Supramolecular Interactions Probed by $^{13}C-^{13}C$ Solid-State NMR Spectroscopy

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Abstract: We present a robust solid-state NMR approach for the accurate determination of molecular interfaces in insoluble and noncrystalline protein–protein complexes. The method relies on the measurement of intermolecular $^{13}C-^{13}C$ distances in mixtures of [1-^{13}C]glucose- and [2-^{13}C]glucose-labeled proteins. We have applied this method to Parkinson's disease-associated α-synuclein fibrils and found that they are stacked in a parallel in-register arrangement. Additionally, intermolecular distance restraints for the structure determination of the fibrils at atomic resolution were measured.

The study of supramolecular interactions is crucial for understanding protein–protein complexes, molecular recognition processes, and formation of large protein self-assemblies such as amyloid fibrils. Biological complexes and assemblies having high molecular weights constitute a challenge to conventional methods for structure determination such as X-ray diffraction and solution NMR spectroscopy. Typical examples are membrane protein complexes and amyloid fibrils that are inherently insoluble and difficult to crystallize. Recent developments in solid-state NMR spectroscopy[1−5](ssNMR) have led to high-resolution spectra with separate signals arising from all ^{13}C and ^{15}N nuclei in the protein. Such studies have aimed at the 3D structure determination at ∼1 Å resolution of microcrystalline proteins,[6−10] membrane systems,[11,12] and amyloid fibrils.[13−18] The study of protein–protein interfaces in supramolecular assemblies by ssNMR has previously been carried out using equimolar mixtures of [U-^{15}N] and [U-^{13}C]-labeled proteins.[19] Intermolecular proximities (up to ∼0.5 nm) that define the molecular interface can be detected in such mixed ^{15}N/^{13}C-labeled samples using heteronuclear ^{15}N-^{13}C recoupling experiments,[20−23] yielding distance restraints for structure calculations[10] of multimeric proteins or for quaternary structure determination.[24,25] To date, the detection of intermolecular contacts by ssNMR has suffered from two disadvantages. First, the low gyromagnetic ratio of ^{15}N spins and the relatively low sensitivity of heteronuclear recoupling experiments compromise the sensitivity of the measurements. Second, the small ^{15}N spectral dispersion (∼40 ppm) restricts the application to small proteins with very high spectral resolution. Here we propose a new method for detection of supramolecular contacts in insoluble and noncrystalline biological assemblies that overcomes these limitations by using solely ^{13}C ssNMR. As an application of our approach, we have determined the supramolecular stacking of Parkinson's disease-associated α-synuclein amyloid fibrils at atomic resolution.

The method presented here relies on the use of only [1-^{13}C]glucose[26] or [2-^{13}C]glucose[27] (Glc) in bacterial growth media as the sole carbon source to produce proteins with different ^{13}C labeling patterns. The two Glc labeling patterns are quasi-complementary,

with many sites that are ^{13}C-labeled with [1-^{13}C]Glc but not with [2-^{13}C]Glc and vice versa. For serine residues, for example, use of [1-^{13}C]Glc as the carbon source produces proteins labeled only on the Cβ site, while [2-^{13}C]Glc provides ^{13}C enrichment for Cα only (Figure 1a). The same Cα/Cβ complementarity is observed for alanine residues. Therefore, while serine/alanine Cα–Cβ cross-peaks can be detected in ssNMR spectra of uniformly ^{13}C-labeled samples, they remain absent in the spectra of [1-^{13}C]Glc- or [2-^{13}C]Glc-labeled samples, provided that there is no scrambling (see below). In homo-oligomeric proteins, microcrystalline materials, and amyloid fibrils, cross-peaks in the spectra of [U-^{13}C]-labeled samples can result from intra- as well as intermolecular contacts (Figure 1b). In contrast, in [1-^{13}C]Glc- and [2-^{13}C]Glc-labeled proteins, neither intra- nor intermolecular Cα–Cβ cross-peaks are detected (Figure 1c). In the case of equimolar mixtures of [1-^{13}C]Glc- and [2-^{13}C]Glc-labeled proteins (denoted as [(1/2)-^{13}C]Glc) introduced here, only intermolecular contacts give rise to cross-peaks. In such mixtures of labeled samples, polarization transfer occurs only between serine/alanine Cα and Cβ sites in the case of an intermolecular proximity. We note that the relatively sparse labeling slows the intramolecular spin diffusion, facilitating the measurement of intermolecular long-range distance restraints.

The application of this method to amyloid fibrils is illustrated in Figure 2. Amyloid fibrils have previously been observed in a parallel in-register or an antiparallel arrangement, and the presence or absence of Cα–Cβ cross-peaks in spectra of a mixed [(1/2)-^{13}C]Glc-labeled sample allows the possible arrangements to be distinguished. Here we applied the method to human α-synuclein (hAS) amyloid fibrils. hAS in its amyloid form is the major component of Lewy bodies involved in Parkinson's disease.[28] hAS amyloid fibrils have been studied by ssNMR,[29−31] but despite their paramount importance, no atomic-resolution structure of hAS amyloids is currently available. In the previous studies, it was shown that the 140 amino acid protein hAS can form fibrils in vitro in several different polymorphic forms. Resonance assignments for these different forms have been presented mostly for the core residues ∼40−100, which are predominantly in the β-sheet conformation. We produced recombinant hAS using either [1-^{13}C]Glc, [2-^{13}C]Glc, or [U-^{13}C]Glc as the carbon source. Subsequently, fibrillized samples were prepared that were either [1-^{13}C]Glc-, [2-^{13}C]Glc-, [U-^{13}C]Glc-, or [(1/2)-^{13}C]Glc-labeled. First, 2D ^{13}C-^{13}C proton-driven spin diffusion (PDSD) spectra of [1-^{13}C]Glc and [2-^{13}C]Glc hAS samples were recorded using a mixing time of 100 ms. The spectra (Figure 2b) displayed high spectral resolution, partially as a result of the removal of one-bond ^{13}C-^{13}C dipolar and J couplings by the use of selective Glc-labeling schemes. The observation of narrow lines and the presence of a unique set of resonances (Figure 2a,b) indicate the absence of polymorphism in the molecular structure and therefore suggest that a unique supramolecular arrangement is present as well. Next, we
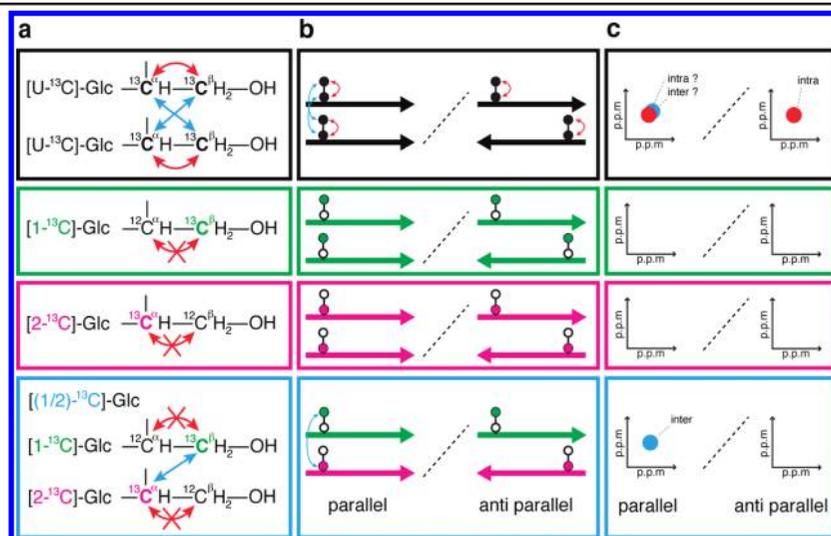
Figure 1. Illustration of the glucose-labeling strategy used for the detection of intermolecular distances by $^{13}C-^{13}C$ ssNMR. (a) Representation of the sites isotopically ^{13}C-labeled for serine using [U-^{13}C]Glc (black), [1-^{13}C]Glc (green), [2-^{13}C]Glc (pink), and mixed [(1/2)-^{13}C]Glc labeling (blue). Arrows represent the possible polarization transfers that can occur during ssNMR experiments (intramolecular, red arrows; intermolecular, blue arrows). (b) Possible polarization transfer between Cα and Cβ spins of a serine residue involved in either a parallel in-register or an antiparallel β-strand arrangement. (c) Corresponding cross-peaks in the $^{13}C-^{13}C$ ssNMR correlation spectra.
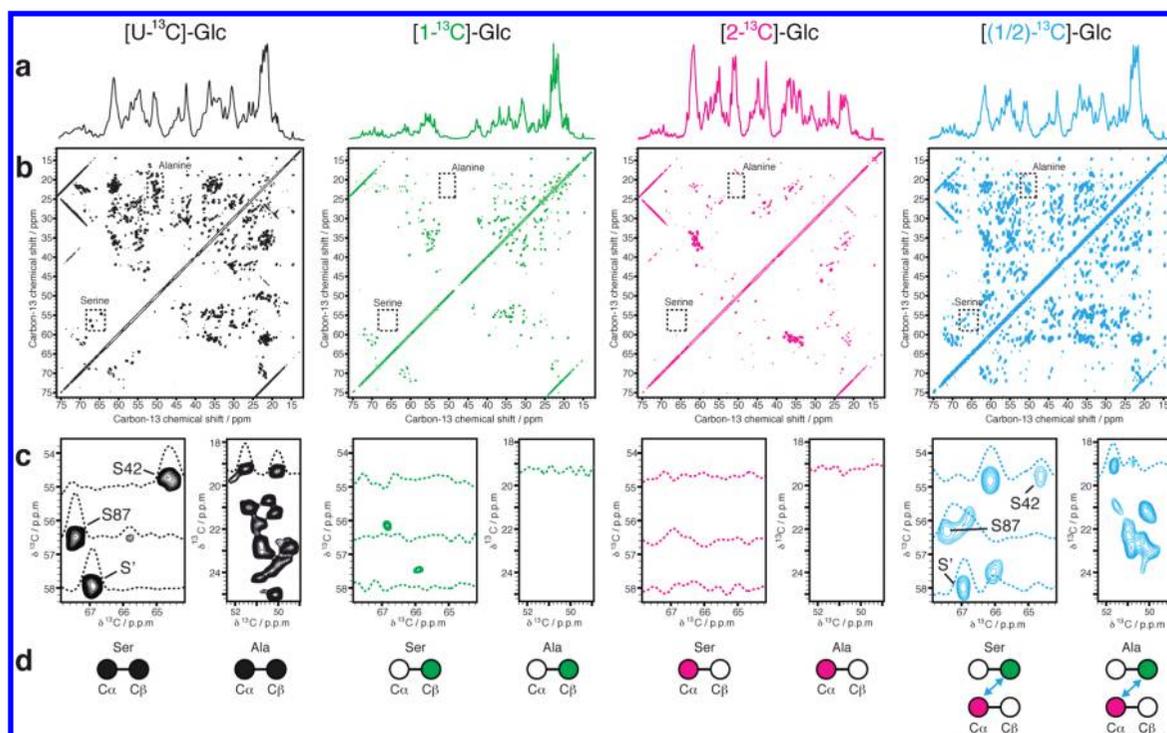

Figure 2. Elucidation of the supramolecular arrangement of hAS amyloid fibrils on the basis of ^{13}C ssNMR spectra recorded on [U-^{13}C]Glc- (black), [1-^{13}C]Glc- (green), [2-^{13}C]Glc- (pink), and [(1/2)-^{13}C]Glc-labeled (blue) hAS samples at a spinning frequency of 11 kHz on a 20.0 T spectrometer. (a) 1D ^{13}C cross-polarization spectra. (b) 2D $^{13}C-^{13}C$ PDSD spectra. Mixing times of 20 ms (for [U-^{13}C]Glc; total experiment time = 3 days) and 100 ms (for [1-^{13}C] and [2-^{13}C]Glc; 6 days) allowed for the detection of $^{13}C-^{13}C$ intraresidue contacts. A mixing time of 850 ms was chosen for the [(1/2)-^{13}C]Glc-labeled hAS sample to detect intermolecular distances (11 days). Alanine and serine regions are highlighted. (c) Excerpts of the $^{13}C-^{13}C$ PDSD spectra for the alanine and serine regions. Intraresidue $^{13}Cα-^{13}Cβ$ contacts were detected in [U-^{13}C]Glc-labeled hAS but were absent in [1-^{13}C]Glc- and [2-^{13}C]Glc-labeled hAS. For [(1/2)-^{13}C]Glc-labeled hAS, $^{13}Cα-^{13}Cβ$ contacts encoding for intermolecular proximities between adjacent hAS molecules in the fibrillar assembly were detected. The 1D traces in the ^{13}C F$_2$ dimension indicated the absence of scrambling. S′ refers to a serine that to date has not been unambiguously assigned. (d) Sketches of different isotopic ^{13}C enrichments for serine and alanine; unlabeled sites are colored in white.

recorded a PDSD spectrum of the mixed [(1/2)-^{13}C]Glc hAS sample using a mixing time of 850 ms (Figure 2b). For comparison, a PDSD spectrum of the [U-^{13}C]-labeled sample was also acquired using a mixing time of 20 ms (Figure 2b). Serines present in the rigid core of hAS fibrils (S42, S87) gave rise to Cα−Cβ cross-peaks in the spectrum of the [U-^{13}C]-labeled sample (Figure 2c).

Importantly, these cross-peaks were absent in the spectra of the [1-^{13}C]Glc- and [2-^{13}C]Glc-labeled samples, demonstrating that the labeling scheme is clean and that no scrambling is observed (traces in Figure 2c). In the spectrum of the mixed [(1/2)-^{13}C]Glc-labeled sample, Cα−Cβ cross-peaks for S42 and S87 were again observed, in this case as a result of intermolecular polarization transfer. The

presence of the S87 Cα–Cβ cross-peak, for example, in the mixed [(1/2)-^{13}C]Glc spectrum revealed a short intermolecular distance between S87 Cα of one hAS molecule and S87 Cβ of an adjacent one (Figure 2d). Additionally, two sequential correlations were observed in this region that were also present in the 100 ms PDSD spectrum of the [1-^{13}C]Glc-labeled sample. Similarly, for alanine (Figure 2c), use of the same method allowed several cross-peaks encoding for intermolecular contacts to be detected as well. Some alanine resonances were missing, which could potentially be attributed to more flexible, less ordered parts of the protein.

Our measurements therefore revealed unambiguously that the stacking of hAS amyloid fibrils takes place in a parallel, in-register manner. The spectrum of the mixed [(1/2)-^{13}C]Glc-labeled sample contained only a single set of cross-peaks with very sharp lines. This reflects a high structural order in the supramolecular arrangement of the intermolecular β-sheets. The presence of this high structural order along the fibril axis is also corroborated by single-line EPR spectra observed in amyloid fibrils of several proteins,[32] including hAS.

In conclusion, we have demonstrated the feasibility of our method for determining the atomic arrangement of supramolecular assemblies. The polarization transfers occurring during ssNMR experiments can be directly converted to short-range distances (<0.7 nm) that can be used as restraints in atomic-resolution structure calculations. As shown in more detail for serine and alanine, intermolecular distance restraints can be obtained for other residue types as well. In comparison with previous methods[19] relying on both ^{13}C and ^{15}N spins, our approach takes advantage of the higher gyromagnetic ratio (γ^{13}C/γ^{15}N $\approx$ 2.5) and larger spectral dispersion ($\sim$190 vs $\sim$40 ppm) of ^{13}C spins and allows for the use of more sensitive ^{13}C–^{13}C experiments for distance-restraint detection in comparison with their heteronuclear ^{15}N–^{13}C counterparts. We have presented here the applicability of our labeling scheme in combination with straightforward experiments such as PDSD at low spinning frequency (ν_r = 11 kHz) and anticipate its application in combination with more sophisticated pulse sequences[33−38] suitable for ssNMR measurements at higher spinning frequencies. Though here it has been demonstrated on amyloid fibrils, the method can readily be applied to a broad range of noncrystalline and insoluble supramolecular assemblies.

Supporting Information Available: Details about hAS sample preparation and solid-state NMR experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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