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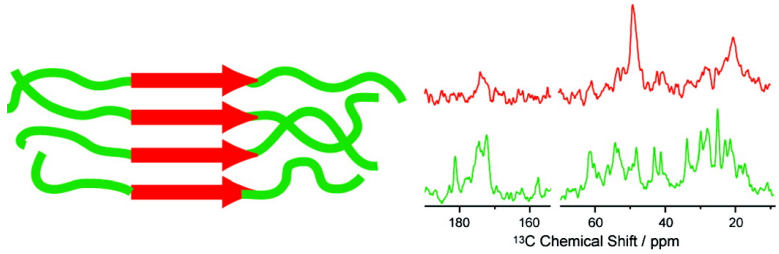

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Structural and Dynamical Characterization of Fibrils from a Disease-Associated Alanine Expansion Domain Using Proteolysis and Solid-State NMR Spectroscopy

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Codon expansions in the gene for the human nuclear poly(A) binding protein PABPN1 result in the extension of a natural oligoalanine stretch of 10 residues positioned directly after the start methionine to a maximum of 17 alanines. Individuals possessing this extension develop the disease oculopharyngeal muscular dystrophy,[1] which is histochemically characterized by amyloid-like intranuclear inclusions, containing PABPN1 as the major constituent.[2] The seven Ala extension of the protein leads to an enhanced propensity to fibrillize, as shown for the N-terminal domain of PABPN1, abbreviated N-(+7)Ala.[3] The fibrils are characterized by extreme robustness similar to spider silk, which also contain oligoalanine stretches. Recently, solid-state NMR was applied to determine the three-dimensional structure of fibrous proteins.[4] Typically, β-strand structures were identified that form the core of the fibrils with intermolecular hydrogen bonds along the filament long axis. Experiments on larger proteins indicated that fibrillar segments are flanked by highly mobile unstructured polypeptide stretches.[5] This property can be exploited as a mobility filter to simplify NMR spectra and to distinguish rigid fibrillar and highly flexible protein parts.[5a] Here, we studied the structure and dynamics of the 152 amino acid N-(+7)Ala protein fibrils. We show that quantitative order parameter measurements represent a valuable tool to identify rigid segments within fibril forming proteins.

Figure 1 shows an electron micrograph displaying the structure of the protein fibrils. Next, we recorded natural abundance ^{13}C MAS NMR spectra (shown in the Supporting Information). Depending on the NMR pulse sequence, either all or only those carbons in rigid structures are excited using direct polarization or cross-polarization (CP), respectively. The NMR spectra show remarkable differences. While a number of well-resolved lines are detected by direct polarization, the CP MAS spectrum only contains three prominent peaks exhibiting typical isotropic shifts of Ala in β-sheet conformation.[6] Indeed, the putative fibril forming part contains the Ala-rich region. A weak signal of Gly can also be identified in the CP MAS spectrum, suggesting that some glycines are part of the fibril core, while the other residues in N-(+7)Ala have to be highly mobile. It is remarkable that coarse information about the structural organization of a large fibril forming protein is available from only ~3 mg of protein and two natural abundance ^{13}C NMR spectra recorded in less than 8 h each.

To corroborate these results, proteolytic cleavage using proteinase K and thermolysin was performed. Since N-(+7)Ala fibrils resist protease digestion, mapping of the peptide sequence that is engaged in the β-sheet structure was performed by the analysis of protease-resistant peptides. Fibrils were incubated with protease and proteolysis products removed by washing with denaturant. The remaining fibrils were solubilized with guanidinium thiocyanate, and peptide fragments were separated by RP-HPLC and characterized by MALDI-TOF mass spectrometry. The major protease-resistant peptides encompassed residues 13/14 to 50−52, confirming that the poly-Ala segment forms the core of the fibril. Proteolysis products are listed in the Supporting Information.

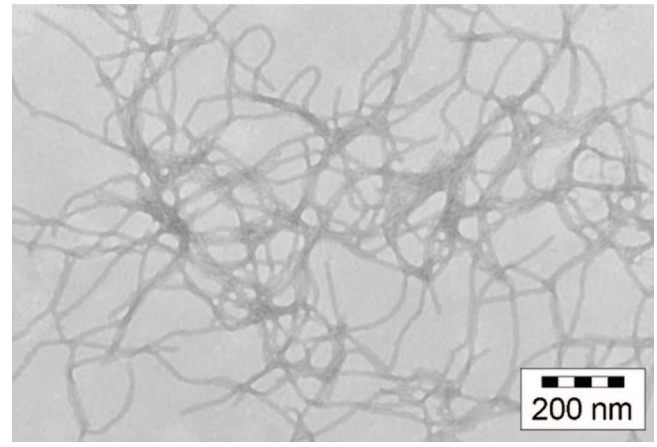

Figure 1. Electron micrographs of fibrils of N-(+7)Ala. The picture was taken after 45 days of fibril formation.

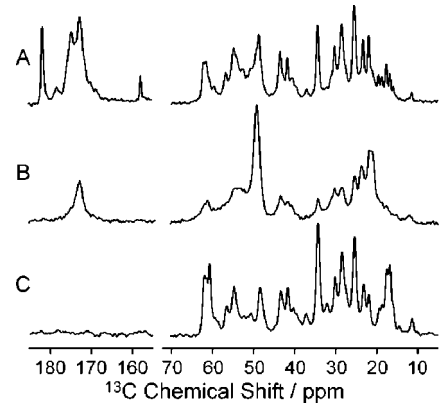

Figure 2. The 188.5 MHz ^{13}C MAS NMR spectra of ^{13}C/^{15}N N-(+7)Ala fibrils (mixture of 2.9 mg labeled and 5.8 mg unlabeled protein), acquired using (A) single pulse excitation, (B) CP, and (C) the INEPT sequence.

To obtain more information about the fibril core of N-(+7)Ala, uniformly ^{13}C/^{15}N-labeled N-(+7)Ala fibrils were prepared. In Figure 2, three ^{13}C MAS NMR spectra of N-(+7)Ala using different excitation schemes are shown. While the directly excited spectrum exhibits all carbons, the cross-polarized or INEPT spectra are sensitive for carbon spins in rigid or mobile protein structures, respectively. Such dynamic filters were already exploited in previous solid-state NMR studies on

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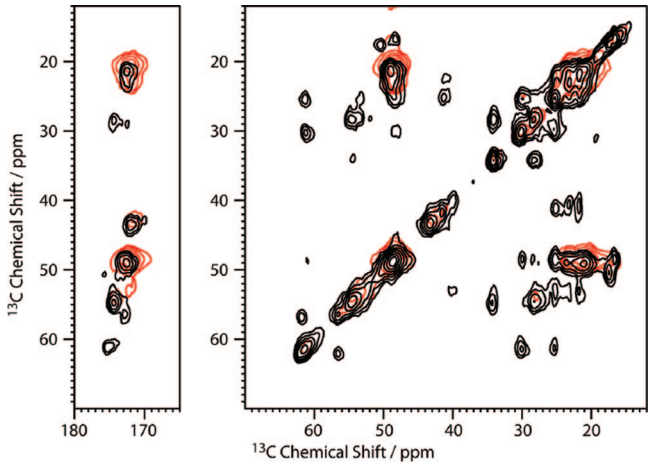

Figure 3. ^{13}C PDSP NMR spectra of N-(+7)Ala fibrils at a mixing time of 500 ms. The 2D spectra were directly polarized (black contours) or cross-polarized (red contour).

fibrillar α-synuclein.[5a] Significant differences in the line shapes of the spectra confirm our results from the natural abundance sample. The CP MAS spectrum shows the dominant Ala signals, but a few other resonances can be identified. To obtain the amino acid type assignments, we recorded ^{13}C−^{13}C proton driven spin exchange (PDSP) spectra with either direct or cross-polarization and CP- or INEPT-based ^{1}H−^{13}C HETCOR spectra (Supporting Information). Typical PDSP spectra are shown in Figure 3. Again, depending on the excitation scheme (CP or direct excitation), very different 2D spectra were obtained. The CP-based PDSP spectrum shows the characteristic cross peak pattern for Ala and Gly only. In contrast, in the directly excited PDSP spectrum, a number of correlation peaks can be assigned to Pro, Ala, Gly, Ser, and Glu. Overall, the number of cross peaks in the PDSP spectra is relatively small, which is typical for largely unstructured and highly dynamic proteins. On the basis of these data, amino acid type assignments for about 88% of the 152 residues in N-(+7)Ala could be carried out.

Full sequential assignment of N-(+7)Ala fibrils as a basis for structure calculation requires specifically labeled protein produced from partially enriched glucose or glycerol.[7] While this work is in progress in our laboratory, here we turn our attention to the molecular dynamics of the fibrils. Since the mobility filtered 1D ^{13}C MAS NMR spectra of N-(+7)Ala fibrils show significant differences, rather drastic alterations in the motional amplitudes of the protein backbone should be detectable. Therefore, we measured ^{1}H−^{13}C diploar couplings in DIPSHIFT[8] experiments either with direct excitation or CP. Only Ala and Gly showed a significant dipolar dephasing in both experiments, while the others produced much attenuated dephasing indicative of small dipolar couplings and low order parameters. The Ala order parameter was 0.77 in CP DIPSHIFT and 0.51 in directly polarized DIPSHIFT spectra. This suggests that the fibril core consists of the Ala and Gly residues, in agreement with the other results. In fact, we found the sequential cross peak between Ala and Gly Cα resonances exclusively in the CP PDSP spectra, indicative of the amino acid stretch 38−42 (AGAAG), which is also part of the fibril (spectrum shown in the Supporting Information). The protease-resistant sequence of N-(+7)Ala fibrils is somewhat larger than what has been observed by solid-state NMR. It is conceivable that the protease cannot access the residues adjacent to the fibril core because of steric hindrance, which has been reported for α-synuclein before.[9]

All other residues showed much lower order parameters (between 0.08 and 0.13). But what about the order parameters of the Ala and Gly residues that are not part of the fibril? In directly polarized DIPSHIFT spectra, all Ala residues are detected in the broad peak, and rigid and mobile residues cannot easily be distinguished. To exclusively measure the NMR signals of the mobile residues, directly excited DIPSHIFT spectra with decreased dipolar decoupling field (∼30 kHz) were acquired. Under these conditions, the rigid signals are much attenuated and only the mobile segments contribute to the NMR signal. Now also the Ala and Gly signals showed very weak order parameters (0.15 and 0.06), similar to those of other mobile residues. Dipolar dephasing curves are shown in the Supporting Information.

In summary, the Ala residues of the fibrillar part show order parameters of 0.77, while the flanking Gly are somewhat more mobile with an order parameter of 0.47. Residues that are not part of the fibril show order parameters between 0.06 and 0.15. Converted to motional amplitude, the rigid Ala undergoes small amplitude fluctuations of ∼30°, but the majority of the protein is highly flexible, undergoing motions with amplitudes between ∼75 and 85°. Motional amplitudes of fibrillar proteins have never been investigated quantitatively before. Previously, the ^{13}C NMR line width was interpreted as mobility marker in Aβ.[4b] Furthermore, mobile sites were found in HET-s by ^{1}H NMR.[5b] So far, site-specific order parameters were only available from solution NMR using Lipari Szabo analysis.[10] This first structural data for the fibrous protein PABPN1 show that dynamic mapping of fibrillar proteins by solid-state NMR may also become a very useful tool as it is for membrane proteins[11] and crystalline proteins.[12]

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Supporting Information Available: Experimental details and additional NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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