Journal of Magnetic Resonance **138,** 193–198 (1999)
Article ID jmre.1999.1709, available online at http://www.idealibrary.com on IDEAL®

Three-Dimensional ^{13}C Shift/^{1}H–^{15}N Coupling/^{15}N Shift Solid-State NMR Correlation Spectroscopy

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Received July 14, 1998; revised September 22, 1998

Triple-resonance experiments capable of correlating directly bonded and proximate carbon and nitrogen backbone sites of uniformly ^{13}C- and ^{15}N-labeled peptides in stationary oriented samples are described. The pulse sequences integrate cross-polarization from ^{1}H to ^{13}C and from ^{13}C to ^{15}N with flip-flop (phase and frequency switched) Lee–Goldburg irradiation for both ^{13}C homonuclear decoupling and ^{1}H–^{15}N spin exchange at the magic angle. Because heteronuclear decoupling is applied throughout, the three-dimensional pulse sequence yields ^{13}C shift/^{1}H–^{15}N coupling/^{15}N shift correlation spectra with single-line resonances in all three frequency dimensions. Not only do the three-dimensional spectra correlate ^{13}C and ^{15}N resonances, they are well resolved due to the three independent frequency dimensions, and they can provide up to four orientationally dependent frequencies as input for structure determination. These experiments have the potential to make sequential backbone resonance assignments in uniformly ^{13}C- and ^{15}N-labeled proteins.

Key Words: **triple-resonance; three-dimensional; cross-polarization; solid-state NMR spectroscopy; assignment; peptide.**

INTRODUCTION

Now that it is possible to obtain highly resolved solid-state NMR spectra of uniformly ^{15}N-labeled membrane proteins in oriented bilayer samples (*1, 2*), efficient sequential resonance assignment methods are needed. In this article, we describe a triple-resonance experiment capable of correlating both directly bonded and proximate carbon and nitrogen backbone sites of uniformly ^{13}C- and ^{15}N-labeled polypeptides in stationary oriented samples. This approach is influenced by the variety of heteronuclear experiments developed for making sequential assignments in solution NMR studies of proteins (*3*), and a method for assigning ^{13}C and ^{15}N resonances of powder samples in magic angle sample spinning experiments demonstrated by Sun *et al.* (*4*).

Both the two- and the three-dimensional versions of the experiment diagrammed in Fig. 1 integrate cross-polarization from ^{1}H to ^{13}C, flip-flop (phase and frequency switched) Lee–Goldburg irradiation (*5–7*) for ^{13}C homonuclear decoupling during t_1, cross-polarization from ^{13}C to ^{15}N (*8*), and, finally, detection of ^{1}H and ^{13}C decoupled ^{15}N signals. The three-dimensional experiment adds an interval of spin exchange at the magic angle (SEMA) (*9*) for measurement of ^{1}H–^{15}N heteronuclear dipolar coupling frequencies. Heteronuclear decoupling is accomplished throughout the experiment with continuous RF irradiation at the ^{1}H, ^{15}N, and ^{13}C resonance frequencies. The resulting spectra have intrinsically high resolution with single-line resonances in all frequency dimensions, correlate ^{13}C and ^{15}N resonances from directly bonded and proximate molecular sites, and provide up to four orientationally dependent frequencies for each residue as input for structure determination (*10*).

The results of the triple-resonance experiment are illustrated with spectra of single crystal samples of ^{13}C- and ^{15}N-labeled *N*-acetylglycine (NAG), which serves as a model for the backbone of uniformly ^{13}C- and ^{15}N-labeled polypeptides. The chemical and crystal structures (*11*) of *N*-acetylglycine are shown in Fig. 2. There are two magnetically inequivalent molecules in each unit cell. The one-dimensional ^{13}C shift spectra demonstrate the ability of the experimental procedures to decouple ^{13}C homonuclear dipolar interactions between directly bonded ^{13}C sites. The two-dimensional ^{13}C shift/^{15}N shift spectrum demonstrates the correlation of the amide ^{15}N resonance with the directly bonded carbonyl carbon and α-carbon ^{13}C resonances. The three-dimensional ^{13}C shift/^{1}H–^{15}N coupling/^{15}N shift spectrum is displayed as a cube and can be analyzed as two-dimensional ^{13}C shift/^{15}N shift planes associated with the ^{1}H–^{15}N coupling frequencies for individual amide sites.

RESULTS AND DISCUSSION

The pulse sequence for the three-dimensional version of the triple-resonance experiment is shown in Fig. 1B. It starts with conventional spin-lock cross-polarization from ^{1}H to ^{13}C, followed by flip-flop Lee–Goldburg irradiation that spin locks the resulting ^{13}C magnetization at the magic angle where it evolves during the t_1 period. The combination of flip-flop Lee–Goldburg irradiation of the ^{13}C spins and continuous irradiation of the ^{1}H and ^{15}N spins suppresses both ^{13}C homonuclear and ^{1}H/^{13}C and ^{15}N/^{13}C heteronuclear dipolar couplings during the t_1 period. Thus, during t_1 the ^{13}C magnetization is affected

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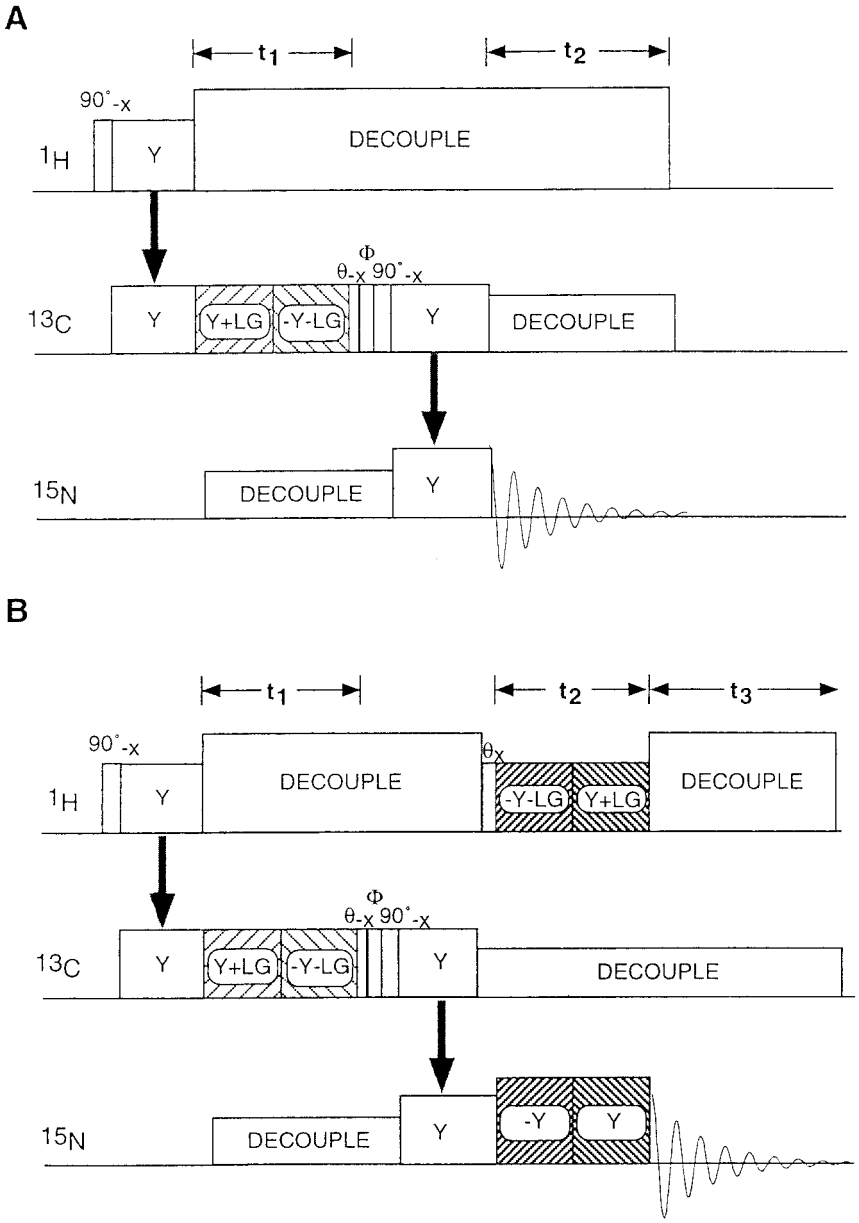

FIG. 1. (A) Pulse sequence for two-dimensional, triple-resonance $^{13}C/^{15}N$ heteronuclear correlation spectroscopy. Each t_1 increment corresponds to an integral number of cycles defined by the flip-flop Lee–Goldburg irradiation. (B) Pulse sequence for a three-dimensional, triple-resonance $^{13}C/^{1}H–^{15}N/^{15}N$ experiment. Each t_1 and t_2 increment corresponds to an integral number of cycles defined by the flip-flop Lee–Goldburg irradation.

only by the ^{13}C chemical shift interaction, which is scaled by 0.58 due to the homonuclear decoupling. Each t_1 increment corresponds to one complete flip-flop Lee–Goldburg cycle; Y + LG indicates that the RF irradiation has Y phase and a positive frequency offset, and $-Y$ − LG corresponds to $-Y$ phase and negative frequency offset. The X-phase pulse labeled θ effects a 35.3° nutation of the ^{13}C magnetization that returns it to the transverse plane where a 90° pulse, labeled ϕ, is phase cycled to achieve quadrature detection in t_1 (*12*), selects the ^{13}C magnetization that is transferred to ^{15}N via spin-lock cross-polarization. The ^{15}N magnetization evolves under only the $^{1}H–^{15}N$ heteronuclear dipolar coupling interaction during the t_2 period because the SEMA procedure removes the effects of ^{1}H homonuclear dipolar couplings and both ^{1}H and ^{15}N chemical shifts. The $^{1}H–^{15}N$ heteronuclear dipolar coupling frequencies are scaled by 0.82 in the SEMA procedure. Finally, unscaled ^{15}N signals are acquired during t_3 in the presence of continuous ^{1}H and ^{13}C irradiation for heteronuclear decoupling. The two-dimensional version of the experiment deletes the $^{1}H–^{15}N$ coupling frequency dimension. The key features in the pulse sequences are the flip-flop Lee–Goldburg irradiation to decouple the homonuclear ^{13}C dipolar

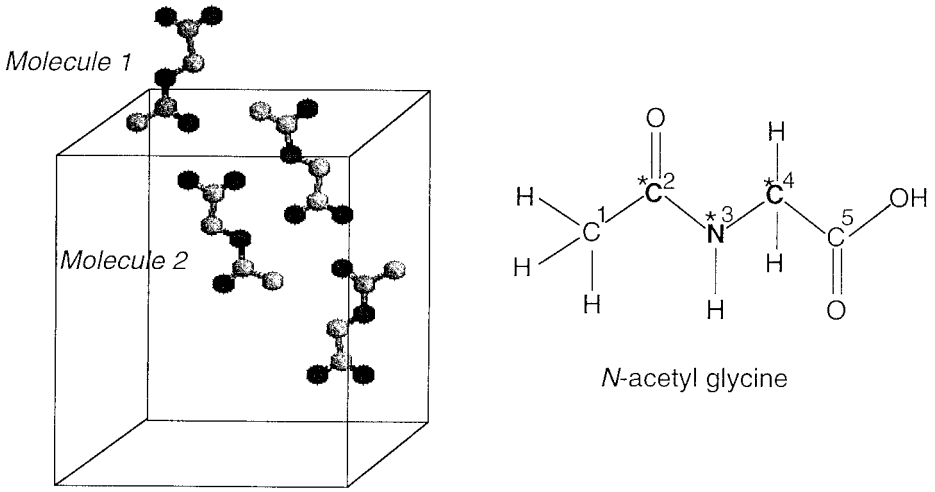

FIG. 2. The chemical and crystal structures of *N*-acetylglycine (*11*). All four molecules in a unit cell are shown and there are two magnetically inequivalent molecules marked as *molecule 1* and *molecule 2.* The chemical structure and its numbering are shown on the right.

interactions and the use of ^{13}C to ^{15}N cross-polarization for sequential transfer of magnetization along the peptide backbone. The field strength of the ^{1}H irradiation must exceed by a substantial margin the field strengths of the irradiations applied to the ^{15}N and ^{13}C spins in order to achieve efficient decoupling and narrow ^{13}C and ^{15}N resonance linewidths (*13*).

Polarization transfer experiments on dilute heteronuclear spin pairs (e.g., ^{13}C–^{15}N) have been demonstrated to provide local structural information (*7, 13, 14*). The MOIST version of spin-lock cross-polarization (*15*) provides reliable and efficient transfer of magnetization from ^{13}C to ^{15}N in the presence of ^{1}H decoupling irradiation. The polarization transfer rate is on the order of heteronuclear dipolar coupling frequency. For example, in the case of a directly bonded ^{13}C–^{15}N spin pair, the dipolar coupling can be as large as 2 kHz, suggesting a cross-polarization mix time of around 0.5 ms for efficient transfer. Polarization transfer between proximate nonbonded ^{13}C–^{15}N spin pairs with larger internuclear distances can be accomplished with longer cross-polarization mix periods. The different responses to cross-polarization mix times can be used to good advantage to separately correlate bonded and more distant heteronuclear spin pairs for both assignment and structure determination purposes. Since the polarization transfer between ^{13}C and ^{15}N occurs in the rotating frame, the resulting cross peaks are always positive. This is advantageous since it eliminates the possibility of mixed positive and negative cross peaks, which limits the applicability of INEPT-type heteronuclear transfer experiments (*16*).

Figure 3 contains a two-dimensional ^{13}C shift/^{15}N shift correlation NMR spectrum of a single crystal of 2,4-^{13}C, 3-^{15}N labeled NAG at an arbitrary orientation with respect to the magnetic field. This spectrum was obtained with the pulse sequence diagrammed in Fig. 1A. The ^{13}C to^{15}N cross-polarization mix time was 1.0 ms. Each NAG molecule contains one ^{15}N amide site and there are two magnetically inequivalent molecules in each unit cell. Two resonances are observed in the ^{15}N chemical shift frequency because each ^{15}N resonance in the spectrum is correlated with the carbonyl carbon and the α-carbon ^{13}C resonances that arise from magnetization transferred from the respective neighboring ^{13}C-labeled sites. The contours describe single-line resonances in both the ^{15}N shift and the ^{13}C shift frequency dimensions, which is a consequence of the effects of full homonuclear and heteronuclear decoupling during both incremented intervals in the pulse sequence.

The cube in Fig. 4 corresponds to a three-dimensional ^{13}C shift/^{1}H–^{15}N coupling/^{15}N shift correlation spectrum obtained

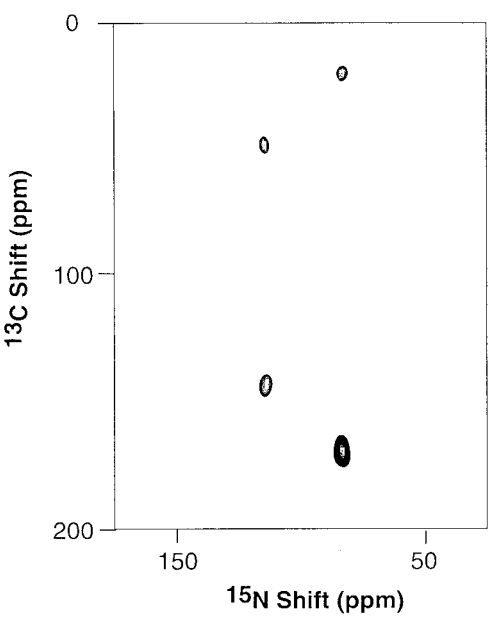

FIG. 3. Experimental two-dimensional ^{13}C shift/^{15}N shift correlation NMR spectrum of a single crystal sample of 2,4-^{13}C, 3-^{15}N NAG at an arbitrary orientation with respect to the magnetic field.

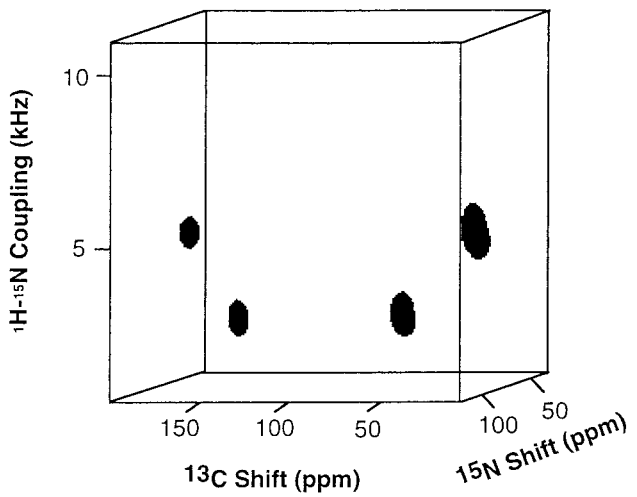

FIG. 4. Experimental three-dimensional ^{13}C shift/^{1}H–^{15}N coupling/^{15}N shift spectrum of the same sample as in Fig. 3.

with the pulse sequence diagrammed in Fig. 1B. The sample and experimental conditions were the same as those used to obtain the two-dimensional spectrum in Fig. 3. There are four signals in the cube, each of which is characterized by three different frequencies, and there are two carbon resonances correlated with each ^{15}N resonance. Each ^{13}C shift/^{15}N shift plane in the spectrum in Fig. 4 corresponds to a magnetically inequivalent molecule that is resolved on the basis of its ^{1}H–^{15}N coupling frequency. The linewidths observed along ^{13}C shift, ^{1}H–^{15}N coupling, and ^{15}N shift frequency dimensions in this spectrum are 2–4 ppm, 400 Hz, and 2–3 ppm, respectively. The linewidths in the ^{13}C shift and ^{1}H–^{15}N coupling frequency dimensions are likely to improve with further development of the experimental methods and instrumentation.

The ^{13}C homonuclear dipolar couplings among carbonyl carbon and α-carbon sites are decoupled during the t_1 interval with flip-flop Lee–Goldburg irradiation at the ^{13}C resonance frequency. Since both ^{1}H and ^{15}N irradiation are applied simultaneously to effect heteronuclear decoupling, the ^{13}C evolution during t_1 reflects only the ^{13}C chemical shift interaction. The implementation of homonuclear ^{13}C decoupling is essential for these experiments to be applicable to uniformly ^{13}C- and ^{15}N-labeled proteins. Unfortunately, this results in substantial scaling (0.58) of the ^{13}C chemical shift frequencies that can only be recovered through the use of higher field magnets. Schmidt-Rohr (*17*) has recently demonstrated an alternative approach to homonuclear ^{13}C decoupling in solids that has the advantage of allowing ^{13}C signals to be directly observed but has the disadvantage of even larger scaling (0.33) of the chemical shift frequencies. The one-dimensional ^{1}H decoupled ^{13}C shift spectra in Figs. 5A and 5B are extracted at the same ^{15}N shift frequency from two-dimensional data sets. The single crystal sample of 1,2,4,5-^{13}C, ^{15}N-labeled NAG differs from the one used to obtain the data in Figs. 3 and 4 in that all four carbon sites are labeled with ^{13}C, and the crystal is at a different orientation in the magnet. The spectrum in Fig. 5A was obtained in the absence of the ^{13}C Lee–Goldburg irradiation during t_1. Both the carbonyl carbon and α-carbon ^{13}C resonances exhibit multiple splittings and complex shapes because of the effects of ^{13}C homonuclear dipolar couplings. The comparison to the spectrum in Fig. 5B shows that the homonuclear decoupling is highly effective. The spectrum in Fig. 5B has well-resolved single-line resonances from each carbonyl carbon and α-carbon site in the molecule.

There are many potential applications of the triple-resonance pulse sequences diagrammed in Fig. 1. It is possible to use these experiments to determine the dihedral angle for the amide backbone site by recording powder spectra on a specifically labeled ^{13}C–^{15}N spin pair. They provide building blocks for a variety of multidimensional solid-state NMR experiments. However, our primary interest is in protein structure determination by solid-state NMR spectroscopy. The pulse sequence shown in Fig. 1B has the potential to yield high-resolution three-dimensional spectra from uniformly ^{13}C- and ^{15}N-labeled proteins. In these spectra, four different orientationally dependent frequencies are associated with each resolved ^{15}N amide site and provide valuable input for structure determination by solid-state NMR spectroscopy of oriented systems.

The pulse sequence also has the potential to be used to make sequential assignments of the peptide backbone resonances by varying the ^{13}C to ^{15}N cross-polarization mix time so that

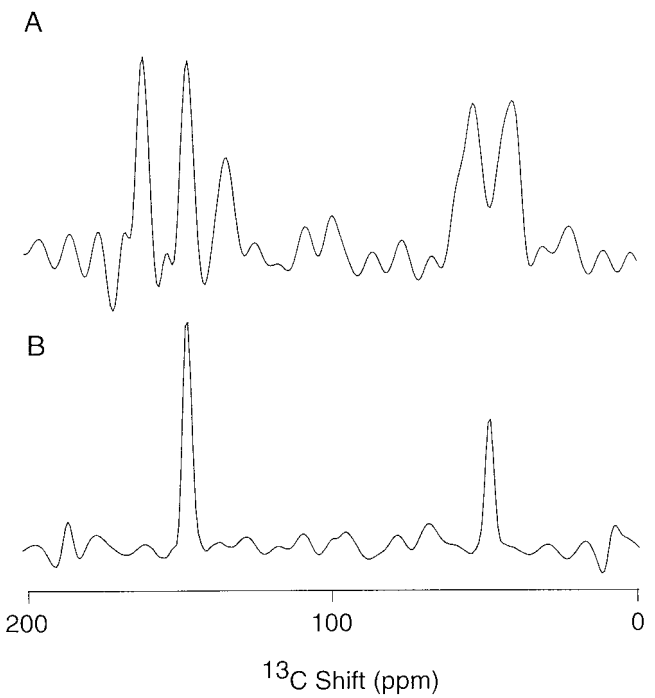

FIG. 5. One-dimensional slices extracted from two-dimensional spectra. (A) Without ^{13}C Lee–Goldburg homonuclear decoupling during t_1. (B) With ^{13}C Lee–Goldburg homonuclear decoupling during t_1 of a single crystal sample of 1,2,3,4–^{13}C, ^{15}N NAG at an arbitrary orientation with respect to the magnetic field.

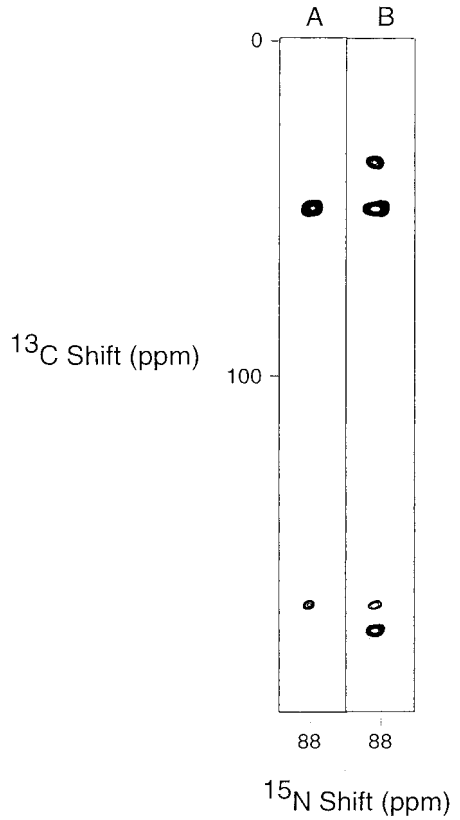

FIG. 6. Experimental ^{13}C spectral strips from two-dimensional spectra. (A) With ^{13}C to ^{15}N mix time of 0.6 ms. (B) With ^{13}C to ^{15}N mix time of 3.0 ms.

directly bonded ^{13}C sites are differentiated from more distant ^{13}C sites on adjacent residues that transfer magnetization to amide ^{15}N sites. A 3-ms ^{13}C–^{15}N cross-polarization mixing time can usually transfer magnetization from ^{13}C of adjacent residues to amide nitrogen. To demonstrate this important application of the pulse sequence diagrammed in Fig. 1A, two-dimensional ^{13}C shift/^{15}N shift correlation spectra of 1,2,4,5-^{13}C, 3-^{15}N-labeled NAG were obtained with two different ^{13}C–^{15}N mix times. The carbon strips shown in Figs. 6A and 6B are extracted at the same ^{15}N shift frequency from equivalent two-dimensional spectra. The data in Fig. 6A were obtained with a 0.6-ms C–N cross-polarization mix time, and those in Fig. 6B were obtained with a 3.0-ms mix time. The two cross peaks observed in Fig. 6A are due to the magnetization transfer from the directly bonded carbonyl carbon (2-C) and methylene carbon (4-C) to the amide nitrogen. With a 3.0-ms mix time, the magnetizations are also transferred to the methyl carbon (1-C) and carboxyl carbon (4-C) sites, therefore four cross peaks corresponding to the four carbons are observed in Fig. 6B. Thus, it is possible to "walk" through the peptide backbone with magnetization transfers through either carbonyl carbon or α-carbons with this approach. The combination of the angular information provided by the frequencies associated with each ^{15}N amide site and the sequential assignments from these correlation experiments has the potential to determine the complete three-dimensional backbone structure of a protein. In this regard, the carbonyl carbon and α-carbon ^{13}C resonance frequencies are a welcome bonus that can lead to more accurate backbone structures.

EXPERIMENTAL

The solid-state NMR experiments were performed at room temperature on a homebuilt triple-resonance spectrometer with a 12.9-T wide-bore Magnex 550/89 magnet. The homebuilt probe had a single 5-mm solenoid coil triply tuned to ^{1}H, ^{13}C, and ^{15}N resonance frequencies of 549.82, 138.27, and 55.72 MHz, respectively. The probe has 30 dB of isolation from the ^{13}C to the ^{15}N channels, and a narrow bandwidth filter was added to the ^{15}N channel to ensure rejection of ^{13}C frequencies and noise during data acquisition. A RF field strength of 50 kHz (90° pulse width of 5 μs) was utilized on the ^{1}H, ^{13}C, and ^{15}N channels and this corresponds to off-resonance Lee–Goldburg frequency jumps of $\pm$35.1 kHz. During the t_2 and t_3 periods, continuous irradiation at the ^{13}C resonance frequency with a RF field strength of 40 kHz was used for heteronuclear decoupling. A cross-polarization mix time of 1.0 ms was used to transfer magnetization from ^{13}C sites to ^{15}N amide sites. During the ^{13}C to ^{15}N cross-polarization mix period, continuous ^{1}H irradiation with a field strength of 83.3 kHz was applied. A total of 32 t_1 and 32 t_2 experiments were performed with dwell times of 32.6 and 40.8 μs, respectively. Two scans were coadded during each increment of t_1 and t_2 in the three-dimensional experiment. Eight scans were coadded during each increment of t_1 in the two-dimensional experiment. A recycle delay of 20 s was used in combination with a flip-back pulse to restore ^{1}H magnetization. The experimental scaling factors were measured to be 0.58 $\pm$ 0.01 during t_1 and 0.81 $\pm$ 0.01 during t_2. Experimental data were processed using Felix (Biosym Technologies). The final processed matrix had 64 $\times$ 128 $\times$ 128 points. The ^{13}C and ^{15}N chemical shifts are referenced with respect 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.

2,4-^{13}C, 3-^{15}N-labeled *N*-acetylglycine was synthesized by coupling 2-^{13}C, ^{15}N-labeled glycine and 2,2′-^{13}C-labeled acetic anhydride (*18*). A 60-mg single crystal of 2,4-^{13}C, 3-^{15}N NAG was crystallized from aqueous solution. 1,2,4,5-^{13}C, 3-^{15}N-labeled *N*-acetylglycine was synthesized by coupling U-^{13}C, ^{15}N-labeled glycine and U-^{13}C-labeled acetic anhydride. A 35-mg single crystal of 1,2,4,5-^{13}C, 3-^{15}N NAG was cocrystallized with natural abundance NAG in the ratio 1:3 from aqueous solution to minimize the intermolecular interaction. The isotopically labeled compounds were obtained from Cambridge Isotope Laboratories (Andover, MA). Figure 2 shows the chemical and crystal structures of NAG, which has two magnetically inequivalent molecules per unit cell (*11*).

ACKNOWLEDGMENTS

We thank Dr. Ronald McNamara for assistance with the instrumentation for the triple-resonance experiments. This research was supported by Grants RO1GM29754 and R37GM24266 from the National Institute of Health, and utilized the Resource for Solid-state NMR of Proteins at the University of Pennsylvania, supported by Grant P41RR09731 from the Biomedical Research Technology Program, Division of Research Resources, National Institute of Health. Z. Gu acknowledges support of postdoctoral Fellowship 5999-99 from the Leukemia Society of America.

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