



Solid-state NMR triple-resonance backbone assignments in a protein

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Abstract

Triple-resonance solid-state NMR spectroscopy is demonstrated to sequentially assign the $^{13}\text{C}'$ and ^{15}N amide backbone resonances of adjacent residues in an oriented protein sample. The observed $^{13}\text{C}'$ chemical shift frequency provides an orientational constraint complementary to those measured from the ^1H and ^{15}N amide resonances in double-resonance experiments.

Introduction

Solid-state NMR spectroscopy is being applied to an increasing number and variety of biological systems, especially membrane proteins (Opella, 1997; Griffin, 1998). Multi-dimensional solid-state NMR correlation experiments enable many individual resonances to be resolved in spectra of uniformly ^{15}N labeled proteins in oriented samples (Jelinek et al., 1995; Marassi et al., 1997; Kim et al., 1998; Opella et al., 1999). Although some resonance assignments have been obtained by comparing spectra obtained from specifically labeled or single-site mutant protein samples, the implementation of generally applicable spectroscopic methods for making sequential resonance assignments is the next essential step in the development of solid-state NMR spectroscopy as a method for determining the structures of proteins in oriented samples. Progress has been made in the application of homonuclear dilute spin-exchange experiments to ^{15}N labeled proteins (Cross et al., 1983; Cross and Opella, 1985; Opella et al., 1999; Marassi et al., 1999). However, triple-resonance methods have great potential as a complementary approach in applications to ^{13}C and

^{15}N labeled proteins, as has been shown in solution NMR studies of proteins (Ikura et al., 1990).

Triple-resonance methods are beginning to be implemented in solid-state NMR spectroscopy (Griffin, 1998). In particular, we have recently utilized multidimensional triple-resonance solid-state NMR experiments for the resolution, assignment, and measurement of resonance frequencies from ^{13}C and ^{15}N labeled single crystal samples of a model peptide (Gu and Opella, 1999). In this article, we demonstrate the successful implementation of a member of this class of experiments on an oriented protein sample, resulting in the sequential assignment of ^{13}C and ^{15}N amide backbone resonances of adjacent residues and the measurement of ^{13}C and ^{15}N amide chemical shift frequencies as orientational constraints for structure determination.

The major coat protein (pVIII) of the filamentous bacteriophage fd is a valuable model system for solid-state NMR experiments, both as structural protein in virus particle (Cross and Opella, 1985; Jelinek et al., 1995; Tan et al., 1999) and as a membrane protein (Marassi et al., 1997). Almost all of the 50 residues in the coat protein are structured and immobile in the 1.6×10^7 Da virus particles, which orient spontaneously in the magnetic field of the NMR spectrometer. We have recently characterized a mutant bacteriophage with a single residue substitution in the major coat protein (Y21M), which has more favorable spectroscopic

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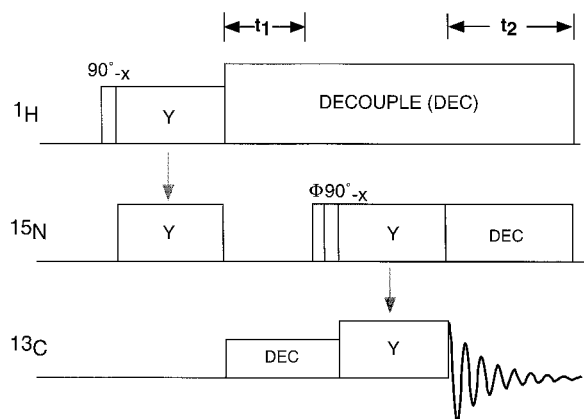


Figure 1. Pulse sequence for the two-dimensional, triple-resonance $^{13}\text{C}/^{15}\text{N}$ heteronuclear correlation experiment. The arrows depict the direction of the magnetization transfer during the experiment. The first ^1H pulse and the receiver are phase cycled to provide spin-temperature alternation.

properties than those of the wild-type coat protein (Tan et al., 1999).

Although many resonances have been resolved in solid-state NMR spectra of oriented samples of uniformly ^{15}N labeled proteins with 25–200 residues (Jelinek et al., 1995; Marassi et al., 1997; Kim et al., 1998; Opella et al., 1999), relatively few have been assigned to specific sites. Several resonance assignments have been made using ^{13}C and ^{15}N double labeled protein samples, albeit with the rudimentary observation of spectral changes due to the splitting of a ^{15}N amide resonance by a directly bonded $^{13}\text{C}'$ (Cross et al., 1985). In a somewhat more sophisticated approach, ^{15}N filtered ^{13}C NMR spectroscopy (Schneider et al., 1987; Oas et al., 1989) enabled the observation of splittings due to the heteronuclear dipolar couplings of $^{13}\text{C}'$ - ^{15}N bonds in the backbone of a double-labeled protein sample (Schneider et al., 1987). The resolution in one-dimensional spectra is severely limited, making multidimensional experiments essential.

The pulse sequence for two-dimensional $^{13}\text{C}/^{15}\text{N}$ heteronuclear chemical shift correlation spectroscopy diagrammed in Figure 1 yields spectra that correlate proximate ^{13}C and ^{15}N labeled sites. The arrows depict the paths of magnetization transfer during the experiment. Magnetization is initially transferred from ^1H to ^{15}N by conventional spin-lock cross-polarization (Pines et al., 1973). During t_1 , the magnetization evolves solely under the influence of the ^{15}N chemical shift because of the heteronuclear decoupling that results from the simultaneous application of continuous irradiation at the ^1H and ^{13}C resonance frequencies.

Two 90° pulses, phase cycled for quadrature detection (States et al., 1982), select the ^{15}N magnetization that is transferred to ^{13}C by spin-lock cross-polarization (Schaefer et al., 1979). ^{13}C magnetization is then detected in the presence of continuous ^1H and ^{15}N irradiation during t_2 . Since there are no ^{13}C nuclei directly bonded to other ^{13}C nuclei in selectively ^{13}C and ^{15}N labeled protein samples, homonuclear ^{13}C decoupling is unnecessary. However, the integration of homonuclear ^{13}C decoupling into the pulse sequences for triple-resonance experiments will enable them to be applied to uniformly ^{13}C and ^{15}N labeled protein samples (Schmidt-Rohr, 1998; Gu and Opella, 1999).

Materials and methods

1- ^{13}C L-phenylalanine and α - ^{15}N L-lysine.2HCl were obtained from Cambridge Isotope Laboratories (Andover, MA). The selectively ^{15}N -Lys and $^{13}\text{C}'$ -Phe labeled protein samples were prepared by infecting *E. coli* TG1 (RecO) cells with Y21M fd bacteriophage grown in minimal media, which was supplemented with 100 mg/L of both of the labeled amino acids, 200 mg/L of all other amino acids except proline, and 1.0 g/L of unlabeled $(\text{NH}_4)_2\text{SO}_4$. A solution containing the amino acids was added just prior to infection of the bacterial growth to minimize scrambling and to maximize incorporation of the labeled amino acids into the viral coat protein. Purification of the virus particles followed the protocol of Malik et al. (1996). The NMR experiments were performed on a concentrated solution of fd phage (40 mg/mL) buffered at pH 8.0 with 5 mM sodium borate with 0.1 mM sodium azide added as a preservative.

The solid-state NMR experiments were performed at 15°C on a home-built triple-resonance spectrometer with a 12.9 T wide-bore Magnex 550/89 magnet. The probe had a single 5 mm ID solenoid coil triple-tuned to the ^1H , ^{13}C , and ^{15}N resonance frequencies of 549.8, 138.3, and 55.7 MHz, respectively. An RF field strength of 50 kHz was utilized on all three channels and this corresponds to an off-resonance Lee–Goldburg (1965) jump frequency of ± 35 kHz. In the $^1\text{H}/^{15}\text{N}$ PISEMA experiment (Wu et al., 1994), continuous ^{13}C irradiation with an RF field strength of 40 kHz was used for heteronuclear decoupling during t_1 and t_2 . In the $^{13}\text{C}/^{15}\text{N}$ correlation experiment, a cross-polarization mix time of 1.0 ms was used to transfer magnetization from the ^{15}N amide of Lys 43 to the $^{13}\text{C}'$ of Phe 42. During the ^{15}N to

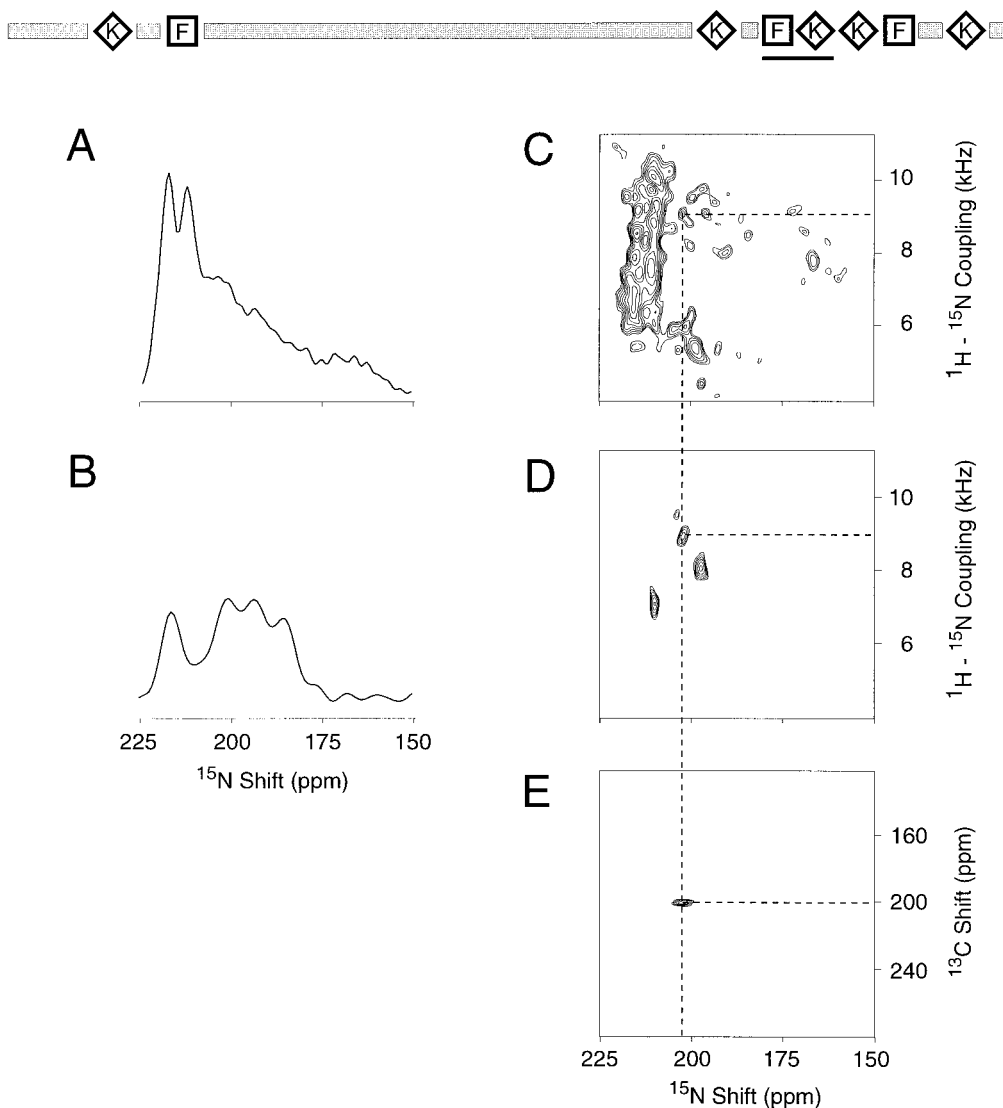


Figure 2. Experimental solid-state NMR spectra of oriented Y21M fd bacteriophage. (A) One-dimensional ^{15}N NMR spectrum of uniformly ^{15}N labeled fd bacteriophage. (B) ^{13}C decoupled one-dimensional ^{15}N NMR spectrum of ^{15}N Lys and $^{13}\text{C}'$ Phe labeled fd bacteriophage. (C) Two-dimensional $^1\text{H}/^{15}\text{N}$ PISEMA spectrum of uniformly ^{15}N labeled fd bacteriophage. (D) ^{13}C decoupled two-dimensional $^1\text{H}/^{15}\text{N}$ PISEMA spectrum of ^{15}N Lys and $^{13}\text{C}'$ Phe labeled fd bacteriophage. (E) Two-dimensional $^{13}\text{C}/^{15}\text{N}$ correlation spectrum of ^{15}N Lys and $^{13}\text{C}'$ Phe labeled fd bacteriophage. A schematic diagram indicating the distribution of the five Lys (residues 8, 40, 43, 44, and 48) and three Phe (residues 11, 42, and 45) is shown at the top. The single $^{13}\text{C}-^{15}\text{N}$ bond in the protein is underlined.

^{13}C cross-polarization mix period, continuous ^1H irradiation with an RF field strength of 83 kHz was applied. 38 t_1 increments of 40.8 μs were used in the PISEMA experiment. 32 t_1 increments of 20 μs were used in the $^{13}\text{C}/^{15}\text{N}$ correlation experiment with 256 scans co-added during each t_1 increment. A recycle delay of 5 s was used in combination with a flip-back pulse (Tegenfeldt and Haerbelen, 1979) to preserve ^1H magnetization. The experimental scale factors were

measured to be $0.81 \pm .01$ during t_1 of the PISEMA experiment. The ^{13}C and ^{15}N chemical shifts were referenced to the ^{13}C frequency of the deshielded peak of adamantane at 38.6 ppm and the ^{15}N frequency of liquid ammonia at 0 ppm, respectively. The experimental NMR data were processed using Felix 95 (MSI, San Diego, CA).

Results and discussion

Solid-state NMR spectra of oriented samples of fd bacteriophage are shown in Figure 2. The approximately 2700 copies of the major coat protein in each bacteriophage particle are symmetrically arranged around the viral DNA along the filament axis parallel to the direction of the applied magnetic field. A total of 44 amide and one indole (Trp-26 side chain) resonances are expected in the displayed region of the spectra of uniformly ^{15}N labeled samples. The first 4–5 residues are mobile and contribute isotropic resonances, Ala-1 and the five Lys side chains contribute amino resonances, Gln-15 a side-chain resonance, and Pro-6 an imino resonance (Cross and Opella, 1985). The sample used in the present study is labeled with ^{15}N -Lys (five sites; diamonds) and $^{13}\text{C}'$ -Phe (three sites; squares); their distribution in the protein sequence is shown schematically at the top of Figure 2. The single $^{13}\text{C}'$ - ^{15}N bond in the protein (underlined) enables this sample to serve as a test case for unambiguous assignment of the ^{15}N amide resonance to a specific residue (n) and the $^{13}\text{C}'$ resonance to the preceding residue ($n - 1$) using triple-resonance solid-state NMR methods (Gu and Opella, 1999).

The one-dimensional ^{15}N NMR spectra in Figures 2A and 2B demonstrate why it is essential to develop multi-dimensional methods for solid-state NMR studies of oriented proteins. fd coat protein is a particularly challenging case because its secondary structure is nearly all α -helix, with the amide N-H bonds aligned approximately parallel to the direction of the applied magnetic field (Banner et al., 1981; Opella et al., 1987). However, this arrangement is representative of membrane proteins with multiple trans-membrane helices in oriented bilayers (Kim et al., 1998). The observed frequency differences arise from variations in molecular orientations; thus, there is very limited dispersion among the ^{15}N chemical shifts of the uniformly ^{15}N labeled sample in Figure 2A. Solid-state NMR spectra of ^{15}N Lys and $^{13}\text{C}'$ Phe labeled fd coat protein in oriented virus particles are shown in Figures 2B, 2D, and 2E. Even with ^{13}C decoupling, only partial resolution is observed among the five ^{15}N labeled sites in the one-dimensional spectrum in Figure 2B.

The combination of ^1H - ^{15}N heteronuclear dipolar coupling and ^{15}N chemical shift frequencies is sufficient to resolve many resonances in the two-dimensional PISEMA spectrum of uniformly ^{15}N labeled coat protein shown in Figure 2C. We have

improved upon our earlier results (Jelinek et al., 1995) largely by taking advantage of the properties of the Y21M mutant coat protein (Tan et al., 1999). Complete spectral resolution can now be obtained in three-dimensional correlation spectra with the ^1H chemical shift as the third frequency dimension (Ramamoorthy et al., 1995).

The comparison of the two-dimensional PISEMA spectra of uniformly ^{15}N (Figure 2C) and selectively ^{15}N Lys (Figure 2D) labeled samples enable the assignment of resonances by residue type. The two-dimensional $^{13}\text{C}'$ / ^{15}N correlation spectrum in Figure 2E was obtained using the pulse sequence diagrammed in Figure 1. As expected, one correlation peak is observed from the $^{13}\text{C}'$ Phe-42- ^{15}N Lys-43 bond. The vertical dotted line shows the similarities of the ^{15}N chemical shifts for resonances identifiable in the two-dimensional spectra in Figures 2C, 2D, and 2E. This enables a resonance in the two-dimensional PISEMA spectrum of uniformly ^{15}N labeled coat protein to be assigned to Lys-43. It also assigns the $^{13}\text{C}'$ resonance in the spectrum in Figure 2E to Phe-42.

The experimental results in Figures 2C-2E not only demonstrate a method for sequential resonance assignment, but also the measurement of both ^{15}N and $^{13}\text{C}'$ chemical shift frequencies in a single two-dimensional spectrum. The two-dimensional PISEMA spectrum then yields the ^1H - ^{15}N dipolar coupling frequency for the amide site. Thus, the combination of data in Figures 2C and 2E yields the sequential assignment and measurement of the ^{15}N chemical shift (202 ppm) and ^1H - ^{15}N dipolar coupling (9.0 kHz) frequencies of the amide of Lys-43 (n residue) as well as the $^{13}\text{C}'$ chemical shift (201 ppm) frequency of Phe-42 ($n - 1$ residue). These frequencies are powerful orientational constraints, and are sufficient to determine the orientation of the peptide plane of Phe-42/Lys-43 with high precision with respect to the direction of the applied magnetic field. This is illustrated graphically in Figure 3.

The orientational constraints contained in the experimental frequencies are expressed as a function of the angles α and β , which describe the orientation of the peptide plane relative to the applied magnetic field, B_0 (Opella et al., 1987). We utilize values for the magnitudes and orientations of the principal values of the chemical shift that are the average of all available for non-glycine L-amino acids in peptide bonds (Asakawa et al., 1992; Hartzell et al., 1987; Lograsso et al., 1989; Mai et al., 1993; Shoji et al., 1990; Teng and Cross, 1989; Teng et al., 1992; Wang et al., 1992; Wu et al.,

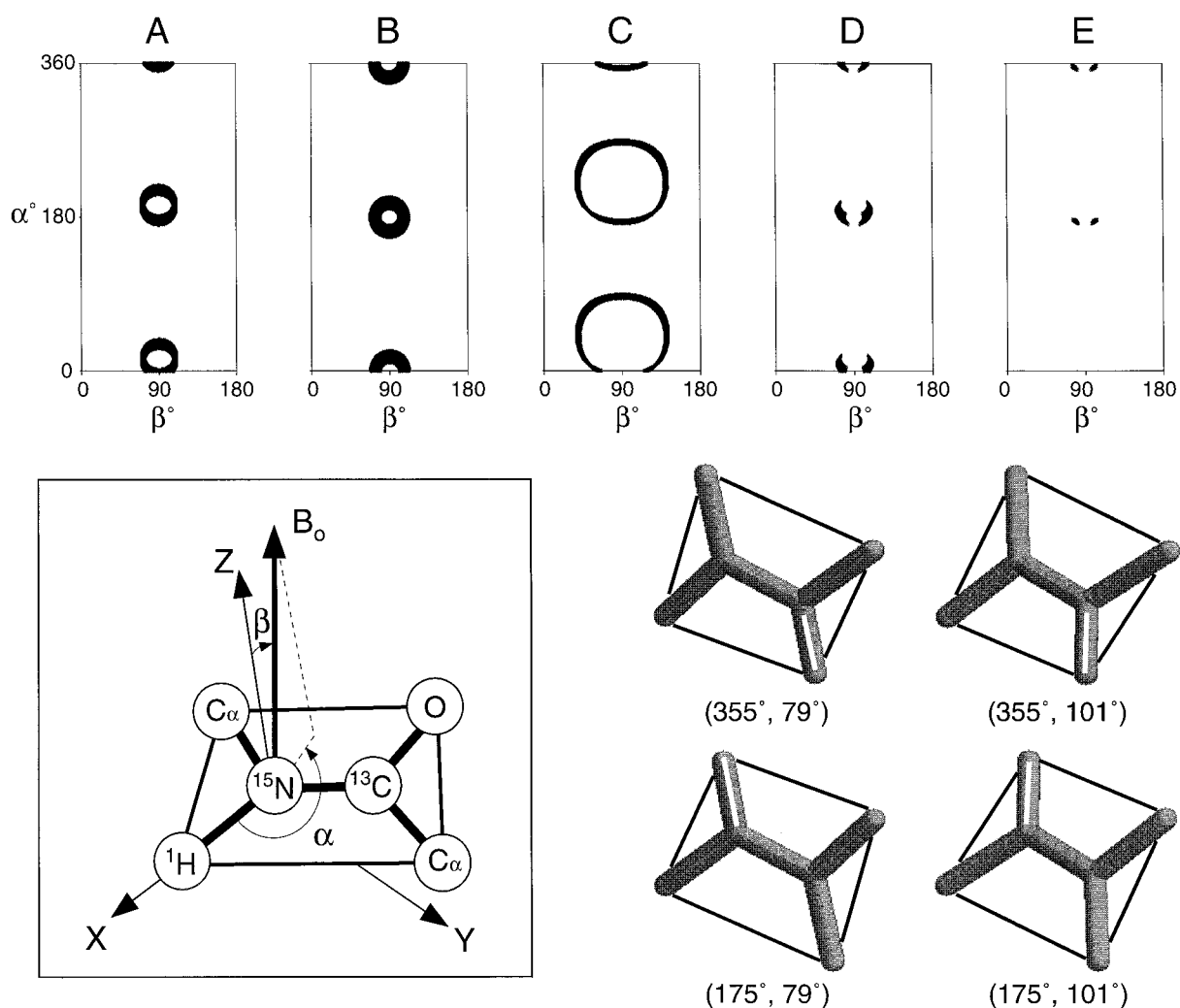


Figure 3. Restriction plots derived from the frequencies measured from the resonances in Figures 2C, 2D, and 2E as shown with the dotted lines: ^{15}N chemical shift of 202 ± 1 ppm; ^1H - ^{15}N dipolar coupling of 18 ± 1 kHz; $^{13}\text{C}'$ chemical shift of 201 ± 1 ppm. The dark areas indicate the combinations of the angles α and β which are consistent with the measured frequencies. The intersection of the restriction plots in A and B and in A, B, and C are given in D and E, respectively. α is the angle between the N-H bond and the projection of B_0 (applied magnetic field) onto the peptide plane. β is the angle between B_0 and the normal to the peptide plane (Opella et al., 1987). The four possible orientations of the peptide plane which satisfy the restriction angles (α, β) in Figure 3E are shown. The NH bond vector is indicated by a white line.

1995). The values for the amide ^{15}N chemical shift tensor are: σ_{11} , 56 ± 2 ppm (in the peptide plane); σ_{22} , 77 ± 4 ppm (orthogonal to the peptide plane); σ_{33} , 217 ± 3 ppm (in the peptide plane and $14^\circ \pm 3^\circ$ away from the N-H bond). The corresponding values for the $^{13}\text{C}'$ chemical shift tensor are: σ_{11} , 244 ± 1 ppm (in the peptide plane); σ_{22} , 175 ± 5 ppm (along the C = O bond); σ_{33} , 90 ± 4 ppm (orthogonal to the peptide plane). The N-H bond length used in the calculations was 1.05 ± 0.04 Å. The ^{15}N chemical shift values are relative to liquid NH_3 , while the ^{13}C values are relative to TMS.

The combinations of α and β angles that are consistent with the ^{15}N chemical shift (Figure 3A), ^1H - ^{15}N dipolar coupling (Figure 3B) and $^{13}\text{C}'$ chemical shift (Figure 3C) frequencies are shown in Figure 3. The axis system is defined and the four peptide plane orientations consistent with the NMR data are also shown in this figure. The intersection of the angular constraints in Figures 3A and 3B are shown in Figure 3D, and those for the constraints in Figures 3A, 3B, and 3C are shown in Figure 3E. The comparison of dark areas in Figures 3D and 3E illustrates the value of the $^{13}\text{C}'$ chemical shift frequency in reducing the

possible orientations consistent with the experimental data. Even though the principal values of the chemical shift tensors have uncertainties of around 5%, the final intersection plot has α and β angles that span no more than 5° and 8°, respectively. More importantly, these three experimental measurements are sufficient to identify four symmetry related peptide plane orientations. Additional restrictions can be derived from the measurement of other frequencies, such as those associated with $^{13}\text{C}'\text{-}^{15}\text{N}$, $^{13}\text{C}'\text{-}^1\text{H}$ dipolar couplings, or ^1H chemical shifts.

The experimental spectra in Figure 2 demonstrate that multidimensional triple-resonance solid-state NMR experiments can be applied to protein samples. In particular, the two-dimensional $^{13}\text{C}/^{15}\text{N}$ correlation spectrum provided the sequential assignment of a resonance in the two-dimensional $^1\text{H}/^{15}\text{N}$ PISEMA spectrum of uniformly ^{15}N labeled coat protein to Lys-43. It also enabled the measurement of the $^{13}\text{C}'$ chemical shift frequency of the preceding residue, Phe-42, which adds a significant orientational constraint for structure determination. These results demonstrate that multidimensional triple-resonance solid-state NMR experiments can be used for enhancing resolution, measuring multiple orientationally dependent spectral parameters, and making sequential assignments with selectively, and potentially uniformly, ^{13}C and ^{15}N labeled proteins in oriented samples.

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