUIT⁶¹ minimization libraries and the SPINEVOLUTION⁶² simulation package.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

AS	α -synuclein
CP-MAS	cross polarization magic-angle spinning
DARR	dipolar assisted rotational resonance
LB	Lewy body
MAS	magic-angle spinning
PD	Parkinson's disease
SSNMR	solid-state nuclear magnetic resonance
ZF-TEDOR	Z-Filtered Transferred Echo Double Resonance

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Figure 1.

Microscopically ordered AS fibrils. (a) ^{13}C - ^{13}C 2D spectrum of U- ^{13}C , ^{15}N AS fibrils with 36% water by mass (50 ms DARR mixing, 750 MHz ^1H frequency and 16.7 kHz MAS rate). (b) Transmission electron micrographs of AS fibrils. (c) Correlations of ^{13}C CA, ^{13}C CB and $^{13}\text{C}'$ AS chemical shifts (CS) from independent sample preparation batches (above) seeded with unlabeled fibrils and (below) unseeded.

Figure 2.

Chemical shift assignments of WT AS fibrils using of U- ^{13}C , ^{15}N and U- ^{15}N , ^{13}C sparsely labeled samples. (a) Strip plot from the 3D NCACX, NCOCX, CANcoCX and CONaCX spectra of U- ^{13}C , ^{15}N AS fibrils showing the sequential backbone connectivity for residues 89 to 95. All spectra were acquired at 600 MHz (^1H frequency) and 13.3 kHz MAS rate. (b) Overlay of ^{13}C - ^{13}C 2D spectra expansions with 50 ms (black) and 250 ms (red) DARR mixing of U- ^{13}C , ^{15}N AS fibrils. Intra-residue (black labels) and inter-residue correlations (gray labels) were used to confirm assignments. (c) Expansion of a ^{13}C - ^{13}C 2D with 250 ms DARR mixing time on U- ^{15}N AS fibrils with 2- ^{13}C glycerol. Black peak labels indicate additional correlations that could be assigned with sparsely labeled samples. Spectra (b) and (c) were acquired at 900 MHz ^1H frequency and 13.3 kHz MAS rate.

Figure 3.

Additional experiments to complete the chemical shift assignments of WT AS fibrils (a) Strip plots of the NCACB with 2.4 ms SPC5₃ mixing at 10.0 kHz MAS rate. (b) CANCO plane of the U-¹⁵N AS fibrils with 2-¹³C glycerol (above) and U-¹⁵N AS fibrils with 1,3-¹³C glycerol (below), showing the complementary labeling pattern to confirm sequential assignments. (c) Planes of the NCACX (250 and 500 ms DARR) on the 2-¹³C-glycerol sample, showing some of the new residues detected (above) and correlations to neighbor residues (below). All spectra were acquired at 600 MHz (¹H frequency) and 13.3 kHz MAS rate (unless indicated).

Figure 4.

Secondary structure of the AS fibrils. (a) TALOS+ predicted backbone dihedral angles ϕ (squares) and ψ (circles) as a function of residue number. Error bars are based on the 10 best TALOS+ database matches. (b) TALOS+ predicted order parameters S^2 . (c) Relative peak heights from the cross peaks of the 3D CANCO spectrum of $U\text{-}^{13}\text{C}, ^{15}\text{N}$ AS fibrils (numbered according to the carbonyl residue). Residues labeled as (#) correspond to residues with overlapped peaks in the CANCO experiment. Error bars correspond to the noise level. (d) Representation of the secondary structure of AS fibrils based on TALOS+ analysis (arrows indicate β -strands; curved lines are turn or loop regions; dashed lines indicated no prediction).

Figure 5.

Site-specific measurements of the AS fibril core dynamics. (a) $^1\text{HN}-^{15}\text{N}$ and (b) $^1\text{HA}-^{13}\text{CA}$ dipolar order parameters and trajectories. (c) ^1HN $R_{1\rho}$ (s $^{-1}$) relaxation rates for selected residues.

Figure 6.

Detection of the mobile regions of AS fibrils. Comparison of (a) CC 2D with initial cross-polarization (CP) to detect rigid regions (750 MHz), (b) IPAP (in-phase anti-phase) CACO and CTUC COSY with initial CP^{44,45} to detect rigid and mobile regions (500 MHz) and (c) IPAP CACO and CTUC COSY with initial DP (direct polarization), low power decoupling and variable-temperature gas temperature set to 30 °C to detect mobile regions (600 MHz). (d) Expansion of the Ala region from the CP-CC 2D (left), CP-CTUC COSY (center) and DP-CTUC COSY (right) spectra. Black labels show residues not detected in the multidimensional dipolar-based experiments, and gray labels show residues assigned with multidimensional dipolar-based experiments. AS sequence (gray residues in the termini with the local structure are underlined with a solid line and residue types only present in one side of the termini underlined with a dashed line).

Figure 7.

Comparison of ^{13}C - ^{13}C 2D spectral expansions (50 ms DARR mixing, 600 MHz ^1H frequency and 13.3 kHz MAS rate) for the wild-type AS fibrils and the three early-onset PD mutants (A30P, E46K and A53T): (a) Thr CA-CG2, (b) Ala CA-CB and (c) Gly CO-CA. Chemical shift perturbations for the mutants versus the WT for selected residues in each area are plotted in the left. Chemical shift assignments of the mutants were obtained with *de novo* sequential backbone walk using 3D experiments as conducted for the WT.

Figure 8.
Comparison of the electron micrographs of (a) wild-type AS fibrils versus the early-onset PD mutants: (b) A30P, (c) E46K and (d) A53T.

Figure 9.

Optimizing hydration of α -synuclein fibril samples for the solid-state NMR experiments (mass of water/total mass). (a) ^1H 1D spectra of U- ^{13}C , ^{15}N AS fibrils at different hydration levels (dry, 16%, 26%, 36%, 44% and 50%) and (b) ^{13}C - ^{13}C 2D spectra with 50 ms DARR mixing. All spectra were acquired under identical conditions at 600 MHz (^1H frequency) and 13.3 kHz MAS rate. (*) Dry fibrils correspond to fibrils dried by N_2 gas until no changes in mass were observed, as previously described.²⁹ Spectra marked in blue correspond to the optimal conditions selected for the multidimensional NMR experiments.

Comparison of ¹H and ¹³C pulse widths (PW), CP enhancement and ¹³C linewidths (Hz) at different percentages of hydration (mass of water/total mass) for U-¹⁵N, ¹³C WT AS fibrils.

Table 1

Hydration (water by mass)	¹³ C PW (μs)	¹ H PW (μs)	CP enhancement	Average ¹³ C LW (Hz)	Normalized signal to noise
Dry fibrils (*)	1.90±0.05	2.00±0.05	1.91±0.07	141±44	0.76±0.06
16%	1.90±0.05	2.05±0.05	1.73±0.06	125±45	0.82±0.05
28%	1.90±0.05	2.05±0.05	1.70±0.06	104±45	0.84±0.05
36%	1.95±0.05	2.15±0.05	1.57±0.06	88±44	0.90±0.05
44%	1.95±0.05	2.15±0.05	1.40±0.06	95±42	1.00±0.04
50%	1.95±0.05	2.20±0.05	1.31±0.07	93±45	0.94±0.04

* Dry fibrils correspond to fibrils dried by N2 gas until no changes in mass were observed, as previously described. 29 Average LW±standard deviation were obtained by measuring the LW for a total of 20 resolved peaks in ¹³C-¹³C 2D experiments at 600-MHz (1H frequency) and 13.3-kHz MAS. Conditions marked in bold correspond to the optimal conditions selected for the multidimensional NMR experiments.