High-Resolution Three-Dimensional Solid-State NMR Spectroscopy of a Uniformly ^{15}N-Labeled Protein

R. Jelinek, A. Ramamoorthy, and S. J. Opella*

Department of Chemistry, University of Pennsylvania Philadelphia, Pennsylvania 19104

Received September 15, 1995

Since the earliest days of protein NMR spectroscopy, isotopic labeling strategies[1,2] have been at least as important as instrumentation and spectroscopic methods in contributing to the success of experimental investigations. Significant progress in these three areas has made NMR spectroscopy a generally applicable method for the determination of the structures of globular proteins in solution.[3] Additional developments are needed so that solid-state NMR spectroscopy can extend the range of proteins whose structures can be determined to include those in membranes and other supramolecular structures. Uniform ^{15}N labeling of proteins, which can be readily and inexpensively accomplished by expression in bacteria grown on a medium with $^{15}NH_4Cl$ as the sole nitrogen source,[4] was originally implemented for solid-state[5] and then extended to solution[6] NMR because of the benefits of having the spin interactions associated with ^{15}N−^{1}H amide sites in each peptide bond. In particular, the high resolution and sensitivity routinely available in three-dimensional (3-D) experiments on uniformly ^{15}N-labeled proteins[7,8] have completely transformed the way protein structures in solution are determined.[9] In this communication, we demonstrate the successful implementation of a newly developed 3-D solid-state NMR experiment[10] on a uniformly ^{15}N-labeled protein. Not only can individual amide NH resonances be resolved and assigned in these spectra, but they also serve as sources of three orientationally dependent spectral parameters used for structure determination of proteins in oriented samples.[11] The combination of uniform isotopic labeling and high-dimensional experiments makes solid-state NMR spectroscopy more generally applicable to protein structure determination: the requirements for multiple labeled samples are reduced; in principle, the spectral parameters needed for structure determination of all sites can be measured in a single experiment; and systematic methods for resonance assignments are feasible.

Several promising solid-state NMR approaches to protein structure determination are being developed.[11−13] They are suitable for different types of samples and utilize measurements of parameters related to distances and orientations; however, they have all relied nearly exclusively on specific or selective isotopic labeling for several purposes, including fundamental sensitivity enhancement, spectral resolution and assignment, and providing the basic spin interactions responsible for the spectral parameters actually used in structure determination. This is at a considerable cost in flexibility, since the multiple roles of the isotopic labels give little room for separating functions. For example, when many resonances are resolved in a spectrum from a uniformly labeled protein, assignments can be readily made by comparisons with spectra from selectively labeled samples; however, this is not possible if adequate resolution is present only in spectra of selectively labeled samples.

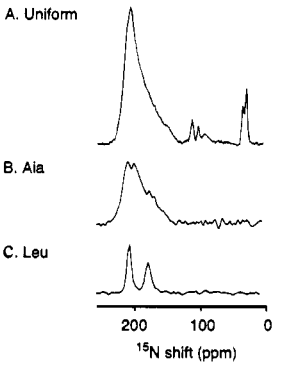

Figure 1. One-dimensional ^{15}N NMR spectra of oriented samples of fd bacteriophage. (A) Uniformly ^{15}N-labeled protein. (B) [^{15}N]Ala-labeled protein. (C) ^{15}N Leu labeled protein. Each spectrum is the result of coadding 5000 transients from cross-polarization with 1 ms mix times. The recycle delay was 4 s. The experiments were performed on a home-built spectrometer with ^{1}H and ^{15}N resonance frequencies of 550 and 55.7 MHz, respectively. The protein has 50 residues, one of which is Pro. The vast majority of the amide resonances are centered around 200 ppm; additional peaks correspond to the amino groups (near 40 ppm) and the mobile N-terminal residues of the protein (near 110 ppm). There are two Leu residues at positions 14 and 41. There are a total of 10 Ala residues, one of which contributes the N-terminal amino group.

Uniaxially oriented samples yield spectra with single line resonances or simple multiplets from each site. The resonance frequencies and splittings reflect the orientations of the anisotropic spin interactions relative to the axis of orientation, which is parallel to the direction of the applied magnetic field, enabling them to be used as the basis for protein structure determination by solid-state NMR spectroscopy.[11] The determination of the backbone structures of oriented proteins relies on the established magnitudes and orientations of the principal elements of the amide ^{1}H chemical shift, ^{1}H−^{15}N dipolar, and ^{15}N chemical shift interaction tensors in the peptide plane.[14]

Filamentous bacteriophages have been extremely useful in the development of solid-state NMR spectroscopy,[11] since the coat protein subunits are immobilized and oriented along with the virus particles by the magnetic field of the NMR spectrometer. Even though each amide nitrogen contributes a single resonance line, the 1-D spectrum of a uniformly ^{15}N-labeled sample (Figure 1A) is an essentially unresolved lump centered around 200 ppm due to severe spectral overlap among the 44 resonances from rigid, helical residues aligned approximately along the direction of the magnetic field.[15] This spectral overlap necessitated the use of many selectively labeled samples in the initial structural studies of fd coat protein,[11,16] which is far from ideal; while the two resonances from the [^{15}N]Leu-labeled sample in Figure 1C are well resolved, the nine amide resonances from the [^{15}N]Ala-labeled sample are highly overlapped in the 1-D spectrum in Figure 1B. A very similar

(1) Markley, J. L.; Putter, I.; Jardetzky, O. *Science* **1968**, *161*, 1249.
(2) LeMaster, D. M. *Prog. NMR Spectrosc.* **1994**, *26*, 371−419.
(3) Wuthrich, K. *Acc. Chem. Res.* **1989**, *22*, 36−44.
(4) Meselson, M.; Stahl, F. W. *Proc. Natl. Acad. Sci. U.S.A.* **1958**, *44*, 671−682.
(5) Cross, T. A.; DiVerdi, J. A.; Opella, S. J. *J. Am. Chem. Soc.* **1982**, *104*, 1759−1761.
(6) Bogusky, M. J.; Tsang, P.; Opella, S. J. *Biochem. Biophys. Res. Commun.* **1985**, *127*, 540−545.
(7) Fesik, S. W.; Zuiderweg, E. R. P. *J. Magn. Reson.* **1988**, *78*, 588−593.
(8) Marion, D.; Driscoll, P. C.; Kay, L. E.; Wingfield, P. J.; Bax, A.; Gronenborn, A. M.; Clore, G. M. *Biochemistry* **1989**, *29*, 6150−6156.
(9) Clore, G. M.; Gronenborn, A. M. *Science* **1991**, *252*, 1390−1399.
(10) Ramamoorthy, A.; Wu, C. H.; Opella, S. J. *J. Magn. Reson., Ser. B* **1995**, *107*, 88−90.
(11) Opella, S. J.; Stewart, P. L.; Valentine, K. G. *Q. Rev. Biophys.* **1987**, *19*, 7−49.
(12) Gullion, T.; Schaefer, J. *J. Magn. Reson.* **1989**, *81*, 196−200.
(13) Levitt, M. H.; Raleigh, D. P.; Creuzet, F.; Griffin, R. G. *J. Chem. Phys.* **1990**, *92*, 6347−6364.
(14) Wu, C. H.; Ramamoorthy, A.; Gierasch, L. M.; Opella, S. J. *J. Am. Chem. Soc.* **1995**, *117*, 6148−6149.
(15) Cross, T. A.; Tsang, P.; Opella, S. J. *Biochemistry* **1983**, *22*, 721−726.
(16) Cross, T. A.; Opella, S. J. *J. Mol. Biol.* **1985**, *182*, 367−381.

0002-7863/95/1517-12348$09.00/0

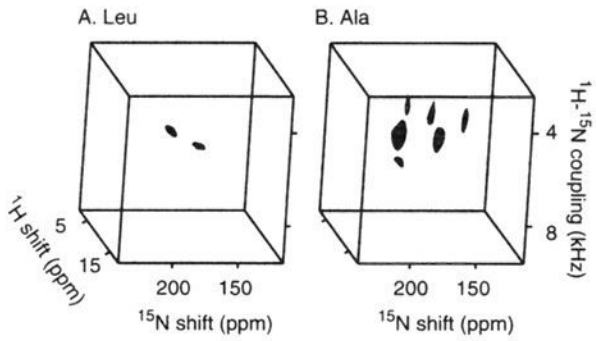

Figure 2. Three-dimensional ^{1}H chemical shift/^{1}H−^{15}N dipolar coupling/^{15}N chemical shift solid-state NMR spectra of oriented samples of ^{15}N-labeled fd bacteriophage. (A) [^{15}N]Leu-labeled coat protein. (B) [^{15}N]Ala-labeled coat protein. 640 transients were coadded for each point in the 3-D experiment. Sixteen and nine points were obtained in the ^{1}H−^{15}N dipolar and ^{1}H chemical shift dimensions, respectively. The final matrix had 128 × 128 × 128 points. The ^{15}N chemical shifts are relative to liquid $^{15}NH_3$, and the ^{1}H chemical shifts are relative to TMS.

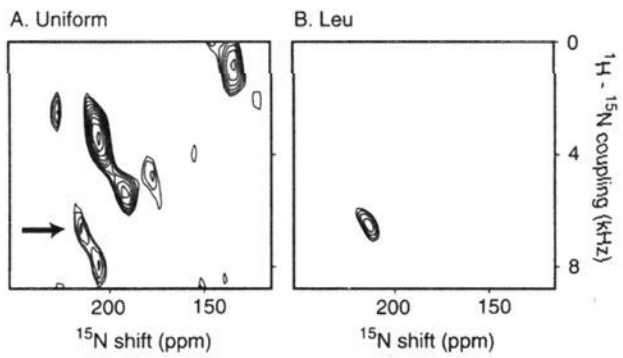

Figure 3. Two-dimensional ^{15}N chemical shift/^{1}H−^{15}N dipolar coupling planes extracted from 3-D correlation spectra of oriented samples of ^{15}N-labeled fd bacteriophage at the ^{1}H chemical shift of 5.5 ppm. (A) Uniformly ^{15}N-labeled coat protein. The resonance corresponding to Leu41 is marked with the arrow. (B) [^{15}N]Leu-labeled coat protein.

situation is encountered with transmembrane helices in oriented bilayers.

The enhanced spectral resolution in 3-D solid-state NMR experiments is a direct consequence of the additional frequency axes as well as the incorporation of spin exchange at the magic angle in the pulse sequence, which gives narrow resonances in the dipolar frequency dimension.[17] Three-dimensional correlation spectra of oriented [^{15}N]Leu- and [^{15}N]Ala-labeled fd samples are presented in Figure 2; the resonances have line widths of ~8 ppm, 600 Hz, and 1.5 ppm, along the ^{15}N chemical shift, ^{1}H−^{15}N dipolar coupling, and ^{1}H chemical shift frequency axes, respectively. The resonances from the two Leu residues are fully resolved in all three dimensions. The nine Ala residues yield six identifiable peaks in the 3-D spectrum of the [^{15}N]-Ala-labeled sample in Figure 2B, which is a notable improvement over the resolution observed in the corresponding 1-D ^{15}N NMR spectrum in Figure 1B.

An even more dramatic improvement in resolution can be seen in the 2-D ^{15}N chemical shift/^{1}H−^{15}N dipolar coupling planes extracted from the 3-D data sets. This is illustrated in Figure 3, where the planes corresponding to the same ^{1}H chemical shift frequency of 5.5 ppm are shown for the uniformly ^{15}N-labeled sample (Figure 3A) and the selectively [^{15}N]Leu-labeled sample (Figure 3B). By comparison with the spectrum in Figure 3B, the peak corresponding to Leu41 is shown to be resolved and assigned in the spectrum in Figure 3A from the uniformly ^{15}N-labeled sample. The ^{15}N chemical shift and ^{1}H−^{15}N dipolar coupling frequencies can be measured, in addition to the ^{1}H chemical shift frequency, for each of the seven resolved resonances in this plane, including that from Leu41; planes at other ^{1}H chemical shift frequencies exhibit many additional resolved resonances. When the spectra are analyzed as 2-D ^{15}N chemical shift/^{1}H−^{15}N dipolar coupling planes at selected

(17) Wu, C. H.; Ramamoorthy, A.; Opella, S. J. *J. Magn. Reson., Ser. A* **1994**, *109*, 270−272.

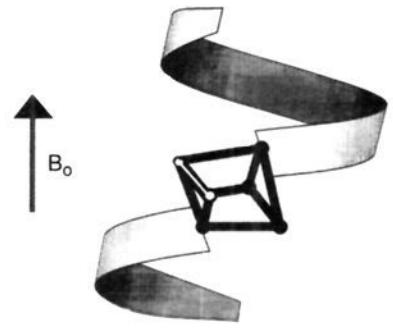

Figure 4. Orientation of the Leu41 peptide plane in the context of the coat protein as determined by spectral parameters of ^{1}H chemical shift of 5.5 ± 0.5 ppm, ^{1}H−^{15}N dipolar coupling of 13 ± 1 kHz, and ^{15}N chemical shift of 210 ± 5 ppm. The N−H bond is indicated in white, and the arrow indicates the direction of the applied magnetic field. The angle between the N−H bond and the magnetic field is 72 ± 3°, and the angle between the N−H bond and the projection of the magnetic field onto the peptide plane is 15 ± 3°.

^{1}H frequencies, all nine amide resonances from the [^{15}N]Ala-labeled sample and >70% of the amide resonances from the uniformly ^{15}N-labeled sample can be resolved. Further improvements in resolution can be expected from higher magnetic fields, especially along the ^{1}H chemical shift dimension, as well as through the implementation of 4-D experiments.[18]

Once resolution is accomplished, the assignment of resonances to specific residues becomes an important issue. As shown with the data in Figure 3 and previous 1- and 2-D experiments,[11,16] it is possible to assign resonances in solid-state NMR spectra of oriented samples using conventional approaches that rely on isotopic labeling or single-site mutations. Significantly, uniformly ^{15}N-labeled proteins enable systematic sequential resonance assignments to be made with homonuclear spin exchange experiments, as demonstrated with 2-D ^{15}N spin exchange experiments on uniformly ^{15}N-labeled fd bacteriophage.[16,19] The [^{15}N]Leu41 resonance resolved in the spectra in Figures 1C, 2C, and 3B was assigned by both conventional and spin exchange methods.[16] Newly developed 3- and 4-D experiments incorporating either dilute spin[18] (^{15}N) or abundant spin[20] (^{1}H) spin exchange are effective in assigning the resonances in 3-D spectra of uniformly ^{15}N-labeled samples.

The three measured spectral parameters provide the angular constraints for determining the orientation of the peptide plane for each amide nitrogen relative to the direction of the magnetic field.[11] Figure 4 displays the orientation of the peptide plane of Leu41 as determined by the frequencies measured from the data shown in Figures 2 and 3. It is shown as part of the helical structure of the coat protein in an oriented virus particle.[11,16]

The successful application of 3-D solid-state NMR experiments on a uniformly ^{15}N-labeled protein is an important step in the development of a general method for protein structure determination. These experiments are directly applicable to membrane proteins[21] and other systems that can be prepared by expression and oriented mechanically or magnetically.

Acknowledgment. This research was supported by Grants R37GM24266 and RO1GM29754 from the General Medical Sciences Institute, National Institutes 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 Institutes of Health. R.J. was supported by a Cancer Research Institute postdoctoral fellowship.

JA953171Q

(18) Ramamoorthy, A.; Gierasch, L. M.; Opella, S. J. *J. Magn. Reson., Ser. B* **1995**, *109*, 112−116.
(19) Cross, T. A.; Frey, M. H.; Opella, S. J. *J. Am. Chem. Soc.* **1983**, *105*, 7471−7473.
(20) Ramamoorthy, A.; Gierasch, L. M.; Opella, S. J. Submitted for publication.
(21) Cross, T. A.; Opella, S. J. *Curr. Opin. Struct. Biol.* **1994**, *4*, 574−581.