Resonance Assignments for Solid Peptides by Dipolar-Mediated $^{13}C/^{15}N$ Correlation Solid-State NMR

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In the past few years, a number of high-resolution solid-state NMR techniques have been developed to determine internuclear distances in biological solids such as peptides, protein aggregates, and membrane proteins.[1−10] More recently, experiments for measuring torsion angles ϕ and ψ along the polypeptide backbone with site-specific resolution were also introduced.[11−17] To utilize the measured distances and torsion angles for structure determination, it is necessary to assign the ^{13}C and ^{15}N spectral lines to specific atoms in the peptide or protein of interest. Approaches to observation of $^{15}N-^{13}C$ correlation spectra based on dipolar-coupling-mediated[18−22] and *J*-coupling-mediated coherence transfer[23,24] were introduced recently and applied to monomeric amino acids and a nucleotide. However, sequential resonance assignment of peptides and proteins in the solid state has not yet been explored experimentally. In this communication, we demonstrate the ^{13}C and ^{15}N resonance assignments of a tripeptide, Formyl-Met-Leu-Phe (MLF), by a $^{13}C-^{15}N$ correlation technique based on dipolar-mediated REDOR-type[4] polarization transfer. The efficient through-space transfer enables us to observe both intraresidue and interresidue cross-peaks in a 2D spectrum and thus achieve both sequential and intraresidue assignments simultaneously. This assignment technique has sufficient sensitivity to yield high signal-to-noise spectra on a peptide containing only ^{15}N labels and ^{13}C at natural abundance.

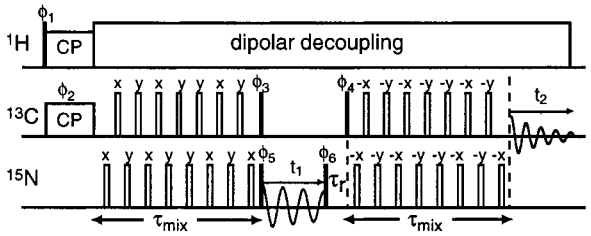

Figure 1. The ^{15}N and ^{13}C correlation sequence for resonance assignment in solid peptides. Filled and open rectangles represent 90° and 180° pulses, respectively. A REDOR sequence with XY-8 phase cycling is used to recouple the N−C dipolar interaction.[27] The ^{15}N evolution period was incremented by one rotor period for consecutive t_1 points. The method of Ruben and co-workers[30] was used to obtain the pure phase ^{15}N chemical shift spectrum in the ω_1 dimension. The phase cycles were $\phi_1 = 13$, $\phi_2 = \phi_3 = 11223344$, $\phi_4 = 33441122$, $\phi_5 = 13243142$, $\phi_6 =$ receiver $= 1324314231421324$, where 1234 represent $xy\bar{x}\bar{y}$.

The pulse sequence for the sequential ^{13}C and ^{15}N assignment of solid peptides is shown in Figure 1. After cross polarization from ^1H to ^{13}C, the ^{15}N and ^{13}C isotropic chemical shifts are probed during the evolution (t_1) and the detection (t_2) periods, respectively. The ^{15}N antiphase magnetization is transferred from ^{13}C via the $^{15}N-^{13}C$ dipolar interaction and a pair of 90° pulses on ^{13}C and ^{15}N.[22,25] The N−C dipolar interaction is prevented from refocusing under magic-angle spinning (MAS)[26] by a REDOR sequence.[4,27] After rotor-synchronous evolution of the ^{15}N chemical shift, the magnetization is transferred back to ^{13}C by a second pair of 90° pulses and an identical REDOR period, with the difference that an additional rotor period (τ_r) is inserted between the two ^{15}N and ^{13}C 90° pulses to compensate for the slight rotor desynchronization caused by the finite pulse lengths. By adjusting the length of the two REDOR mixing periods, we can selectively recouple N−C spin pairs separated by different distances and/or numbers of bonds. For example, directly bonded $^{15}N-^{13}C$ spins have a dipolar coupling constant of about 1 kHz and thus require a mixing time of about 500 μs to reach maximum signal intensity. A two-bond N−C spin pair such as $N-C^{\beta}$ also has a fixed internuclear distance as determined by the bond angle and the bond lengths, and a mixing time of about 2.5 ms is optimal for observing the corresponding cross-peaks with maximum intensity.

The 2D $^{15}N-^{13}C$ correlation experiment is applied to the ^{15}N-enriched chemotactic peptide, Formyl-Met-Leu-Phe.[28] The experiment was initially motivated by a number of distinct differences between the CPMAS ^{13}C spectrum (Figure 2a) and the solution ^{13}C spectrum of the tripeptide.[29] For example, in the solution spectrum, the middle C^{α} resonance is shifted upfield while it is equidistant from the two other C^{α} resonances in the solid spectrum. Another example is the carbonyl region around 173 ppm, where three peaks are clearly resolved in the solid

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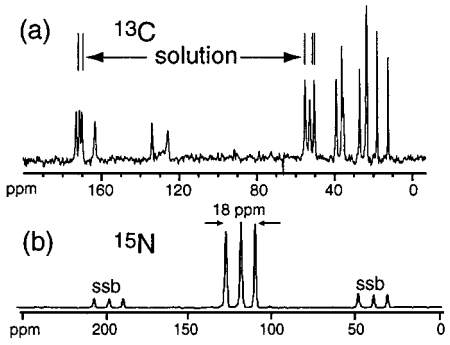

Figure 2. CPMAS spectra of Formyl-Met-Leu-Phe. (a) ^{13}C spectrum, obtained at a spinning speed of $\omega_r/2\pi = 8000$ Hz. The chemical shift scale is set relative to the most downfield C^α resonance, which is 55 ppm according to the solution ^{13}C spectrum. The positions of several peaks in the solution ^{13}C spectrum[29] are shown as vertical lines at the top of the spectrum. (b) ^{15}N spectrum, obtained at $\omega_r/2\pi = 3255$ Hz. Three backbone amide resonances with line widths of ca. 40 Hz are resolved in the centerband and the spinning sidebands (ssb). The chemical shifts are referenced to liquid NH_3 assuming that solid NH_4Cl has a chemical shift of 38.5 ppm. The peptide was custom synthesized using Fmoc chemistry (American Peptide Company, Sunnyvale, CA) from ^{15}N-labeled amino acids (Cambridge Isotope Laboratory, Andover, MA). The lyophilized powder was recrystallized by slow evaporation from 2-propanol. The spectra were obtained on a custom-designed spectrometer operating at 100.6 MHz for ^{13}C and 40.6 MHz for ^{15}N and with a triple-resonance transmission-line probe with a 5 mm Chemagnetics (Fort Collins, CO) MAS spinning module. Proton rf fields of ~100 kHz were used for excitation and decoupling.

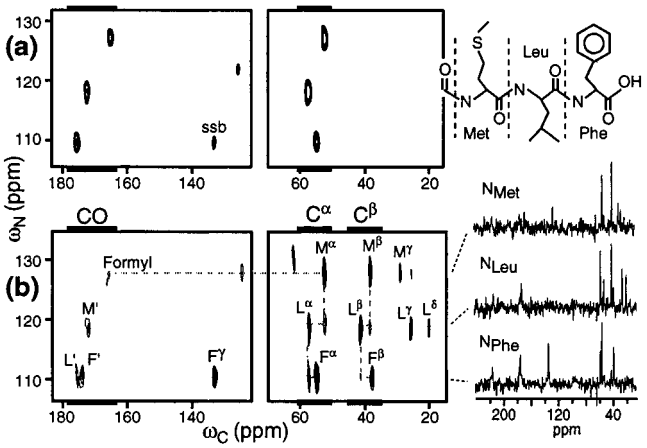

Figure 3. 2D ^{15}N−^{13}C chemical shift correlation spectra of Formyl-Met-Leu-Phe, obtained with N−C mixing times of (a) 614.4 μs ($=2\tau_r$) and (b) 2.46 ms ($=8\tau_r$). The assignments of C^α, C^β, C′, and ^{15}N resonances are illustrated by dashed lines connecting peaks with a common ^{15}N or ^{13}C frequency in spectrum (b). Horizontal bars indicate the C^α, C^β, and C′ chemical shift regions. The sample was spun at 3255 ± 2 Hz. The maximum ^{15}N evolution time was 30 rotor periods. The spectra were obtained on a custom-designed spectrometer operating at 79.7 MHz for ^{13}C and 32.1 MHz for ^{15}N. Each spectrum was acquired in 22 h.

spectrum while only two can be seen in the solution ^{13}C spectrum. Clearly, independent solid-state ^{13}C resonance assignment is required. In addition to the ambiguity of the ^{13}C spectral assignment, the ^{15}N spectrum of this compound has not been reported. Figure 2b displays the CPMAS ^{15}N spectrum, where three well-resolved peaks corresponding to the backbone amide sites are observed but the identity of each resonance awaits determination.

Figure 3 shows two 2D ^{15}N−^{13}C correlation spectra of MLF acquired with two different mixing periods. At the shorter mixing time (614 μs), each ^{15}N cross section in the spectrum (Figure 3a) exhibits two ^{13}C peaks. These correspond to the one-bond N−C connectivities including the intraresidue $N_i-C^\alpha_i$ spin pairs and interresidue $N_i-C'_{i-1}$ pairs. At the longer mixing time (2.46 ms), more aliphatic ^{13}C resonances appear in each ^{15}N cross section, with varying intensities depending on the distances of these carbons from the closest ^{15}N atom (Figure 3b). The assignment begins with the most upfield C^α peak due to an asymmetry present among the three ^{15}N cross sections: the most downfield (127 ppm) ^{15}N slice exhibits only one C^α peak while the other two slices each exhibit two C^α cross-peaks. Since the only residue in a peptide that does not experience both an intraresidue $N_i-C^\alpha_i$ dipolar coupling and an interresidue $N_i-C^\alpha_{i-1}$ coupling is the N-terminal residue, we conclude that Met accounts for the downfield ^{15}N peak and its associated C^α peak. From this starting point, it is straightforward to combine the connectivities observed in spectrum (b) with the primary sequence of the peptide to assign the rest of the N and C^α resonances. The middle ^{15}N resonance (118 ppm) results from the Leu residue while the most upfield (110 ppm) ^{15}N resonance belongs to the Phe residue.

Once the ^{15}N resonances are assigned, the carbonyl resonances C′ can also be identified. The peak at 165 ppm in the N_{Met} cross section is attributed to the formyl carbon, while the peak in the N_{Leu} slice results from C'_{Met}. The two resolved carbonyl resonances in the N_{phe} slice (Figure 3b) are assigned based on the fact that the carbonyl peak at the corresponding position in the short-mixing-time spectrum (Figure 3a) must result from the directly bonded C'_{Leu}. Thus the upfield C′ resonance in the N_{phe} slice (Figure 3b) must result from C'_{Phe}. The peak at 133 ppm in the N_{phe} cross section (Figure 3b) is attributed to a combination of the spinning sidebands of the carbonyl carbons and the quaternary carbon of the phenyl ring, since the intensity of this peak is far stronger than the corresponding first-order sideband downfield from the centerband (1D cross section). Other aliphatic ^{13}C resonances can be assigned similarly to the C^α resonances. All β carbons, C^γ_{Met}, C^γ_{Leu}, and C^δ_{Leu}, are observed, with the more intense peaks corresponding to carbons closer to the ^{15}N atoms in the covalent structure of the peptide.

In conclusion, we have shown that it is possible to resolve and assign the ^{13}C and ^{15}N resonances of a solid oligopeptide with a dipolar-mediated ^{15}N−^{13}C correlation technique. Our ^{13}C assignment differs from the solution-state assignment, which highlights the necessity of carrying out independent resonance assignment of biological solids in their native states. By using dipolar mixing periods of variable duration, both intraresidue and sequential ^{15}N−^{13}C connectivities can be obtained to yield the assignment of most ^{13}C resonances and all ^{15}N resonances. It is important to realize that although this technique relies on through-space interactions, it is suitable for revealing through-bond connectivities due to the short mixing times (<3 ms) used and the fact that tertiary-structure-induced close spatial contacts rarely form between ^{13}C and backbone ^{15}N sites. The dipolar polarization transfer is extremely efficient, as evidenced in the cross-peaks arising from ^{15}N−^{13}C spin pairs separated by as many as four bonds (Leu: N−C^δ) after merely 2.5 ms of polarization transfer. Such efficiencies in correlating distant spins in the covalent structure circumvent the use of long mixing periods that would be required for *J*-coupling-based correlation techniques. Moreover, the technique is sufficiently sensitive for a ^{13}C-unlabeled tripeptide ($M_r = 438$). Variants of the technique where the ^{15}N−^{13}C recoupling method may be optimized are anticipated to be useful for resonance assignment of larger peptides and proteins that are doubly ^{15}N/^{13}C enriched.

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