A COMPARISON OF RESULTANT FIBRILS UPON ENVIRONMENTAL AND SEQUENTIAL MANIPULATIONS OF α-SYNUCLEIN, A PARKINSON’S DISEASE ASSOCIATED PROTEIN

BY

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DISSESSATION

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ABSTRACT

α-Synuclein (AS) fibrils are the main component of Lewy bodies, filamentous inclusions known as the pathological hallmark of Parkinson’s disease (PD). The normal function, toxic state and detailed fibrillation pathway of AS are not well understood. However, AS is further implicated in PD by the presence of three single point mutations—A53T, A30P and E46K—and allele duplication or triplication in the familial form. One unifying difference between the single point mutations is their relative fibrillation rates; while E46K and A53T fibrillate more quickly, A30P fibrillates more slowly relative to the wild-type. Other factors that dictate the fibrillation rate of AS include: protein and salt concentration, agitation, temperature and pH. Therefore, carefully controlling fibrillation conditions is essential for making fair comparisons between wild-type AS fibrils and any sequential or environmental manipulations. This allows for the distinction between significant perturbations and slight differences from one sample batch to the next. Environmental manipulations of interest include: (1) the addition of metals and (2) a decrease of pH. Although there is no direct evidence linking metals to neurodegenerative diseases, evidence of elevated levels of metals are found in healthy aging brain and in brain of patients with neurodegenerative diseases. In addition, mitochondrial deficiencies and oxidative stress caused by cytosolic acidification have been associated with several PD cases. Several efforts have been made to identify the effects of these sequential and environmental manipulations; however, the techniques used were of low resolution or poor quality. Here, we have used the well-established, high-resolution methodologies of solid-state NMR to investigate
the anomalies and differences between standard wild-type AS fibrils and those that have been sequentially or environmentally manipulated.
Para mi mamá y querido esposo
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ABBREVIATIONS

PD: Parkinson’s disease
AS: α-synuclein
WT: wild-type
SSNMR: magic-angle spinning solid-state NMR
DSS: 4,4-dimethyl-4-silapentane-1-sulfonic acid
DARR: Dipolar Assisted Rotational Resonance
DP: direct polarization
CP: cross polarization
CS: chemical shifts
PW: pulse widths
LW: linewidths
HSQC: Heteronuclear single-quantum correlation spectroscopy
BMRB: Biological Magnetic Resonance Data Bank
CTUC: constant-time uniform-sign cross-peak
COSY: correlation spectroscopy
CHAPTER 1

INTRODUCTION

1.1 Connection between α-synuclein and neurodegenerative diseases

Parkinson’s disease (PD), multiple system atrophy and many other neurodegenerative diseases have similar pathologies that manifest as filamentous inclusions termed Lewy bodies (Figure 1.1) (1). The principal proteinaceous component of Lewy bodies is aggregated α-synuclein (AS), a 140 amino acid protein arranged in an unknown fibrillar structure (2). The normal functions of AS are also unknown, but AS shares conserved features with the exchangeable apolipoproteins (3); in addition, several lines of evidence suggest a role for AS in presynaptic vesicle trafficking (4-6). The toxic state of AS, whether the protofibrils or the mature fibrils, and how this fibrillation mechanism relates to PD is an ongoing debate (7-11). The detailed mechanism of how AS fibrils assemble from monomers through protofibrils to mature fibrils is also poorly understood (Figure 1.1) (12). Investigating the structure of AS fibrils and the effect of sequential and environmental manipulations on the structure in great detail may further illuminate its role in PD pathology. In addition, this knowledge will lead to rational development of diagnostics and therapies for PD.

1.2 Propensity to self-associate

Several sequential characteristics are predicted to influence fibril formation. One of these characteristics includes the appearance of consecutive amino acids containing hydrophobic side-chains. Evidence of this was shown to increase aggregation upon mutating to more hydrophobic
amino acids of nucleation sites (13-15). Correspondingly, mutating to hydrophilic amino acids decreases the rate of aggregation. The region believed to be essential for the aggregation in AS is said to be 71-82 (16). This stretch is located inside the proposed fibril core 30-98 (17-20) and is also a part of the sixth characteristic KTKEGV imperfect repeat found in AS (Figure 1.2). AS contains seven of these KTKEGV imperfect repeats and after mutation of the sixth repeat (69AVVTG74V to KTKEGV), the rate of fibrillation decreases (21, 22). Finally, AS possesses a highly negatively charged C-terminus (Figure 1.2), which upon neutralization or truncation increases the rate of AS fibrillation (23, 24). Similarly, in other aggregation systems, a high net charge inhibits the propensity to aggregate (14, 25). Analogously, the C-terminus of AS is proposed to inhibit aggregation by long-range electrostatic interactions between itself, the N-terminus and the fibril core (26-28). Furthermore, mutations, A30P and A53T are reported to disrupt these long-range interactions (29). However, in a separate investigation by solution NMR, the mutant E46K increases the affinity of this interaction, while mutants A30P and A53T have no effect (30). Initial structural studies of AS fibrils support the existence of a central β-sheet core, but did not provide detailed information on the extent of the structured regions nor the topology (7, 31). Further investigation into the long-range interactions and the wild-type (WT) structure will provide a fundamental understanding regarding the mechanism of AS fibrillation. The recent chemical shift assignments reported for full-length WT AS fibrils (32) is the first step in solving the high-resolution structure of WT AS fibrils.

1.3 Early onset Parkinson’s disease mutants

While PD is typically sporadic, several mutations in the SNCA gene encoding AS are associated with familial PD, including single point mutations—A53T (33), A30P (34) and E46K
— and allele duplication (36) or triplication (37). Although the PD-associated AS mutations are extremely rare, analysis of their pathogenicity could significantly illuminate the mechanisms underlying sporadic disease. Like the wild-type (WT) protein expressed in *E. coli*, all mutant forms of AS are intrinsically unfolded in aqueous solution, but adopt an α-helical secondary structure within their *N*-termini upon binding to phospholipid vesicles or detergent micelles (38, 39). Within A30P, the helical domain is partially disrupted by proline substitution, with a consequential decrease in lipid affinity (38), while the A53T and E46K mutants exhibit similar or enhanced lipid binding when compared to WT AS (38, 40). Thus, altered lipid affinity is not a unifying phenotype for disease-associated mutants.

While each of the three point mutations A53T (33), A30P(34) and E46K (35) cause early onset PD (Figure 1.1), each mutant has different properties related to fibrillation kinetics and fibril structure. A53T exhibits a faster fibrillation rate (40-44) and promotes more oligomeric species (42, 45, 46). In addition, A30P promotes more oligomeric species (42, 45, 46) and overall aggregation (47) and has a slower fibrillation rate relative to the WT (41). Conversely, E46K has a shorter lag phase, which increases the fibrillation rate (40-44). However, E46K does not promote the oligomeric species (30). On the other hand, A30P AS has been reported to fibrillate more slowly than WT AS *in vitro* (42) and to preferentially populate a soluble, protofibrillar intermediate, whereas WT readily progresses to mature, insoluble fibrils (46). This has fueled speculation that the mechanism of A30P toxicity may be fundamentally different than WT AS. Only recently has tissue from a familial PD patient with the A30P mutation become available for analysis. This individual displayed neuropathology typical of idiopathic PD, but
with a greater-than-typical load of insoluble fibrillar aggregates (48). This is a surprising result that strongly implicates fibrillar AS in the pathogenesis of A30P-dependent PD.

Although the A30 and E46 positions are conserved across a variety of species, the A53 position is not. A threonine is present instead of an alanine at position 53 for non-primate mammals along with six other substitutions: S87N, L100M, N103G, A107Y, D1221G and N122S (33). Elevated neurodegenerative effects are displayed in mice expressing human A53T AS (49-55); this suggests that these additional substitutions may add a complementary physiological effect to A53T. Similarly, mice expressing human E46K AS form inclusions closely resembling those of human Lewy bodies (56). These results further support the relationship between AS fibrils and PD pathology.

Regardless of their relative fibrillation rates (7, 46, 57), the mutants and WT AS fibrils have similar morphologies when viewed by low resolution techniques, such as electron microscopy (58). Heise et al. also reported an extended β-sheet core in A53T AS fibrils (59). However, this study reported problems in reproducing samples that yielded different fibril polymorphs between different batches of WT sample preparations (18, 59). Therefore, the irreproducible sample preparations make it difficult to obtain an accurate structural comparison between the WT and A53T mutant. Recent solution NMR studies of quenched hydrogen/deuterium (H/D) exchange have suggested that the A30P mutation does not perturb the location or arrangement of β-strands in WT AS fibrils (60). However, it is not possible to draw site-specific conclusions regarding the structure from H/D exchange experiments alone; for example, some β-sheet regions may be more exposed than others or protected regions may not exhibit a β-sheet secondary structure. In addition, these indirect measurements rely on low molecular weight samples, which require
fibrils to be broken down into smaller units because of their high molecular weight. Therefore, it is essential to achieve reproducible fibrils preparations in the solid-state in order to investigate the anomalies and differences between the PD-related mutants and WT fibrils.

1.4 Significance of low pH fibrils

Mitochondrial deficiencies (61-63) and oxidative stress caused by cytosolic acidification have been associated with several PD cases (64). In addition, lowering pH causes the rate of AS fibrillation to increase (65, 66). The accelerated fibrillation rate may be caused by a neutralization of the highly negatively charged C-terminus of AS (Figure 1.2), (23, 24). This is consistent with other aggregation systems where a high net charge inhibits the propensity of aggregation (14, 25). However, there has yet to be a detailed investigation to validate this hypothesis. The isolation of differences between physiological and low pH AS fibrils will provide insight into the driving force of fibril stability.

1.5 Metal-binding association to α-synuclein

Although there is no direct evidence linking metals to neurodegenerative diseases, evidence of elevated levels of metals, such as Fe$^{+3}$ (67, 68), Zn$^{+2}$ (69) and Cu$^{+2}$ (70), are found in healthy aging brain and in brain of patients with neurodegenerative diseases (71). In addition, the rate of AS fibrillation increases in the presence of certain metal ions, such as Cu$^{+2}$ and Fe$^{+3}$ (72, 73). In particular, Cu$^{+2}$ has a significantly high binding affinity to AS (73). It is proposed that the induction of nucleation by various metals, including Cu$^{+2}$, has the effect of neutralizing the repulsion of negatively charged amino acids primarily found in the C-terminus, which causes the unstructured protein to collapse (72, 74). However, a truncation of the C-terminus showed high
affinity Cu\(^{+2}\) binding in the \(N\)-terminus (residues 3-9 and 49-52, containing H50) in solution (73). Further evidence has indicated that each Cu\(^{+2}\) binding-site is independent with no effect on Cu\(^{+2}\) binding affinity upon mutation of residue 50 from His to Ala (75). Currently, bound and unbound AS monomer to Cu\(^{+2}\) is established to have similar structures through electron paramagnetic resonance, circular dichroism and solution NMR (72, 76). Conversely, the fibril form of AS was observed to be macroscopically different in the presence of metals, as observed by transmission electron microscopy and far-UV circular dichroism (72, 77). Nevertheless, these techniques lack the ability to evaluate site-specific structural differences between AS fibrils bound and unbound to Cu\(^{+2}\). Identifying the region where metals cause conformational changes to the fibril structure may help reveal the role metals play in neurodegenerative diseases, such as PD.

1.6 Magic-angle spinning solid-state NMR

Protein fibrils, such as AS, are insoluble, which precludes formation of high quality crystals. Hence, commonly used structure determination techniques cannot be used to obtain atomic level structure information of these systems. However, magic-angle spinning (78, 79) solid-state NMR (SSNMR) (80-90) is a well-established technique for investigating the structure, dynamics and function of a wide variety of biological systems, regardless of molecular weight, solubility and ability to form crystals without altering sample integrity. The SSNMR community has made significant progress in the study and understanding of amyloid fibrils (91, 92), such as \(\beta\)-2-microglobulin (93, 94), Het-s(218-298) (95-100) and Transthyretin(105-115) (101, 102). There are SSNMR methods established to study protein-protein interactions through chemical shift perturbations (103), as well as to investigate structure-based drug design (103-106) and
interactions between proteins and a membrane surface (107, 108). These advances and the current progress in the Rienstra group towards solving the structure of WT AS by SSNMR (32, 89, 109, 110) has helped systematically identify site-specific changes in secondary structure upon sequential and environmental manipulations of AS fibrils.

### 1.7 References


1.8 Figures

**Figure 1.1** General schematic of fibril formation of amyloid fibrils. Lewy body immunostained for AS fibrils. Figure adapted from Dobson, C.M. Nature (2003) 426, 884-890.
**Figure 1.2** Pictorial depiction of the amino acid sequence of AS, natively unfolded and linear for clarity. Highlighted in red are the three pathogenic mutants A30P, A53T and E46K. In yellow, the GxxxG motif is highlighted and the negatively charged amino acids of the C-terminus in magenta. The sixth characteristic KTKEGV imperfect repeats are highlighted in dark blue.
CHAPTER 2

MATERIALS AND METHODS

2.1 Notes and acknowledgements

All experiments in the subsequent chapters were conducted by the following protocol unless otherwise indicated in the relevant chapter. I would like to thank Deborah Berthold and all of the undergraduate students for their help around the wet laboratory and for all the helpful discussions.

2.2 Protein expression and purification

Uniformly-$^{15}$N, $^{13}$C labeled wild-type (WT), A30P, A53T and E46K $\alpha$-synuclein (AS) samples were prepared by previously published protocols (1). Recombinant protein expression in *E. coli* BL21(DE3) proceeded in high yield (~60 mg per L) using minimal media supplemented with BioExpress (Cambridge Isotopes). The purification was performed by chemical lysis and two chromatographic steps (hydrophobic interaction and gel exclusion) a third of a liter at a time. The sample purity was confirmed by gel electrophoresis and mass spectrometry. This purification protocol was optimized by using larger chromatographic columns with the capacity of purifying a liter at a time, significantly reducing the protein purification time. In addition, Amicon stirred cell concentrators are now used to reduce loss from fibril formation due to over concentration. The revised protocol has reduced the bottleneck of AS fibril sample preparation.
2.3 \(\alpha\)-Synuclein fibrillation

Solutions of monomeric AS (1 mM, 50 mM phosphate buffer, pH 7.5, 0.02% azide, 0.1 mM EDTA) were syringe filtered (0.22 \(\mu\)m filters) into parafilmed, 1.7 mL Eppendorf ultracentrifuged tubes and samples were seeded with 15 \(\mu\)L of natural abundance, pre-generation AS fibrils per 500 \(\mu\)L of AS sample. Samples were then incubated with shaking (200 rpm) at 37 °C as previously described (1). Low pH fibrils were prepared in 50 mM acetate buffer, pH 5.0, 0.02% azide and 0.1 mM EDTA.

2.4 Thioflavin T fluorescence

Solutions of monomeric WT and A30P AS (1 mM protein, 10 mM phosphate buffer at pH 7.4, 2.7 mM KCl and 137 mM NaCl) were filtered, and fibril formation was measured by monitoring Thioflavin T (15 \(\mu\)M, Sigma Aldrich) fluorescence using established protocols (2). Control wells were prepared to account for light scatter and possible quenching. Ninety-six well plates were incubated at 37 °C and agitated for 16 minutes prior to each reading with four minutes of no agitation. Seven replicates were performed for each condition.

2.5 Electron microscopy

WT and A30P AS fibril samples were treated with Karnovsky’s fixative and negatively stained with 2% ammonium molybdate (w/v). Samples were applied on Formvar carbon coated grids (300 mesh) and were viewed with a Hitachi H600 Transmission Electron Microscope operating at 75 kV.
2.6 Solution NMR spectroscopy

Monomeric AS samples were prepared for solution NMR experiments as 400 µL solutions containing 1 mM \(^{15}\text{N}\) labeled monomeric AS in 50 mM phosphate buffer (pH 7.5, 0.02% NaN\(_3\) w/v, 0.1 mM EDTA), 10% \(\text{D}_2\text{O}\) and 1 mM DSS. A two-dimensional (2D) BEST \(^1\text{H}-^{15}\text{N}\) heteronuclear single-quantum correlation (HSQC) spectrum (3, 4) was acquired of WT and A30P AS monomeric samples, Figure 4.1. Solution NMR experiments were conducted on a Varian INOVA 14 Tesla (600 MHz, \(^1\text{H}\) frequency) spectrometer using a triple resonance (\(^1\text{H}-^{13}\text{C}-^{15}\text{N}\)), triaxial gradient probe utilizing VNMRJ version 2.1B software with BioPack. All solution NMR spectra were acquired at 0 °C. Solution NMR chemical shift assignments were based on published assignments posted on the Biological Magnetic Resonance data Bank (BMRB) with the accession numbers #16300 (5, 6) and #16546 (7, 8) for the WT and A30P, respectively.

2.7 \(\alpha\)-Synuclein fibril preparation and packing for solid-state NMR experiments

All samples were washed by the following protocol with the exception of low pH AS fibril samples. After three weeks of fibrillation, samples were ultra-centrifuged for 1 h at 100,000 g. The resultant pellet from a 500 µL sample was washed with three aliquots of 100 µL Milli-Q water and ultra-centrifuged again for 1 h at 100,000 g. The supernatant was removed.

AS fibril pellets were dried under a stream of \(\text{N}_2\) (g) until the final mass was unchanged (9). The sample was contained in an Eppendorf tube and left unexposed to its surroundings by poking two holes in the tube cap and directly allowing the \(\text{N}_2\) (g) stream to enter through one of the holes.
The dried pellets were packed into 3.2-mm (thin or standard wall) 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 four weeks.

2.8 Solid-state NMR data collection and analysis

A 14.1 Tesla (600 MHz, $^1$H frequency) Varian Infinity Plus spectrometer equipped with a 3.2-mm T3 Varian Balun™ $^1$H-$^{13}$C-$^{15}$N MAS probe, in triple resonance mode, was utilized to perform all SSNMR experiments. The experiments were acquired using tangent ramped cross polarization (10) and SPINAL-64 (11, 12) $^1$H decoupling with a field strength of ~75 kHz during acquisition and evolution periods. For 3D $^{15}$N-$^{13}$C-$^{13}$C and $^{13}$C-$^{15}$N-$^{13}$C correlation experiments, a band-selective SPECIFIC CP (13) was utilized for the heteronuclear polarization transfer between $^{15}$N and $^{13}$C and DARR (14) mixing for $^{13}$C homonuclear polarization transfer. All experiments were acquired under 13.3 kHz magic angle spinning and at a variable temperature of 10 °C. The adamantane downfield peak was assumed to resonate at 40.48 ppm to externally reference chemical shifts (15).

Data were processed with back linear prediction applied to the direct dimension. Zero filling, Lorentzian-to-Gaussian apodization and/or cosine bells were applied for each dimension before Fourier transformation using nmrPipe (16). Peak picking, assignments and peak heights were obtained with Sparky software (17) and NMRViewJ (18) using Gaussian peak integration methods.
2.9 References


CHAPTER 3

OPTIMIZATION OF THE α-SYNUCLEIN FIBRIL PREPARATION FOR SOLID-STATE NMR

3.1 Notes and acknowledgements

This chapter is adapted from Springer Science and Business Media: *Journal of Molecular Biology* (2011), “Structured regions of alpha-synuclein fibrils include the early-onset Parkinson's disease mutation sites,” 411, 881-895, authors: Gemma Comellas, Luisel R. Lemkau, Andrew J. Nieuwkoop, Kathryn D. Kloepper, Daniel T. Ladror, Reika Ebisu, Wendy S. Woods, Andrew S. Lipton, Julia M. George and Chad M. Rienstra. This work was supported by the National Institutes of Health (R01-GM073770, R01-GM073770 ARRA supplement and NCRR Instruments Grant S10 RR025037-01). Gemma Comellas was a Caja Madrid Foundation Graduate Fellow. A portion of the research was performed in the Environmental Molecular Sciences Laboratory, a national scientific user facility sponsored by the Department of Energy's Office of Biological and Environmental Research and located at Pacific Northwest National Laboratory. Electron micrographs were obtained in the Frederick Seitz Materials Research Laboratory Central Facilities, University of Illinois, which are partially supported by the U.S. Department of Energy (funded under grants DE-FG02-07ER46453 and DE-FG02-07ER46471). The authors thank Dr. Robert Tycko for advice regarding fibril re-hydration, Dr. Lou A. Miller for help and advice regarding EM, and Dr. Anna E. Nesbitt for careful reading and discussions of the manuscript.
3.2 Abstract

Sample reproducibility is very important for comparing different samples. Fibrillation conditions and hydration are two factors in sample reproducibility and spectral quality. We have carefully controlled buffer, pH, temperature and agitation conditions during fibrillation, which contribute to spectrally identical samples. We also found that washing samples to remove excess salt and soluble oligomeric species increases spectral resolution and facilitates the acquisition of multidimensional solid-state NMR experiments and the assignment of site-specific chemical shifts. In addition, we have optimized the hydration level for AS fibril samples and determined that 36% water by mass is a fair compromise between sensitivity and resolution.

3.3 Improved spectral quality

We have confirmed the well-known relative rates of fibrillation among WT, E46K, A53T and A30P, as discussed in more detail above (1-3). The time required to yield >90% of the protein as mature fibrils, starting with unseeded ~500 mM solutions, varies from a few days (A53T) to 3-4 weeks (A30P). Significant morphological differences have previously been observed as a function of AS mutation, concentration and time (1). However, we have succeeded in seeding samples to avoid the lag time associated with nucleation; this, along with careful control of the incubation conditions, has resulted in morphologically and spectroscopically identical samples from one batch to the next (4), Figure 3.1. This represented an essential step for the comparison of samples, as it is essential to distinguish between significant perturbations upon mutations and those caused by slight differences in sample preparation. Initially, after mature fibrils were formed, fibrils were dried manually with N$_2$ (g) and required four workdays of manual time to avoid sample loss, because this method required the sample tube to be open
and exposed to its surroundings as fibrils were dried. Static and overestimations of high gas pressure caused sample to become airborne and lost. The development of a simple device keeps the sample contained in the sample tube and left unexposed to its surroundings. Therefore, supervision is no longer required and sample loss is no longer an issue due to overestimation of gas pressure or static. Furthermore, the sample was dried within a few hours (dependent on sample quantity).

These improved sample preparation methods have resulted in improved SSNMR spectral resolution and sensitivity, Figure 3.2. Initially, fibril solutions were directly packed into 3.2-mm standard wall rotors (Varian, Inc., Fort Collins, CO) after fibrillation (5). Data required extremely low temperature (-40 °C) and longer acquisition time due to less sample mass per volume and rigidity (6), Figure 3.2a. This preparation was improved by drying samples under a stream of N₂ (g) until the mass was unchanged allowing for more protein capacity and less water in a 3.2-mm standard rotor (7), Figure 3.2b. The resolution was improved by re-hydrating the dry fibrils with 36% water by volume, Figure 3.2c. The latest optimal preparation consists of increased sensitivity when fibrils are washed (8) and double the sample quantity is packed into a 3.2-mm thin wall rotor (Varian, Inc., Fort Collins, CO), Figure 3.2d; thin wall rotors hold 28 μL of material compared to standard wall rotors that only hold 18 μL. Fibrils were washed by re-suspending the dry fibrils in water and then dried and re-hydrated. Washing samples removes monomer, soluble oligomeric species and excess salt. By removing monomer and oligomeric species from the sample, the sample homogeneity was increased and resulted in the sample’s natural linewidths to decrease. By removing excess salt from the sample, the tuning profile of the sample became stable and more decoupling power could be applied during acquisition and
evolution. To assess the extent of oligomerization that was removed during the wash, the supernatant of the wash was run through a size exclusion column and evidence of oligomers and monomer was present. Overall, these advances increased spectral resolution and helped with the acquisition of experiments that require a higher decoupling power.

3.4 Optimization of sample hydration

Previous studies from our group have demonstrated the important effect of hydration to the resolution and sensitivity of the SSNMR spectra of full-length, α-synuclein fibrils (7). Here, we optimized the hydration level of AS fibrils to maximize the resolution and sensitivity of SSNMR multidimensional experiments, which are essential to complete chemical shift assignments. To optimize the hydration level, fibrils were dried by N₂ (g) until no changes in mass were observed, and ¹H 1D and ¹³C-¹³C 2D spectra were acquired (Figure 3.3). The hydration of the sample was gradually increased and ¹H 1D and ¹³C-¹³C 2D spectra were acquired at each hydration level up to 50% (mass of water/total mass), as shown in Figure 3.3.

As reported in Table 3.1, the pulse width for both for ¹H and ¹³C became longer upon hydration and the overall cross-polarization efficiency decreased as expected. However, we observe significant improvement in the ¹³C linewidths and signal-to-noise of individual peaks (Table 3.1) that we hypothesize to be attributed to changes in the timescale of the dynamics of certain regions of the full-length protein. Average linewidths ± standard deviation and normalized signal-to-noise were obtained by measuring the linewidths and signal-to-noise for a total of 20 resolved peaks in ¹³C-¹³C 2D experiments. Conditions marked in bold correspond to the optimal conditions selected for multidimensional NMR experiments for all samples.
3.5 Conclusion

AS is highly sensitive to fibrillation conditions, such as buffer, temperature pH and agitation, as seen in Hoyer et al. (9). Here we found that after carefully controlling fibrillation conditions, samples were highly reproducible. We also found that washing samples to remove excess salt and soluble oligomeric species increased sample homogeneity. This increases spectral resolution facilitating the acquisition of multidimensional SSNMR experiments and the assigning of site-specific chemical shifts. Chemical shifts are highly sensitive to their environment, especially hydration (7). Thus, we have optimized the hydration level for AS fibril samples and found that 36% water by mass was the best compromise between resolution and sensitivity.

3.6 References


3.7 Figures

**Figure 3.1** Microscopically ordered AS fibrils. (a) $^{13}\text{C}-{^{13}\text{C}}$ 2D spectrum of U-$^{13}\text{C}$, $^{15}\text{N}$ AS fibrils with 36% water by mass (50 ms DARR mixing, 750 MHz $^1\text{H}$ frequency and 16.7 kHz MAS rate). (b) Transmission electron micrographs of AS fibrils. (c) Correlations of $^{13}\text{CA}, ^{13}\text{CB}$ and $^{13}\text{C'}$ AS chemical shifts (CS) from independent sample preparation batches (above) seeded with unlabeled fibrils and (below) unseeded.
Figure 3.2  Comparison of $^{13}$C-$^{13}$C 2D spectra of AS fibrils. (a) Fully hydrated AS fibrils acquired on a 600 MHz spectrometer, 7 mg, 2.2 h. (b) Dried AS fibrils acquired on a 500 MHz spectrometer, 10 mg, 1.7 h. (c) Re-hydrated AS fibrils acquired on a 500 MHz spectrometer, 10 mg, 1.8 h. (d) Re-hydrated AS fibrils acquired on a 600 MHz spectrometer, 25 mg, 2.2 h. All spectra were acquired at 0 °C with the exception of (a), which was acquired at -40 °C. All spectra were acquired with 10 ms DARR mixing with the exception of (c), which was acquired with 25 ms DARR mixing. Spectra were processed identically, with 0.26 ppm line broadening in both dimensions, and drawn with contours 5 times the noise level. A and B are figures adapted from Kloepper, K. D.; Hartman, K. L.; Ladror, D. T.; Rienstra, C. M.; (2007) J. Phys. Chem. B, 111, 13353-13356.
Figure 3.3  Optimization of hydration for α-synuclein fibril samples for SSNMR experiments (mass of water/total mass). (a) $^1$H 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 ($^1$H 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 (6). Spectra marked in blue correspond to the optimal conditions selected for the multidimensional NMR experiments.
3.8 Tables

Table 3.1 Comparison of $^1$H and $^{13}$C pulse widths (PW), CP enhancement and $^{13}$C linewidths (Hz) at different percentages of hydration (mass of water/total mass) for U-$^{15}$N, $^{13}$C WT AS fibrils. (*) Dry fibrils correspond to fibrils dried by N$_2$ gas until no changes in mass were observed, as previously described (6). Average linewidths (LW) ± standard deviation were obtained by measuring the LW for a total of 20 resolved peaks in $^{13}$C-$^{13}$C 2D experiments at 600 MHz (1H frequency) and 13.3 kHz MAS.

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<th>$^{13}$C PW (µs)</th>
<th>$^1$H PW (µs)</th>
<th>Cross Polarization enhancement</th>
<th>Average $^{13}$C LW (Hz)</th>
<th>Normalized signal-to-noise</th>
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CHAPTER 4

A30P α-SYNUCLEIN ADOPTS THE WILD-TYPE FIBRIL STRUCTURE, DESPITE SLOWER FIBRILLATION KINETICS

4.1 Notes and acknowledgements

This chapter is adapted has been submitted to the *Journal of Biological Chemistry* for peer review, A30P α-synuclein adopts the wild-type fibril structure, despite slower fibrillation kinetics, authors: Luisel R. Lemkau, Gemma Comellas, Kathryn D. Kloeppep, Wendy S. Woods, Julia M. George and Chad M. Rienstra. We thank Kem Winter and Shin Lee for assisting with sample preparation and Andrew J. Nieuwkoop for technical assistance with NMR data collection. L.R.L. thanks the National Institutes of Heath Research Supplements to Promote Diversity in Health-Related Research (GM073770-02 S1 SUPP) and the Novartis Pre-doctoral Fellowship. G.C. thanks the Caja Madrid Foundation Graduate Fellowship.

4.2 Abstract

α-Synuclein (AS) is associated with both sporadic and familial forms of Parkinson’s disease (PD). In sporadic disease, wild-type AS fibrillates and accumulates as Lewy bodies within dopamine neurons of the substantia nigra. The accumulation of misfolded AS is associated with the death of these neurons, which underlies many of the clinical features of PD. In addition, a rare missense mutation in AS, A30P, is associated with highly penetrant, autosomal dominant PD, although the pathogenic mechanism is unclear. A30P AS fibrillates more slowly than the wild-type protein *in vitro*, and has been reported to preferentially adopt a soluble, protofibrillar conformation. This has led to speculation that A30P forms aggregates that are distinct in
structure as compared to wild-type AS. Here we perform a detailed comparison of the chemical shifts and secondary structures of these fibrillar species, based upon our recent characterization of full-length wild-type fibrils. We have assigned A30P AS fibril chemical shifts *de novo* and used them to empirically determine its secondary structure. Our results illustrate that although A30P forms fibrils slower than WT, their chemical shifts and secondary structure are in high agreement, demonstrating a conserved β-sheet core.

4.3 Introduction

Alpha-synuclein (AS) is centrally implicated in Parkinson’s disease (PD) and several other neurodegenerative disorders (1). The unifying feature of the “synucleinopathies” is aggregation and accumulation of AS protein within intracellular inclusions (2). While these diseases are typically sporadic, several mutations in the SNCA gene encoding AS are associated with familial PD, including single point mutations—A53T (3), A30P (4) and E46K (5)—and allele duplication (6) or triplication (7).

Although the PD-associated AS mutations are extremely rare, analysis of their pathogenicity could significantly illuminate the mechanisms underlying sporadic disease. The normal function of AS is not precisely known, but it shares conserved structural features with the exchangeable apolipoproteins (8), and several lines of evidence suggest a role for AS in presynaptic vesicle trafficking (9-11). Like the wild-type (WT) protein, all mutant forms of AS are intrinsically unfolded in aqueous solution, but adopt an α-helical secondary structure within their N-termini upon binding to phospholipid vesicles or detergent micelles (12, 13). Within A30P, the helical domain is partially disrupted by proline substitution, with a consequent decrease in lipid affinity (12), while the A53T and E46K mutants exhibit similar or enhanced lipid binding when
compared to WT AS (12, 14). Thus, altered lipid affinity is not a unifying phenotype for disease-associated mutants.

One hypothesis to account for the pathogenicity of the PD-related mutations is that they promote pathological AS aggregation. However, A30P AS has been reported to fibrillate more slowly than WT AS in vitro (15), and to preferentially populate a soluble, protofibrillar intermediate, whereas WT readily progresses to mature, insoluble fibrils (16). This has fueled speculation that the mechanism of A30P toxicity may be fundamentally different than that of WT AS. Only recently has tissue from a familial PD patient with the A30P mutation has become available for analysis. This individual displayed neuropathology typical of idiopathic PD, but with a greater-than-typical load of insoluble fibrillar aggregates (17). This is a surprising result that strongly implicates fibrillar AS in the pathogenesis of A30P-dependent PD.

A30P and WT AS fibrils have similar morphologies when viewed by low resolution techniques, like electron microscopy (18). Although recent solution NMR studies of quenched hydrogen/deuterium (H/D) exchange have suggested that the A30P mutation does not perturb the location or arrangement of β-strands in WT AS fibrils (19), it is not possible to draw site-specific conclusions regarding structure from H/D exchange experiments alone; for example, some β-sheet regions may be more exposed than others or protected regions may not exhibit a β-sheet secondary structure. In addition, these indirect measurements rely on low molecular weight samples, which require fibrils to be broken down to smaller units because of their high molecular weight. In contrast, solid-state NMR (SSNMR) is uniquely positioned to obtain atomic-resolution structural information of systems that are of high molecular weight, not soluble and do not form crystals, such as AS fibrils, without altering sample integrity. In this study, we sought to
determine whether A30P AS fibrils differ at an atomic level in secondary structure from WT AS fibrils. Our results, based on chemical shift analysis obtained with multidimensional SSNMR experiments, illustrate that A30P and WT AS fibrils are highly similar in secondary structural details.

4.4 Materials and methods

4.4.1 Protein sample preparation

Natural abundance and uniformly $^{13}$C, $^{15}$N labeled WT and A30P full-length, monomer samples were expressed and purified as previously described (20). Briefly, recombinant protein was expressed in *E. coli* BL21(DE3), while grown in minimal media supplemented with $^{13}$C, $^{15}$N BioExpress (Cambridge Isotopes). Purification was performed by thermal lysis, hydrophobic interaction and size exclusion chromatography resulting in high yield (>40 mg per liter). The sample purity was confirmed by gel electrophoresis and mass spectrometry. To verify that the mutation was present, the plasmid was sequenced and $^{1}$H-$^{15}$N HSQC spectra of the purified monomer samples were acquired, as shown in Figure S1.

4.4.2 Thioflavin T fluorescence

Solutions of monomeric WT and A30P AS (1 mM, 10 mM phosphate buffer, 2.7 mM KCl, 137 mM NaCl, pH 7.4) were filtered, and fibril formation was measured by monitoring Thioflavin T (15 μM, Sigma Aldrich) fluorescence using established protocols (21). Control wells were prepared to account for light scatter and possible quenching. Ninety-six well plates were incubated at 37 °C and agitated for 16 minutes prior to each reading with 4 minutes of no agitation. Seven replicates were performed for both A30P and WT AS.
4.4.3 *Electron Microscopy*

WT and A30P AS fibril samples were treated with Karnovsky’s fixative and negatively stained with 2 % ammonium molybdate (w/v). Samples were applied on formvar carbon coated grids (300 mesh) and were viewed with a Hitachi H600 Transmission Electron Microscope, operating at 75 kV.

4.4.4 *Solution NMR spectroscopy*

Monomeric AS samples were prepared for solution NMR experiments as 400 µL solutions containing 0.33 mM ¹⁵N labeled monomeric AS in 50 mM phosphate buffer (pH 7.5, 0.02% NaN₃ w/v), 10% D₂O and 1 mM DSS. A two-dimensional (2D) BEST ¹H-¹⁵N heteronuclear single-quantum correlation (HSQC) spectrum (22, 23) was acquired of WT and A30P AS monomeric samples, Figure 3.1. Solution NMR experiments were conducted on a Varian INOVA 14 Tesla (600 MHz, ¹H frequency) spectrometer using a triple resonance (¹H-¹³C-¹⁵N) triaxial gradient probe, utilizing VNMRJ version 2.1B software with BioPack. All solution NMR spectra were acquired at 0 °C. Chemical shift assignments labeled in Figure S1 are based on the published assignments that are deposited on the Biological Magnetic Resonance data Bank (BMRB) with the accession numbers #16300 (24, 25) and #16546 (26, 27) for the WT and A30P, respectively. The amide proton chemical shift of A30 is reported to be 8.26 ppm and 123.54 ppm of the amide nitrogen for the WT monomer. We did not see a correlation at these frequencies in either spectrum. However, we found that the chemical shifts of A29 and G31-T33 had been perturbed as previously described upon mutation.
4.4.5 Solid-state NMR spectroscopy

An initial solution of monomeric, natural abundance A30P AS (1 mM protein, 50 mM phosphate buffer, pH 7.5, 0.02% azide and 0.1 mM EDTA) was filtered with a 0.22 µm syringe filter. This solution was incubated with shaking (200 rpm) at 37 °C for 3 weeks to produce mature fibrils. These fibrils were then used to seed future uniformly 13C, 15N labeled A30P AS fibrils. After the allotted time, fibril solutions were washed, dried, packed into 3.2 mm standard or thin wall rotors (Varian, Inc., Fort Collins, CO) and rehydrated with 36% (m/v) of water as per previously described protocols (28).

A 14.1 Tesla (600 MHz, 1H frequency) Varian Infinity Plus spectrometer equipped with a 3.2 mm T3 Varian Balun™ 1H-13C-15N MAS probe, in triple resonance mode, was utilized to perform all SSNMR experiments. Experiments employed tangent ramped cross polarization (29) and SPINAL-64 (30, 31) 1H decoupling with a field strength of ~75 kHz during acquisition and evolution periods. For 3D 15N-13C-13C and 13C-15N-13C correlation experiments, band-selective SPECIFIC cross polarization (CP) (32) was utilized for heteronuclear polarization transfer between 15N and 13C and DARR (33) mixing for 13C homonuclear polarization transfer. All experiments were acquired under 13.3 kHz magic angle spinning and at a variable temperature of 10 °C. The adamantane downfield peak was assumed to resonate at 40.48 ppm to externally reference chemical shifts (34). All 2D and 3D spectra used for chemical shift assignments are listed in Table S1.

Data were processed with back linear prediction applied to the direct dimension. Zero filling, Lorentzian-to-Gaussian apodization and/or cosine bells were applied for each dimension before
Fourier transformation using nmrPipe (35). Peak picking, assignments and peak heights were obtained with Sparky software (36) using Gaussian peak integration methods.

4.5 Results and discussion

4.5.1 A30P α-synuclein fibrillates slower than WT

WT and A30P AS solutions were prepared, as previously described (20), to monitor fibril formation with Thioflavin T fluorescence. An increase in lag time indicates a decrease in the fibrillation rate, as shown for A30P when compared to WT (Figure 3.2a). Our data show WT AS to propagate after 24 hours and A30P after 72 hours. These results are in agreement with those of Li et al. (37) and Meuvis et al. (38). Although A30P and WT fibrillate at different rates, a comparison of electron micrographs of mature fibrils, formed after three weeks of incubation, exhibit no distinguishable changes in the fibril morphology upon mutation (Figure 3.2b).

4.5.2 A30P α-synuclein fibrils are highly reproducible

SSNMR has proven to be a useful structural biology technique in exploring the structure and dynamics of amyloid fibrils, like AS fibrils (39-41). Certain amino acids (i.e., Gly, Ala, Thr, Ser, Ile, and Pro) are distinctively identifiable by their unique chemical shift patterns and are highly sensitive to secondary structure (42-46). Therefore, to evaluate slight variations between one fibril batch to the next, $^{13}$C-$^{13}$C 2D spectra with 50 ms DARR (47) mixing were acquired of three different batches of A30P AS fibrils; prepared as previously described for uniformly $^{13}$C, $^{15}$N labeled WT AS fibrils (20, 28). The linear regression analysis of two individual batches showed $R^2$ values of 0.996, 0.999 and 0.999 for $^{13}$C’, $^{13}$CA and $^{13}$CB, respectively (Figure 3.2c-e). Independent batches also exhibit average chemical shift variations of less than 0.2 ppm. Our
A30P fibril samples are microscopically well-ordered, evidenced by the narrow line widths, averaging 0.2 ppm (Figure 3.2f). Many of the spectral fingerprints of A30P AS fibrils are identical to those of WT; for example, the highly resolved Thr and Ser regions, shown in Figure 1g and 1h.

4.5.3 A30P α-synuclein fibril chemical shift assignments

SSNMR heteronuclear (\(^{15}\text{N}-^{13}\text{C}\)) and homonuclear (\(^{13}\text{C}-^{13}\text{C}\)) 2D experiments with longer DARR mixing times were acquired on uniformly \(^{13}\text{C},^{15}\text{N}\) labeled A30P AS fibrils to detect intra- and inter-residue correlations and confirm pair-wise assignments. Some signal patterns could be assigned immediately, but the majority of correlation patterns were degenerate due to the presence of sequential, imperfect repeats (KTKEGV) in the AS N-terminus. Accordingly, 3D experiments (Table 3.1) were acquired to obtain unambiguous, site-specific chemical shift assignments.

\(^{15}\text{N}-^{13}\text{C}-^{13}\text{C}\) 3D experiments detect a common nitrogen frequency to associate two neighboring residues (the \(i\) and \(i-1\) residues). Additionally, \(^{13}\text{C},^{15}\text{N}-^{13}\text{C}\) 3D experiments can be used to gain an added common frequency such as \(^{13}\text{CA}\) or \(^{13}\text{C’}\) in the second dimension. For example, a CAN(co)CX starts with \(^{13}\text{C}\) polarization on \(^{13}\text{CA}\) nuclei and is transferred to \(^{15}\text{N}\) nuclei; this is followed by a polarization transfer from \(^{15}\text{N}\) to \(^{13}\text{C’}\) nuclei (where no chemical shift evolution takes place, hence the parentheses) using specific cross polarization (48). Once these cross polarization transfers have occurred, the resulting polarization on the \(^{13}\text{C’}\) nucleus is transferred dispersedly through space to the side chain \(^{13}\text{C}\) nuclei using the DARR mixing scheme. Using these three multidimensional experiments, we conducted chemical shift assignments of sequential residues using the backbone walk method, a well-established
technique for making *de novo* site-specific chemical shift assignments (28, 49-55). Figure 3.3 illustrates how the backbone walk method is used to assign seven consecutive residues in A30P AS fibrils. Sequential backbone assignments were achieved for the stretches of V40-V49, G51-V55 and E57-D98 (Figures 3.4 and 3.5). A total of 63 unique *de novo* resonance assignments were possible for A30P AS fibrils (Table 3.2) without relying upon the WT AS chemical shift lists.

In fact, the resolution and sensitivity of A30P AS fibril spectra were so favorable that a number of signals were detected in the loop regions that were not evident in comparable spectra of WT AS fibrils: E57, E61 and D98. Residues Y39 to K43, K58 to K60, Q62 and K97, highlighted in Table 3.2, were previously assigned for WT AS fibrils from data acquired on \(^{13}\)C sparsely labeled samples (28), which provide higher resolution data compared to uniformly \(^{13}\)C labeled samples (56). These residues were detected and assigned using uniformly \(^{13}\)C labeled A30P fibril samples. We attribute these results to technical improvements in the data collection and potential contributions from differences in the dynamics of the loop regions.

4.5.4 *A30P a-synuclein forms fibrils that are structurally similar to wild-type*

We applied linear regression analysis to the complete set of WT (28) and A30P AS fibril chemical shifts (Figure 3.6). The chemical shifts of \(^{13}\)CA and \(^{13}\)CB are highly sensitive to secondary structure confirmation; thus, \(R^2\) values of 0.999 and 0.998, respectively, confirm nearly identical secondary structures between A30P and WT AS fibrils. The \(^{15}\)N and \(^{13}\)C\(^{'}\) chemical shifts exhibit \(R^2\) values of 0.998 and 0.991, respectively, which are consistent with modest perturbations in hydrogen bonding and electrostatics upon mutation (57, 58).
As previously demonstrated (59), the dipolar-based CANCO experiment produces a correlation for the most rigid residues in a given sample. Amyloid fibrils contain a rigid core, where dipolar-based experiments transfer polarization with high efficiency, and mobile regions, where polarization transfer is difficult or not allowed in dipolar-based experiments (40, 60). Thus, the signal intensities report qualitatively on rigidity. Sixty-one correlations were identified in the CANCO spectrum of A30P AS fibrils of the expected 140 residues in AS. Of the correlations detected in the CANCO spectra, 91% were unambiguously assigned. Figure 3.7c demonstrates the trend in signal intensity by residue number for A30P AS fibrils. The region with the greatest intensity is found for residues 68 to 94, which includes the most hydrophobic stretch, 71-82 (61). We attribute the missing signals in the CANCO spectra of full-length A30P AS fibrils to mobility in dipolar-based experiments. Polarization is transferred through-space in dipolar experiments, which is more efficient between two rigid nuclei. Therefore, more mobile regions like the termini and those in loop confirmations are less likely to be detected by dipolar-based experiments.

SSNMR detects differences in chemical environments with high sensitivity (43-46, 62). This allows for the identification of amino acids and prediction of backbone dihedral angles (Φ and Ψ) and secondary structure with the TALOS+ program, illustrated in Figures 3.7a and 3.7b (44, 63). Our results pertaining to A30P AS fibrils indicate a highly similar β-sheet secondary structure relative to the WT (28), Figure 3.7a. This is consistent with the localization of A30 outside the stable β-sheet core for the WT fibril structure, thus exerting no major effect on the β-sheet secondary structure of the core upon mutation (28). The improvement in data quality of A30P AS fibrils allowed for the detection of additional residues, which also allowed for an
extended, empirically determined secondary structure compared to the WT in the V55-V63 region. In addition, when the chemical shifts of this region were compared between A30P and WT AS fibrils, perturbations greater than 0.5 ppm for V55, K58, K60 and V63 were found, which are greater than the variations found between individual sample preparations, < 0.2 ppm. Considering that these perturbations correspond to a similar region of the protein, these results suggest that the location of A30 in the WT AS fibril structure alters the packing of the fibril in this region. Presume three possible scenarios: (1) this region and A30 are in close proximity with in the WT fold, (2) there is an intermolecular interaction between this region on one molecule and position A30 of a second molecule or (3) the N-terminal domain is perturbed relative to the WT. In the latter of these possibilities, the N-terminal domain is more dynamic than the rest of the fibril core; therefore, such perturbations are not detected in dipolar experiments, as we do not observe these signals. Significantly, Goedert and coworkers report that the truncation of the N-terminal domain of AS causes an increase in the fibrillation lag time of WT AS (65). This demonstrates that the intact N-terminal domain plays a role in promoting fibrillation, and suggests a mechanism whereby disruption of the N-terminal domain (via proline substitution at A30) might inhibit fibrillation kinetics without altering the fibril structure. This together leads to the hypothesis that the A30P mutation affects the susceptibility to form fibrils, hence the propensity to form more oligomeric species than mature fibrils (66). Although in vitro fibril formation is slower than for the WT, one could also envision differences in the fibrillation kinetics in cellular environments. This would also be consistent with the presence of a greater-than-typical load of insoluble fibrillar aggregates in brain of the familial PD patient with the A30P mutation (17).
4.6 Conclusion

We have performed a site-specific comparison between the secondary structures of full-length WT and A30P AS fibrils. Chemical shift assignments and the empirically determined secondary structure demonstrate that A30P adopts the wild-type fibril structure, despite the fact that A30P forms fibrils more slowly. Our results show that the A30P mutation does not substantially perturb the resulting AS fibril secondary structure at an atomic level.

4.7 References


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4.8 Figures

**Figure 4.1** Solution NMR demonstrates the A30P mutation was present before fibrillation. (a) Overlaid 1H-15N HSQC spectra of A30P (blue) and WT (red) purified AS monomer. Expansions of residues near mutation site: (b) G31, (c) T33, (d) K32 and (e) A29. Assignments based on the deposited solution assignments from the BMRB for WT # 16300 (25) and A30P #16546 (27).
Figure 4.2 In vitro fibrillation conditions provide microscopically well-ordered A30P AS fibrils that form slower than WT. (a) Average fibril formation assay of (red triangles) WT and (blue circles) A30P AS fibrils monitored by Thioflavin T fluorescence. Error bars were determined from seven replicates for each. (b) Comparison of the electron micrographs of (left) WT and (right) A30P AS fibrils. (a) $^{13}$C', (b) $^{13}$CA and (c) $^{13}$CB chemical shift plots between two individual batches of A30P AS fibrils. (d) $^{13}$C-$^{13}$C 2D with 50 ms DARR mixing of A30P AS fibrils. Overlaid expansions of WT (red) on to A30P (blue) AS fibrils for the (e) Thr/Ser (CB-C') and (f) Thr/Ser (CB-CA) regions.
Figure 4.3 Multidimensional spectra with high sensitivity and resolution allowed for the chemical shift assignments of A30P AS fibrils. (left) Backbone walk schematic. (right) Illustration of backbone connectivity through the NCACX (red), NCOCX (blue) and CAN(co)CX (orange) spectra of residues V71-N65 for A30P AS fibrils. All spectra acquired with 50 ms DARR mixing and processed with 0.5 ppm of line-broadening in each dimension.
Figure 4.4  Backbone walk scheme using NCACX (red) with 50 ms DARR, CAN(CO)CX (orange) with 50 ms DARR and NCO CX (blue) with 100 ms DARR spectra of A30P AS fibrils; residues D98 to T72. Spectra were acquired at 10 °C and 13.3 kHz MAS. Acquisition and processing details are described above.
**Figure 4.5** Backbone walk scheme using NCACX (red) with 50 ms DARR, CAN(CO)CX (orange) with 50 ms DARR and NCOCX (blue) with 100 ms DARR spectra of A30P AS fibrils; residues V71 to G41. Spectra were acquired at 10 °C and 13.3 kHz MAS. Acquisition and processing details are described above.
Figure 4.6 Comparison of the chemical shift assignments of A30P and WT AS fibrils (28) demonstrates that the fibril is mostly unchanged upon A30P mutation. (a) $^{15}\text{N}$, (b) $^{13}\text{CA}$, (c) $^{13}\text{C'}$, and (d) $^{13}\text{CB}$ chemical shift plots of WT versus A30P AS fibrils. Residues that differ by more than 0.5 ppm are labeled and highlighted with blue circles.
Figure 4.7 Comparison of the secondary structures between WT (28) and A30P AS fibrils demonstrates that the fibril core is mostly unchanged upon A30P mutation. (a) Representation of the secondary structure of WT (red) and A30P (blue) AS fibrils based on TALOS+ analysis (β-strands, arrows; turn or loop curved lines; not predicted, dashed lines). (b) TALOS+ predicted backbone dihedral angles $\phi$ (black squares) and $\psi$ (gray circles), error bars based on the 10 best TALOS+ database matches and (c) the normalized peak heights from CANCO as a function of residue number for A30P.
Table 4.1 Description of the 2D and 3D experiments acquired in order to obtain the \textit{de novo} $^{13}$C, $^{15}$N chemical shift assignments for U-$^{13}$C, $^{15}$N A30P AS fibrils. Chemical Shifts were deposited to the BMRB, entry #17214. The total amount of protein was calculated based on the intensity of a $^{13}$C 1D DP experiment versus that of adamantane (the mass for adamantane was determined before packing the sample in the rotor).

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Table 4.2 $^{13}$C and $^{15}$N chemical shift assignments of A30P AS fibrils (with 36% water by mass) using only uniformly labeled samples. Chemical shifts were deposited to the BMRB, entry #17214. WT-AS fibril assignments were made with data acquired on samples with selected $^{13}$C labeling indicated by an asterisk (*) and assignments not possible with either selected or uniform labeling are indicated in bold. Assignments in italics are tentative for A30P fibrils.

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CHAPTER 5

STRUCTURAL PERTURBATIONS FOUND UPON MUTATION OF ALPHA-SYNUCLEIN TO A53T AND E46K IN THE FIBRIL STATE

5.1 Notes and Acknowledgements

This chapter is based on a manuscript with the same title that is currently under preparation. L.R.L. thanks the National Institutes of Heath Research Supplements to Promote Diversity in Health-Related Research (GM073770-02 S1 SUPP) and the Novartis Pre-doctoral Fellowship. G.C. thanks the Caja Madrid Foundation Graduate Fellowship. Funding for the 750 MHz $^1$H frequency spectrometer used to acquire the A53T AS fibril data were provided by Award Number S10RR025037 from the National Center For Research Resources. The content is solely the responsibility of the authors and does not necessarily represent the official view of the National Center For Research Resources or the National Institutes of Health.

5.2 Abstract

Parkinson’s disease (PD) is associated with an aggregated protein, α-synuclein (AS), found in Lewy bodies within dopamine neurons of the substantia nigra of PD patients. AS is further related to PD by the presence of three single point mutations A30P, E46K and A53T in the familial form. Of these the A53T mutation is unique in that there is a threonine instead of an alanine at position 53 for non-primate mammals, while the other two single point mutations are conserved. Mice expressing human E46K or A53T AS form display AS inclusions closely resembling those of Human Lewy Bodies. This evidence further supports the relationship between AS fibrils and PD pathology, which has lead to several investigations into the anomalies
and similarities between the wild-type (WT) and mutants in hopes to acquire knowledge related to PD etiology. Here, we have compared in detail the chemical shifts, secondary structures and conformational dynamics of the E46K and A53T AS fibrillar species with our recent characterization of full-length wild-type fibrils. We have acquired multidimensional solid-state NMR experiments in order to assign the E46K and A53T AS fibril chemical shifts *de novo* and use them to empirically determine their secondary structure. Our detailed comparison illustrates large perturbations upon the E46K mutation that leads to major changes in secondary structure. In addition we find a similar secondary structure between A53T and WT AS fibrils, with slight perturbations near the mutation site, the 60s and 80s.

### 5.3 Introduction

Alpha-synuclein (AS) is a widely studied protein for its relevance to Parkinson’s disease (PD) and other neurodegenerative diseases (1). Aggregated AS is the main proteineous component found in Lewy bodies, the pathological hallmark of PD (2). The native function of AS is not well understood, however, it has conserved structural motifs found in exchangeable apolipoproteins (3) and several results point towards a role in presynaptic vesicle trafficking (4-6). In addition, AS is further implicated in PD pathology through the finding of three single point mutations—A30P (7), E46K (8) and A53T (9)—and allele duplication (10) and triplication (11) in the familial form of PD.

Several investigations into the anomalies and similarities between the wild-type (WT) and mutants have been conducted in hopes to acquire knowledge related to PD etiology. The A30P and E46K mutations are conserved across a variety of species, however A53T is not. A threonine is present instead of an alanine at position 53 for non-primate mammals along with six other
substitutions: S87N, L100M, N103G, A107Y, D1221G and N122S (9). Elevated neurodegenerative effects are displayed in mice expressing human A53T AS (12-18); this suggests that these additional substitutions may add a complementary physiological effect to A53T. Similarly, mice expressing human E46K AS form AS inclusions closely resembling those of Human Lewy Bodies (19). These results further support the relationship between AS fibrils and PD pathology.

All three familial mutants, along with the WT, are unstructured in solution with the exception of residual secondary structure near the mutation sites (20-22). In addition, these mutants illustrate an α-helical N-terminal domain upon binding to phospholipid vesicles or detergent micelles, with an affinity similar to that of WT (22-25). However, the mutants differ from the WT when considering their fibrillation kinetics. A53T (26-29) and E46K (22) form fibrils faster than WT (24, 30), while A30P fibrillates much slowly (27, 31). Through atomic force and electron microscopy, the WT and mutant AS fibrils have been shown to have an overall similar morphology (24, 32, 33). However, these methods do not give atomic level structural information on how the wild-type fibril structure is perturbed upon mutation.

An emerging technique in structural biology is magic angle spinning solid-state NMR (SSNMR). Because amyloid fibrils, like AS, are not soluble and do not form x-ray diffractable quality crystals, many scientist resort to SSNMR, indifferent to these issues, to investigate such systems. Much progress has been made towards solving amyloid fibril structures using SSNMR(34-38). For example, the latest being that of amyloid β (39, 40).

Most recently we used SSNMR to report that despite the slower kinetics of A30P AS fibril formation, the A30P mutation does not affect the WT secondary structure at an atomic level (31).
Heise et al. also reported an extended $\beta$-sheet core in A53T AS fibrils (41). However, this study reported encountering problems in reproducing samples yielding different fibril polymorphs between different batches of WT sample preparations (42). Therefore, the irreproducible sample preparations make it difficult to obtain an accurate structural comparison between the WT and A53T mutant fairly. On the other hand, we have reproduced spectrally identical AS fibril samples from one batch to the next for the wild-type and mutants (31, 43, 44) that we have used here to investigate the anomalies and differences between two of the mutants, E46K and A53T, and the WT in the fibril state using SSNMR.

5.4 Materials and Methods

5.4.1 Protein sample preparation

Uniformly-$^{15}$N, $^{13}$C labeled WT, A53T and E46K AS were prepared following previously published protocols (43). In summary, recombinant protein expression was performed in *E. coli* BL21(DE3) using minimal media supplemented with BioExpress (Cambridge Isotopes). Purification, proceeds in high yield (~60 mg per L), is performed with chemical lysis and two chromatographic steps (hydrophobic interaction and gel exclusion). Sample purity was confirmed by SDS-PAGE gel electrophoresis and mass spectrometry.

5.4.2 $\alpha$-Synuclein fibrillation

Solutions of monomeric AS (1 mM, 50 mM phosphate buffer, pH 7.5, 0.02% azide, 0.1 mM EDTA) were syringe filtered (0.22 $\mu$m filters) and seeded with natural abundance pre-generation AS fibrils. Samples were then incubated with shaking (200 rpm) at 37 °C as previously described (43).
5.4.3 Electron microscopy

A53T, E46K and WT AS fibril samples were treated with Karnovsky’s fixative. After negatively staining with 2 % ammonium molybdate (w/v), samples were applied on formvar carbon coated grids (300 mesh). Samples were viewed, operating at 75 kV, with a Hitachi H600 Transmission Electron Microscope.

5.4.4 α-Synuclein fibril preparation for solid-state NMR experiments

After 3 weeks of fibrillation, samples were ultracentrifuged for 1 hr at 100,000 g. The resultant pellets were washed and re-ultracentrifuged again for 1 hr at 100,000 g. The supernatant was removed. AS fibril pellets were dried under a stream of N₂ (g) until the final mass was unchanged (45). The dried pellets were packed into 3.2 mm (thin or standard wall) 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 (44).

5.4.5 Solid-state NMR data collection and analysis

A 14.1 Tesla (600 MHz, ¹H frequency) Varian Infinity Plus spectrometer equipped with a 3.2 mm T3 Varian Balun™ ¹H-¹³C-¹⁵N MAS probe, in triple resonance mode, was utilized to perform all E46K SSNMR experiments. A 17.6 Tesla (750 MHz, ¹H frequency) Varian Unity Inova spectrometer equipped with a Varian 3.2 mm Balun™ ¹H-¹³C-¹⁵N MAS probe, in triple resonance mode, was utilized to perform all A53T SSNMR experiments. All experiments utilized tangent ramped cross polarization (46) and SPINAL-64 (47, 48) ¹H decoupling during acquisition and evolution periods with ~75 kHz of field strength. For 3D ¹⁵N-¹³C-¹³C and ¹³C-¹⁵N-¹³C correlation experiments, band-selective SPECIFIC CP (49) was employed for
heteronuclear polarization transfer between $^{15}\text{N}$ and $^{13}\text{C}$ and DARR (50) mixing for $^{13}\text{C}$ homonuclear polarization transfer. All E46K experiments were acquired under 13.3 kHz magic angle spinning and at a variable temperature of 10 °C. All A53T experiments were acquired under 10.0 kHz magic angle spinning and at a variable temperature of 0 °C. Chemical shifts were externally referenced using adamantine; the downfield peak was assumed to resonate at 40.48 ppm (51).

Data were processed by applying back linear prediction to the direct dimension. Zero filling, Lorentzian-to-Gaussian apodization and/or cosine bells were applied for each dimension before Fourier transformation using nmrPipe (52). Peak picking, assignments and peak heights were obtained with NMRViewJ software (53) using Gaussian peak integration methods.

5.5 Results and discussion

5.5.1 Sample characteristics

Recombinant protein expression was performed with *E. coli* BL21(DE3) cells and purified yielding ~60 mg of uniformly-$^{15}\text{N}$, $^{13}\text{C}$ labeled WT, A53T and E46K per liter of media (43). Monomer samples were set to aggregate and the mutant fibrillation kinetics were confirmed to be faster than WT AS (Figure 5.1a), as previously reported by monitoring Thioflavin T fluorescence (22, 24, 26-30). Fibrils were also found to have similar fibril morphology using electron microscopy (24, 32, 33), as shown in Figure 5.1b. Hydration levels were measured to be 36% water by mass as per previously published protocols for each of the samples (38). $^{13}\text{C}$-$^{13}\text{C}$ 2D experiments were acquired of each of the mutant fibril samples (Figure 5.1c,d). The spectra exhibit narrow linewidths, <0.2 ppm, indicative of well ordered and homogeneity throughout the fibrils, as previously observed for the WT (38).
5.5.2 De novo chemical shift assignments of the α-synuclein fibril mutants

*De novo* chemical shifts were assigned using multidimensional SSNMR experiments for both mutant fibrils, listed in Table 5.1. The same amide frequency in three of the spectra—NCACX, NCOCX and CAN(co)CX—can be used to make a sequential connection, resulting in site-specific chemical shift assignments, illustrated in Figure 5.2. The same can be performed using the alpha carbon frequency in the NCACX and CAN(co)CX experiments or carbonyl carbon frequency in the NCOCX and CAN(co)CX experiments. Sequential assignments for E46K were possible from K46-V49, T59-Q79, G84-F94 and G106-P108. Sequential assignments for A53T were also possible from S42-V49, V52-V55 and T59-K97. Chemical shift assignments are listed in Table 5.2 and 5.3 for E46K and A53T respectively.

5.5.3 Major chemical shift perturbations upon E46K mutation

We recently published the secondary structure of the WT, which possesses a repeated β-sheet motif: long (L38-V49), short (V52-V55), short (V63-V66), long (G68-Q79), short (T81-G84) and short (A89-K97) (44). In the WT secondary structure, E46 is located in the first of the two long β-sheet strands. In addition, a major replacement in the side-chain charge of position 46 is made upon the E46K mutation: negative to positive. These two factors may cause disruptions in salt bridges, electrostatic interactions and hydrogen bonds established in the WT structure upon fibrillation. Therefore, it is not surprising that upon mutation, the chemical shift perturbations are largely evident across the amino acid sequence, as shown in Figure 5.3.

Carbon thirteen chemical shifts are highly susceptible to secondary structure making them a useful tool for measuring perturbations upon mutation (54-58). A \(^{13}C-{^{13}}C\) 2D spectrum of E46K exhibits major perturbations compared to the WT (Figure 5.3). Correlations corresponding to
residues I88 and T92 have shifted downfield, while S87, A89, A90, A91 and G93 have all shifted upfield in the E46K spectrum relative to the WT.

5.5.4 Minor chemical shift perturbations upon A53T mutation

We find that the perturbations upon the A53T mutation are minor relative to the E46K mutation. In the WT secondary structure, A53 is located in the first of the two short β-sheet strands, where upon mutation may not cause a major change in secondary structure relative to E46K. The Ala region of the A53T AS fibril $^{13}$C-$^{13}$C 2D spectrum overlays nicely with that of the WT, of course with the exception of A53, at 50 and 21.5 ppm (Figure 5.4). The mutated Thr correlation now is detected at 61.8 (CA), 70.4 (CB) and 173.6 ppm (C’) found in the Thr region where perturbations to T54 and T59 are also evident. The CA and N chemical shift differences between WT and A53T AS fibrils are found near the mutation site, the 60s and 80s.

It is interesting to note the differences for the N, C’, CA and CB chemical shifts of residue E83: 0.2, 0.0, 0.1 and 1.4 ppm respectively (Figure 5.4). The difference between the E83 CG chemical shifts is 1.0 ppm. Figure 5.5 illustrates a possible model describing how the chemical shifts corresponding to the CB and CG of E83 can be perturbed more than 1.0 ppm while those of the N, C’ and CA are not effected within error.

5.5.5 Conformational dynamics and secondary structure comparison between the mutants and WT

In addition to chemical shifts, we can also compare the relative rigidity of the fibril core using SSNMR. The signal intensity of a dipolar-based CANCO experiment reports on dynamics qualitatively, where the higher the signal intensity the more rigid the region in question (59). It is not surprising that the region responsible for fibrillation, reported by Giasson et al. as residues
71-82 for the WT, exhibits high signal intensity regardless of mutation (Figure 5.6). However, it is of note that in the WT and A53T the signal intensity of the 90s are also strong while, this is not true for E46K. This suggests that the 90s for the E46K fibril are relatively more dynamic, which might suggest some possible differences in the fibrillation pathway.

Because chemical shifts are highly sensitive to atomic environment (54-58), backbone dihedral angles (Φ and Ψ) and secondary structure can be predicted. Chemical shift assignments were imputed into the TALOS+ program (57, 60) to obtain the backbone dihedral angels and secondary structure for E46K and A53T AS fibrils. E46K exhibits a long β-sheet strand between residues K60-Q79 and three short β-sheet strands located at residues G47-V49, G84-A85 and A89-G93. This is a large change in secondary structure compared to the WT. This is likely the result of changing a negatively charged amino acid, positioned centrally in the WT fibril core, to positive. These differences may also be related to the accelerated aggregation rate upon mutation to E46K (22). The secondary structure of A53T is similar to the WT secondary structure (44), with the exception of the placement of the last β-sheet A91-K97. The small change in amino acid—Ala to Thr—may not be enough to disrupt the packing of the fibril; thus minor perturbations are observed.

5.6 Conclusion

AS fibrils are the main component of the pathological hallmark of PD, Lewy Bodies. AS is further related to PD by the presence of three single point mutations A30P (7), E46K (8) and A53T (9). Here, we have compared the secondary structure of E46K and A53T to the WT. Our results indicate a similar secondary structure between A53T and WT AS fibrils, with slight perturbations near the mutation site, and in the 60s and 80s. We find large chemical shift
perturbations upon the E46K mutation and major perturbations in the final fibril secondary structure. The level of structural perturbation is in high agreement with our secondary structure determined for the WT (44).

5.7 References


5.8 Figures

Figure 5.1 Carefully controlled in vitro fibrillation conditions provide microscopically well-ordered mutant AS fibrils that form more rapidly than WT. (a) Average fibril formation assay of (blue squares) WT, (orange circles) E46K and (red triangles) A53T AS fibrils monitored by Thioflavin T fluorescence. Error bars were determined from seven replicates for each. (b) Comparison of the electron micrographs of (top) WT, (middle) E46K and (bottom) A53T AS fibrils. $^{13}$C-$^{13}$C 2D with 50 ms DARR mixing of (c) E46K and (d) A53T AS fibrils.
**Figure 5.2** Multidimensional spectra with high sensitivity and resolution allowed for the chemical shift assignments of A53T AS fibrils. (left) Backbone-walk schematic. (right) Illustration of backbone connectivity through the NCACX (red), NCOCX (blue) and CAN(co)CX (orange) spectra of residues A90-E83 for A53T AS fibrils. All spectra acquired with 50 ms DARR mixing acquired at 750 MHz $^1$H frequency and 12.5 kHz MAS spinning.
Figure 5.3 The E46K mutation causes major chemical shift perturbations throughout the AS fibril sequence. (left) Expansions of $^{13}\text{C}-^{13}\text{C}$ 2D spectral overlays (50 ms DARR mixing, 600 MHz $^1\text{H}$ frequency and 13.3 kHz MAS rate) of WT (blue) and E46K (orange) AS fibril samples. (right) Plot of chemical shift perturbations between WT and E46K chemical shifts versus residue number. Residues labeled as (*) correspond to perturbations above the y-axis scale. Residues labeled as (#) correspond to glycines. The mutation is indicated with (†). Error bars correspond to the chemical shift variations from one WT batch to another. WT chemical shift assignments
were obtained from the Biological Magnetic Resonance Bank #16939. E46K chemical shift assignments were obtained de novo.
Figure 5.4 The A53T mutation causes minor perturbations. (left) Expansions of $^{13}\text{C}-^{13}\text{C}$ 2D spectral overlays (50 ms DARR mixing, 600 MHz $^1\text{H}$ frequency and 13.3 kHz MAS rate) of WT (blue) and A53T (red) AS fibril samples. (right) Plot of the chemical shift perturbations between WT and A53T chemical shifts versus residue number. Residues labeled as (*) correspond to perturbations above 1 ppm. Residues labeled as (#) correspond to glycines. The mutation is indicated with (†). Error bars correspond to the chemical shift variations from one WT batch to another. WT chemical shift assignments were obtained from the Biological Magnetic Resonance Bank #16939. A53T chemical shift assignments were obtained de novo.
Figure 5.5  A possible model describing the interaction between residues E83 and A53T.
Figure 5.6  Normalized peak heights from CANCO experiment as a function of residue number for (orange) E46K, (red) A53T and WT (blue). Representation of the secondary structure of WT AS fibrils based on TALOS+ analysis (β-strands, arrows; turn or loop curved lines; not predicted, dashed lines) from Comellas et al. (2011) J. Mol. Biol. 411, 881-895. Region responsible for fibrillation highlighted with a black outlined box. Grey boxes highlight WT β-strands.
Figure 5.7 TALOS+ predicted backbone dihedral angles $\psi$ (circles) and $\phi$ (squares), error bars based on the 10 best TALOS+ database matches as a function of residue number for (a) E46K and (b) A53T. Representation of the secondary structure of WT (blue), E46K (orange) and A53T (red) AS fibrils based on TALOS+ analysis ($\beta$-strands, arrows; turn or loop curved lines; not...
5.9 Tables

Table 5.1 Description of the 2D and 3D experiments acquired in order to obtain the de novo $^{13}$C, $^{15}$N chemical shift assignments for U-$^{13}$C, $^{15}$N E46K and A53T AS fibrils. The total amount of protein was calculated based on the intensity of a $^{13}$C 1D DP experiment versus that of adamantane (the mass for adamantane was determined before packing the sample in the rotor).

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Table 5.2 $^{13}$C and $^{15}$N chemical shift assignments of E46K AS fibrils (with 36% water by mass) using only uniformly labeled samples. Mutation designated by (*).

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CHAPTER 6

FIBRILS FORMED UNDER ACIDIC CONDITIONS

6.1 Notes and Acknowledgements

I would like to thank Dr. Trent Franks, Dr. Heather Frericks Schmidt and Dr. Lindsay Sperling for helpful discussions about data collection and analysis.

6.2 Abstract

Mitochondrial deficiencies and oxidative stress caused by cytosolic acidification have been associated with Parkinson’s disease patients. This evidence motivates the isolation of differences between physiological and low pH α-synuclein (AS) fibrils. Here we have used the advantages of solid-state NMR to illustrate such differences and our results illustrate an overall perturbation to the fibril secondary structure. In particular, the chemical shifts that correspond to side-chain carboxyl groups shifted upfield; this was consistent with a decrease in pH causing the protonation of these side-chains. Investigating how the fibril structure is perturbed upon decreasing pH can provide insight into the driving force of fibril stability.

6.3 Introduction

Parkinson’s disease (PD), multiple system atrophy and many other neurodegenerative diseases have shown similar pathologies including filamentous inclusions termed Lewy bodies (1). The principal proteinaceous component of Lewy bodies is aggregated α-synuclein (AS), a natively unfolded, 140 residue protein (2). Mutations in the AS gene (A30P, E46K and A53T) have been found to lead to the familial form of PD (3-5). Protein fibrils, such as AS, are
predominantly composed of a β-sheet secondary structure. Initially, AS forms a prefibrillar intermediate termed protofibrils, which further assemble into mature fibrils (6). The detailed mechanism of how AS fibrils assemble—from monomers through protofibrils to mature fibrils—is poorly understood. The toxic state of AS, whether it is the protofibrils or the mature fibrils, and how the mechanism relates to PD, is an ongoing debate (7-9). Structural information on fibrils could facilitate answering these questions.

Different factors have been shown to significantly affect the rate of fibrillation in vitro, such as metals (10), oxidation (11), phosphorylation (12) and nitration (13, 14). In particular, lowering the pH the monomer solution causes the rate of fibrillation to increase (15). A useful consequence of this property is that reduced pH may be used to decrease sample preparation times; however, whether the structure remains the same at an atomic level requires further investigation. Decreasing pH may also cause residues with carboxylic acid side chains to become protonated. This would principally affect the C-terminus (96-140), which is predominantly composed of acidic residues (Figure 6.1). The C-terminus, in neutral pH samples, is charged and inhibits fibril aggregation (16-18); in addition, it is proposed to be unfolded in the fibril state. Recently, mitochondrial deficiencies (19-21) and oxidative stress caused by cytosolic acidification have been associated with PD cases (22). This evidence motivates the isolation of differences between physiological and low pH AS fibrils. In addition, this can provide insight into the driving force of fibril stability.
6.4 Results and discussion

6.4.1 Behavioral and physical differences between pH 5.0 and pH 7.5 AS fibrils

Experiments were performed at pH 5.0 to take advantage of the increase in fibrillation rate at a decreased pH (Figure 6.2) (15). In addition to an increased fibrillation rate, other physical properties of pH 5.0 fibrils were determined. At pH 7.5, the AS monomer required a minimum of 10 days of shaking at 37 °C to fully form fibrils. In contrast, at pH 5.0, the monomer only required 48 h. By visual inspection, fully formed pH 7.5 fibrils consisted of a highly viscous, clear gelatinous substance, where pH 5.0 fibrils appeared as an opaque white precipitate in solution (Figure 7.3). The end products were identical by visual inspection after drying (Figure 6.4); nonetheless, both had different properties. By decreasing the pH of the solution, the C-terminus became more protonated as the pH approached the pKa, 4.8, of the carboxyl side-chain groups. We hypothesize that making the C-terminus more hydrophobic will result in an attraction towards the N-terminus and drive the increase of fibril formation into a different structure.

6.4.2 Solid-state NMR spectral comparison of AS fibrils at pH 5.0 versus pH 7.5

One-dimensional (1D) $^{13}$C-detected direct-polarization (DP) magic-angle spinning (MAS) solid-state NMR (SSNMR) spectra of both uniformly-$^{13}$C, $^{15}$N labeled AS fibril samples were acquired for comparison (Figure 6.5). The pH 5.0 fibril spectra have similar chemical shift characteristics to pH 7.5 fibrils; however, slight differences are evident throughout the spectra. Spectral differences are evident through chemical shift changes and the presence or absence of certain peaks in the spectra. The slight upfield shift of the acidic residue side chain correlations are due to the protonation of those sites. When pH is decreased close to the pKa of carboxyl groups, acidic residue side chains gain a proton on the highly electron dense oxygen, and therefore, electron density is decreased. Consequentially, the carbonyl carbon became more
shielded resulting in a more upfield carbon chemical shift. In AS, the majority of glutamates and aspartates are located in the C-terminus (Figure 6.1). Thus, the side-chain carbonyl cross peaks were shifted in the pH 5.0 spectrum. Nevertheless, the resolution present in the 1D spectra is not adequate to obtain any site-specific information.

For an initial comparison, several $^{13}$C-$^{13}$C and $^{15}$N-$^{13}$C 2D correlation experiments were acquired on pH 5.0 and pH 7.5 fibrils varying in DARR (23) mixing times from 10 to 250 ms. Shorter mixing times, i.e., 10 ms, primarily result in intra-residue correlations between one or two bonds observed in the spectrum. In the case of longer mixing times, the polarization travels further resulting in longer-range correlations in the spectra. Differences between the two fibril preparations were observed in the $^{13}$C-$^{13}$C 2D spectra with 50 ms DARR mixing (Figure 6.6c, d). Side-chain carbonyl cross peaks appear to shift upfield overlapping a highly congested region in the pH 5.0 fibril spectrum compared to the pH 7.5 fibril spectrum (Figure 6.6a, b), as a result of protonation of these sites. In addition, the pH 5.0 alanine region (Figure 6.2f) displays an outlier peak at 53 and 16 ppm not found in comparable pH 7.5 fibril spectra at 0 °C. However, spectra acquired of pH 7.5 fibrils at lower temperatures exhibit the outlier peak (24). As temperature is decreased, rigid regions become more CP efficient, demonstrating this residue is mobile in pH 7.5 fibrils compared to pH 5.0 fibrils. Furthermore (Figure 6.2e), both isoleucine spin systems are prominent in the spectrum of pH 5.0 fibrils compared to the pH 7.5 fibril spectrum. Conversely, the second isoleucine spin system, Ile 112, appears in more advanced experiments, such as TEDOR (25) for pH 7.5 fibrils, where polarization transfer is less dependent on dynamics. This points towards a rigid Ile 112 in the pH 5.0 fibrils relative to the pH 7.5 fibrils. These spectral changes may be attributed to the increased propensity of the C-terminus to self-associate at pH 5.0, where a larger population of the acidic residue side-chains are protonated.
This observation further validates the possible role of the C-terminus inhibiting fibril formation in pH 7.5 fibrils (17, 18, 26).

6.4.3 Rigidity in low pH AS fibrils

Another SSNMR experiment that provides information on structural differences is the constant-time J-based experiments (27, 28). The previous dipolar-based experiments, involved polarization transfers occurring through space, which produce both intra- and inter-residue correlations. Constant-time, J-based homonuclear experiments utilize polarization transfer through scalar couplings (or through bond) solely producing correlations from single bond transfers. This produced a highly resolved spectrum, which had fewer correlations and allows for a simplified analysis. Therefore, constant-time uniform-sign cross-peak (CTUC) correlation spectroscopy (COSY) spectra of both fibril types, optimized for both the aliphatic (Figure 6.7b) and CA-CO correlations, (Figure 6.7a) were acquired.

An additional advantage to the CA-CO optimized spectrum is that it resolves correlations of the carbonyl side chains of acidic residues, which in turn provide more detailed information about the protonation state of these carbonyl side chains. The spectra of pH 7.5 fibrils were acquired with cross polarization (CP) (29), producing cross peaks from more rigid regions. The pH 5.0 fibril spectra were acquired with direct polarization (DP), producing cross peaks for the more dynamic regions. Ideally, increasing sample temperature increases the motion of dynamic regions and improves polarization transfer through random coil regions in DP experiments. Conversely, decreasing the temperature of CP experiments would increase polarization transfer through rigid regions, such as β-sheets and the fibril core of AS. Similar to the side-chain carbonyl region of the $^{13}$C-$^{13}$C 2D spectra (Figure 6.6a), upfield shifted pH 5.0 fibril cross peaks are found in the CA-CO optimized CTUC COSY spectra (Figure 6.7a). Although better resolved,
a fair comparison between the pH 5.0 fibril spectrum and the pH 7.5 spectrums could not be reached due to the differences in acquisition technique (i.e., DP versus CP). For example, the outlier peaks between ~60-50 ppm (Figure 6.7a) in the indirect dimension, characteristic of acidic CA chemical shifts, maybe attributed to the protonation of the C-terminus or regions of pH 5.0 fibrils having different dynamic properties. This, when combined with the other apparent differences, may indicate an overall structural difference of the pH 5.0 fibril and not just the C-terminus. Acquiring a DP spectrum of the pH 7.5 fibrils and a CP spectrum of the pH 5.0 fibrils can aid in defining the differences between the rigid and dynamic regions of the fibril.

Similar differences are seen in the aliphatic optimized CTUC COSY spectrum (Figure 6.6b). The pH 5.0 fibrils were acquired with DP (red) and CP (green) both at 0 °C, while the pH 7.5 fibrils were acquired with CP (blue). The DP spectrum of pH 5.0 fibrils was useful in resolving 13 new cross peaks (per side of the diagonal) in the methyl region, which contains glutamate and lysine side chain correlations (Figure 6.7c). The resonance at 30 ppm in the direct and 35 ppm in the indirect dimension is consistent with the chemical shifts of Glu C\(^{\gamma}\)-C\(^{\delta}\). In the pH 5.0 fibril spectrum this cross peak shifted slightly upfield in the indirect dimension compared to the same resonance in the pH 7.5 fibril spectrum. This is similar to the spectral differences found in the CA-CO optimized spectra (Figure 6.7a) and the \(^{13}\)C-\(^{13}\)C 2D spectra for the carbonyl side chain regions (Figure 6.6a, b). The above-mentioned outlier peak, found in the alanine region of the \(^{13}\)C-\(^{13}\)C 2D spectrum (Figure 6.6f), appears in the CP spectrum and not in the DP spectrum (Figure 6.7d) of the pH 5.0 fibril CTUC COSY experiments, which further confirms this residue is in a rigid part of the pH 5.0 fibril in contrast to the pH 7.5 fibril. Familiarization of how protonation affects regions of the fibril, by comparing site-specific information, could help understand the driving force of fibril formation. A more detailed comparison of the two fibril
preparations would be possible if the spectra were acquired with similar conditions, such as with the same hardware, temperature, pulse sequence, decoupling power and acquisition time.

6.4.4 Assigning low pH AS fibril chemical shifts site-specifically

$^{13}$C-$^{13}$C 2D spectra provide abundant information, including amino acid type and assignments. However, site-specific amino acid assignments are necessary to fully understand the structural differences between the two types of fibril preparations. Moving to further dimensions, such as NCACX and NOCCX three-dimensional (3D) spectra (Figure 6.8b, c), can be used to assign site-specific chemical shifts. These experiments possess a common $^{15}$N frequency relating two adjacent residues, i.e., the $i$ and $i\pm1$ residue (Figure 6.8a). Because $^{15}$N chemical shifts are highly dependent on solvent conditions, rigid regions of the fibril not affected by pH would display similarities in $^{13}$C frequencies and differences in $^{15}$N frequencies. However, differences can be observed in both $^{15}$N frequencies and $^{13}$C frequencies (Table 6.1). The residue side-chains exposed to solvent are more likely to experience an environmental change causing changes in chemical shifts. Being that the variations between the chemical shifts are primarily present in the fibril core; thus, differences in the homogeneity of the fibril core are likely.

6.4.5 Chemical shift differences between low pH and pH 7.5

The majority of the fibril core backbone chemical shift assignments for pH 7.5 fibrils are confirmed; however, the majority of pH 5.0 fibrils are tentatively assigned (Table 6.1). Assuming all assignments are confirmed in both types, ~30% of the chemical shifts coincide with both fibril types and ~70% of those are different within ± 0.6 ppm, Figure 6.8. Further analysis of current data sets is needed to confirm assignments and make a fair comparison between pH 5.0 and pH 7.5 fibrils. However, even in the NCC 3D experiments there are highly
degenerate planes, where foreseeable ambiguity will hinder the chemical shift assigning process. Therefore, acquiring experiments with higher resolution (further dimensions) and higher sensitivity (improved pulse sequences) will aid in identifying site-specific assignments and differences between the two types of fibrils.

6.4.6 Possible future experiments

We have observed noteworthy physical property and spectral differences between pH 5.0 and pH 7.5 fibrils. However, the SSNMR experiments acquired have only provided some site-specific information due to congestion in numerous planes, similar to other proteins studied by SSNMR (30, 31). Using an additional dimension (CNCC 4D) (32) has shown promising results by increasing resolution and facilitating site-specific assignments. Nevertheless, when DARR (23) based experiments are used, the polarization transfer efficiency from one $^{13}$C resonance to another can be 10% or less, making multidimensional experiments extremely difficult. The application of rotational resonance tickling ($R^2T$) (33) or super-cycled POST-C5 (SPC-5) (34) have both shown to increase the homonuclear polarization transfer efficiency to 50%. By increasing the polarization transfer efficiency, sensitivity is increased and acquisition times are reduced. These experiments also decrease ambiguity by eliminating long-range interresidue correlations, which appear in longer mixing DARR experiments (23). A 4D CANCOCA spectrum using $R^2T$ and a 4D CONCACB spectrum using SPC-5 of fibrils at both pH values will aid in the progress of site-specific assignments, ultimately facilitating a more detailed structural comparison between the two fibril preparations.
6.5 Conclusion

The number of neurodegenerative disease cases is steadily increasing, while the cause of such diseases is still not well understood. Furthermore, the toxic state of AS is not well defined (7-9). We anticipate that an atomic resolution structural understanding of low pH AS fibrils in comparison to physiological pH AS fibrils may lead to important insights into the mechanism of AS fibrillation and the development of new PD therapies.

6.6 References


6.7 Figures

Figure 6.1  Sequence of wild-type AS. Residues with negatively charged side chains (at pH 7) are highlighted in red, the GxxxG region is highlighted in blue and the \textit{KTKEGV} imperfect repeats are underlined in black.
Figure 6.2  AS fibrils form faster at lower pH. Comparison of pH 5.0 (blue circles) versus pH 7.5 (black squares) fibrillation rates monitored by Thioflavin T fluorescence.
Figure 6.3  Low pH AS fibrils are different than pH 7.5 fibrils by visual inspection before drying. A photograph of pH 7.5 fibrils after aggregation (a), after ultracentrifugation (b) and pH 5.0 fibrils before ultracentrifugation (c).
Figure 6.4  Low pH AS fibrils are similar to pH 7.5 fibrils by visual inspection after drying. A photograph of pH 7.5 fibrils (a) and pH 5.0 fibrils (b) after drying under a stream of nitrogen gas.
**Figure 6.5** $^{13}C$ 1D spectral comparison of pH 5.0 versus pH 7.5 fibrils illustrates differences throughout the spectra. One-dimensional (1D) $^{13}C$-detected direct-polarization (DP) spectra of WT AS (pH 5.0) spinning at 20.08 kHz MAS (red) overlaid over pH 7.5 spinning at 13.33 kHz MAS (blue). Both samples were ~10 mg and were acquired at 0 °C on a 600 MHz $^1H$ frequency spectrometer.
Figure 6.6 \(^{13}\)C-\(^{13}\)C 2D spectral comparison of WT AS (pH 7.5 fibrils, blue) with 50 ms DARR mixing (11 mg, 1.5 hrs, -10 °C, 500 MHz \(^{1}\)H frequency, 11.111 kHz MAS) versus pH 5.0 WT AS (red) (10 mg, 2.5 hrs, 0 °C, 600 MHz \(^{1}\)H frequency, 13.333 kHz MAS). The acidic residue side carbonyl region (a, b), Ile CD region (e), and Ala CA-CB region (f) are shown as expansions of the carbonyl (c) and aliphatic (d) regions.
Figure 6.7  CTUC COSY spectra optimized for the CA-CO (a) and aliphatic correlations (b) of WT AS at pH 5.0 with DP (red) and with CP (green) ($\tau = 3.6$ ms, $600$ MHz $^1$H frequency, $25$ h, $20.83$ kHz, $0$ °C) overlaid with pH 7.5 (cross polarization, $\tau = 4.5$ ms, $500$ MHz $^1$H frequency, $17$ h, $22.22$ kHz spinning, $10$ °C) (blue). Expansions of the methyl region (c) and the alanine region (d) are also shown. The lack of signal in the threonine CA-CB regions is due to a shorter $\tau$ value used in the pH 5.0 fibril spectra, where the scalar transfer is not as optimal.
Figure 6.8  Schematic of backbone walk in NCACX and NOCXL 3D experiments (a). A selected plain from both the NCACX (b) and NCACX (c) 3D experiments acquired on pH 7.5 (600 MHz 1H frequency, 74 h, 0 °C, 50 ms DARR mixing) (blue) and pH 5.0 (500 MHz 1H frequency, 60 h, -50 °C, 18 ms DARR mixing) (red) AS fibrils.
Figure 6.9  Chemical shift perturbations found throughout the AS sequence when comparing low pH to pH 7.5 AS fibrils. CA (green) and CB (purple) chemical shift differences between low pH and pH 7.5 plotted versus residue number.
### 6.8 Tables

**Table 6.1** AS pH 5.0 fibril *de novo* chemical shift assignments (ppm). Residues in italics represent low confidence in assignments.

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CHAPTER 7

THE EFFECT OF METALS ON $\alpha$-SYNUCLEIN FIBRILLATION

7.1 Notes and acknowledgements

I would like to thank Professor Chad Rienstra for help with data acquisition and Professor Julia George for helpful discussions.

7.2 Abstract

$\alpha$-Synuclein (AS) fibrils are the main component of Lewy bodies, which are indicative of Parkinson’s disease (PD) pathology. Although there is no direct evidence linking metals to neurodegenerative diseases, elevated levels of metals are found in both healthy aging brain and brain of patients with neurodegenerative diseases. However, the rate of AS fibrillation increases in the presence of certain metal ions, such as $\text{Cu}^{+2}$ (1, 2), suggesting that metals catalyze in PD susceptible patients. Identifying the region where metals cause conformational changes to the fibril structure may help reveal the role metals play in neurodegenerative diseases, such as PD.

7.3 Introduction

The rate of $\alpha$-synuclein (AS) fibrillation increases in the presence of certain metal ions, such as $\text{Cu}^{+2}$ and $\text{Fe}^{+3}$ (1, 2). In particular, $\text{Cu}^{+2}$ has a significantly high binding affinity to AS (2). The induction of nucleation by various metals including $\text{Cu}^{+2}$ has been proposed as an effect of neutralizing the repulsion by the negatively charged amino acids primarily in the C-terminus, causing the unstructured protein to collapse (1, 3). However, a truncation of the C-terminus has shown high affinity $\text{Cu}^{+2}$ binding at the N-terminus (residues 3-9 and 49-52, containing H50) in
solution (2). Further evidence has been found indicating that each Cu$^{+2}$ binding-site is independent from each other with no effect on binding affinity upon mutation of residue 50 from His to Ala (4). Currently, the AS monomer bound and unbound to Cu$^{+2}$ has been established to have a similar morphology through electron paramagnetic resonance, circular dichroism and solution NMR (1, 5). However, these techniques lack the ability to evaluate site-specific structural differences between AS bound and unbound to Cu$^{+2}$ in the fibril state. Although there is no direct evidence linking metals to neurodegenerative diseases, evidence of elevated metal levels are found in healthy aging brain and in brain of patients with neurodegenerative diseases (6), such as Fe$^{+3}$ (7, 8), Zn$^{+2}$ (9) and Cu$^{+2}$ (10). Identifying the region where metals cause conformational changes to the fibril structure may help reveal the role metals play in neurodegenerative diseases, such as Parkinson’s disease (PD).

7.4 Proposed project methodology

To investigate the effects of certain metals on AS fibrils, two types of preparations will be utilized. Water is routinely used to remove salt; however, the control samples will utilize a metal solution to identify any non-specific interactions, which can lead to false positives. Second, Cu$^{2+}$ and Fe$^{3+}$ will be added to WT AS monomer solutions before starting the fibrillation process. Another consideration should be that Cu$^{2+}$ and Fe$^{3+}$ are both paramagnetic, which may cause short T2 relaxation (11, 12) and hyperfine shifts (12, 13). Zn$^{2+}$ is commonly used to substitute or dilute paramagnetic ions to reduce these effects (14, 15). In addition, Zn$^{2+}$ is known to bind AS without perturbing the fibrillation kinetics (1) and may serve as a positive control. Short T2 relaxation results in extreme line broadening for amino acids near the mental center, making them difficult to assign. Washing paramagnetic samples with EDTA will remove excess amounts
of unbound metal, therefore, reducing the paramagnetic relaxation effect. In addition, analyzing the effects of Zn$^{2+}$ can facilitate the identification of amino acids corresponding to hyperfine shifts.

7.5 Preliminary results

7.5.1 Sample characterization

Uniformly $^{13}$C, $^{15}$N labeled wild-type AS samples were expressed and purified as per previously established protocols (16). Two samples were prepared: AS washed with a 10:1 (1) ZnCl$_2$: AS and (2) CuCl$_2$: AS molar ratio. While the zinc washed samples did not appear different from samples not washed by zinc by visual inspection, the copper washed sample exhibited a bluish-green color. Both samples were packed in 3.2-mm rotors and evaluated on a 600 MHz $^1$H frequency spectrometer.

7.5.2 Differences found after exposing $\alpha$-synuclein fibrils to copper

Although the $^{13}$C1D spectra of both samples exhibited narrow line widths, the copper sample possessed a tuning profile indicative of a salty sample, also known as the “bow-tie” effect. Therefore, more advanced experiments could not be conducted due to this limitation. Differences in peak height are evident in the CA, CB and side-chain carbonyl regions, as shown in Figure 7.1. The decrease in signal intensity of the side-chain carbonyl regions is likely due to binding of the positively charged copper cations to the negatively charged side-chain carbonyl carbons primarily found in the C-terminus. This is not surprising because it is well established that the C-terminus is mobile and free from the fibril core. However, deviations in the other regions of the spectrum are likely caused by an interaction between the copper and the protein.
7.5.3 Differences found after exposing α-synuclein fibrils to zinc

Certain amino acids (Gly, Ala, Thr, Ser, Ile, and Pro) are distinctively identifiable in two-dimensional (2D) spectra by their unique chemical shift patterns and their corresponding chemical shifts are further dispersed by secondary structure (17-21). Therefore, to effectively compare the standard AS fibril samples to those prepared with metals, $^{13}$C-$^{13}$C 2D spectra with 50 ms DARR (22) mixing were acquired to identify differences in individual amino acids and adjacent residues, eluding to structural differences. The Zn$^{+2}$ sample displayed a stable tuning profile; therefore, acquisition of multidimensional experiments on this sample was possible. The Zn$^{+2}$ sample exhibits three additional Ala residues, as shown in Figure 8.2a. No major differences were seen in the Gly region (Figure 7.2b). However, the Thr regions exhibit the emergence of five new correlations in addition to a possible Ser correlation (Figure 7.2c, d). These results suggest a stabilization of the N-terminal domain of the fibrils.

7.6 Future direction

The chemical shift differences between AS and metals (e.g., Cu$^{2+}$ and Fe$^{3+}$) (1, 2) can be assessed with samples where the addition of metals are added before and after AS fibril formation. After samples are prepared, three-dimensional experiments should be acquired on each sample to obtain chemical shift assignments. Chemical shifts can then be compared to those of standard AS fibrils to identify where these metals are binding. The chemical shift information can be further used to empirically determine the secondary structure for each of the samples. These results will lead to site-specific knowledge, which may aid in a fundamental understanding of fibril formation relevant to PD.
7.7 References


7.8 Figures

Figure 7.1 Differences found after washing α-synuclein fibrils with copper. $^{13}$C 1D spectra of α-synuclein fibrils with (green) and without (black) washing with copper.
Figure 7.2 Perturbations after washing α-synuclein fibrils with Zn⁴⁺. Expanded fingerprint regions, Ala CA-CB (a), Gly CO-CA (b), Thr CB-CA (c) and CB-CG (d) in ¹³C-¹³C 2D spectra of AS fibrils washed with zinc (red) overlaid with standard AS fibrils (blue). All spectra were acquired with 50 ms DARR mixing, at 10 °C, on a 600 MHz (¹H frequency) spectrometer and processed with 0.2 ppm of line broadening.
In Loving Memory

Luis Rodriguez

1931-2006