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Three-Dimensional Solid-State NMR Spectroscopy Is Essential for Resolution of Resonances from In-Plane Residues in Uniformly ^{15}N-Labeled Helical Membrane Proteins in Oriented Lipid Bilayers

Francesca M. Marassi,* Che Ma,† Jennifer J. Gesell,† and Stanley J. Opella†

**The Wistar Institute, Philadelphia, Pennsylvania 19104-4268; and †Department of Chemistry, University of Pennsylvania, Philadelphia, Pennsylvania 19014-6323*

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Uniformly ^{15}N-labeled samples of membrane proteins with helices aligned parallel to the membrane surface give two-dimensional PISEMA spectra that are highly overlapped due to limited dispersions of ^{1}H–^{15}N dipolar coupling and ^{15}N chemical shift frequencies. However, resolution is greatly improved in three-dimensional ^{1}H chemical shift/^{1}H–^{15}N dipolar coupling/^{15}N chemical shift correlation spectra. The 23-residue antibiotic peptide magainin and a 54-residue polypeptide corresponding to the cytoplasmic domain of the HIV-1 accessory protein Vpu are used as examples. Both polypeptides consist almost entirely of α-helices, with their axes aligned parallel to the membrane surface. The measurement of three orientationally dependent frequencies for Val17 of magainin enabled the three-dimensional orientation of this helical peptide to be determined in the lipid bilayer. © 2000 Academic Press

Key Words: **PISEMA; magainin; membrane protein; solid-state NMR; protein structure.**

INTRODUCTION

The structures of membrane proteins can be determined in phospholipid bilayers by solid-state NMR spectroscopy (*1*, *2*). The approach we are developing takes advantage of the high resolution and both angular and distance information available in stationary oriented samples. Although even the simplest one-dimensional solid-state ^{15}N NMR spectra of oriented bilayer samples provide valuable information about the architecture of membrane peptides and proteins (*3–7*), complete structure determination requires the measurement of multiple orientationally dependent frequencies for each residue. For example, the three-dimensional structures of the gramicidin ion channel peptide (*8*) and of a functional peptide corresponding to the M2 segment of the acetylcholine receptor (AChR) (*9*) have been determined with this method. In order for this approach to be generally applicable, it is essential to be able to resolve resonances from uniformly isotopically labeled proteins prepared by expression in bacteria or other organisms that can grow on minimal medium. Using the PISEMA (polarization inversion spin exchange at the magic angle) experiment (*10*), which is a high-resolution version of separated local field spectroscopy (*11*), many individual resonances have been resolved in the multidimensional solid-state NMR spectra of uniformly ^{15}N-labeled membrane proteins with between 25 and 200 residues in oriented bilayer samples (*7*, *9*, *12–14*). The observed frequencies of the single-line resonances depend on the orientation of the molecular sites relative to the direction of the applied magnetic field and provide both the mechanism for resolution among resonances and the orientational constraints used for structure determination. The ability to measure multiple orientationally dependent frequencies for each resonance is an important feature of this approach because, in principle, it enables a protein structure to be determined from a single assigned spectrum.

Transmembrane helices give well-resolved two-dimensional ^{1}H–^{15}N dipolar coupling/^{15}N chemical shift PISEMA spectra (*7*, *9*, *12*, *13*). This fortunate situation happens largely because transmembrane helices typically cross the membrane at an angle (*15–17*). Solid-state NMR experiments have been used to characterize the orientations of transmembrane helices in a number of polypeptides, including AChR M2 (*9*), the HIV-1 accessory protein Vpu (*4*, *13*, *14*), and influenza M2 (*5*). The tilt leads to variations in the angles formed by the backbone amide NH bond vectors relative to the magnetic field, and this is manifest in the dispersion of both the ^{1}H–^{15}N dipolar coupling and the ^{15}N chemical shift frequencies (*16*, *17*).

In contrast to transmembrane helices, surface bound helices generally have their axes parallel to the plane of the bilayer and orthogonal to the direction of the applied magnetic field. Consequently, they exhibit much smaller angular dispersions of their amide NH bond vectors relative to the magnetic field (*16*). This leads to a high degree of overlap in the spectral region associated with in-plane helices in two-dimensional PISEMA spectra of uniformly ^{15}N-labeled proteins (*7*, *13*, *14*, *16*). The results described in this Communication demonstrate the crucial role played by the ^{1}H chemical shift interaction in the

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resolution of resonances from in-plane helices of membrane proteins (*14*). Three-dimensional ^{1}H chemical shift/^{1}H–^{15}N dipolar coupling/^{15}N chemical shift correlation spectra (*18*) can give very favorable results in situations where the two-dimensional spectra are seemingly intractable.

Magainin, an antibiotic peptide isolated from frog skin (*19*), has only 23 residues (GIGKFLHSAKKFGKAFVGEI MNS), yet its one- and two-dimensional solid-state NMR spectra display poor resolution because the peptide is a nearly linear α-helix oriented in the plane of the membrane. The structure of magainin, determined by multidimensional solution NMR spectroscopy in lipid micelles (*20*), is an amphipathic α-helix with slight curvature. Our earlier solid-state NMR experiments were consistent in showing that the helix axis lies in the plane of the phospholipid bilayer (*21*). These studies utilized specifically ^{15}N-labeled synthetic peptides, which limited the investigation to only a few sites in the peptide. Expression in bacteria allows us to prepare uniformly ^{15}N-labeled samples suitable for complete structure determination as long as all of the resonances can be resolved.

Similarly, Vpu_{28-81}, a polypeptide corresponding to the cytoplasmic domain of the HIV-1 accessory protein Vpu (*22*), interacts with membrane bilayers as in-plane α-helices (*4, 13, 14*), and uniformly ^{15}N-labeled samples give poorly resolved one- and two-dimensional solid-state NMR spectra. Full-length Vpu is an 81-residue membrane protein (MQPIQIAIVA LVVAIIIAIV VWSIVIIEYR KILRQRKIDR LIDRLIERAE DSGNESEGEI SALVELGVEL GHHAPWDVDD L). Using NMR experiments in micelles and in lipid bilayers, we have demonstrated that full-length Vpu folds into two distinct structural domains: a single transmembrane N-terminal hydrophobic helix that makes an angle of about 15° with the bilayer normal and two amphipathic in-plane helices in the cytoplasmic C-terminal domain (*4, 13, 14*).

RESULTS AND DISCUSSION

Solid-state NMR spectra of uniformly ^{15}N-labeled magainin and Vpu_{28-81} in oriented lipid bilayers are shown in Fig. 1. The ^{31}P NMR spectra of the lipids in both samples (Figs. 1A and 1B) display a single peak near 30 ppm, as expected for highly aligned bilayers with this lipid composition (phosphatidylcholine:phosphatidylglycerol, 4:1 molar ratio) and peptide:lipid molar ratios (magainin, 1.6:100; and Vpu_{28-81}, 0.5:100). Although the one-dimensional ^{15}N chemical shift and two-dimensional PISEMA spectra of magainin (Figs. 1C and 1E) and Vpu_{28-81} (Figs. 1D and 1F) have limited resolution, they provide sufficient information to map out the architecture of the polypeptides illustrated at the top of Fig. 1 (*4, 13, 14*). The orientational dependence of the frequencies results in the overlap of resonances in these highly symmetric polypeptides containing only in-plane helices. The resonance intensity is highly concentrated near the ^{1}H–^{15}N dipolar coupling (5 kHz) and ^{15}N chemical shift (70 ppm) frequencies associated with NH bonds parallel to the membrane surface and perpendicular to the direction of the applied magnetic field.

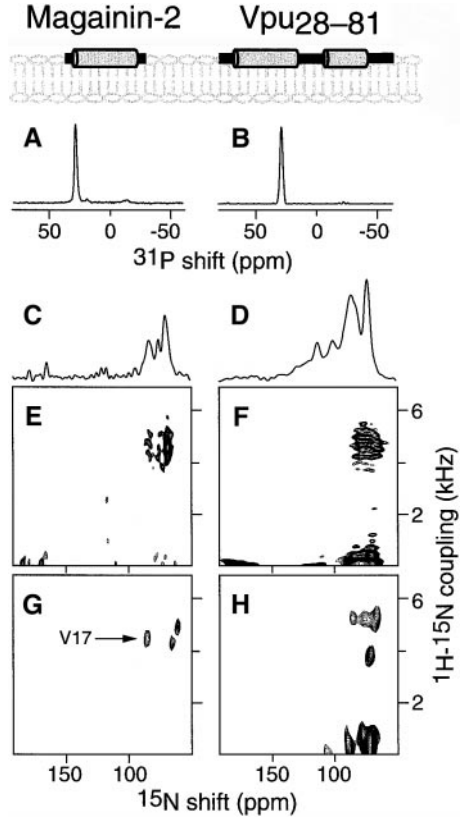

FIG. 1. Solid-state NMR spectra of uniformly ^{15}N-labeled magainin (left) and Vpu_{28-81} (right) in oriented lipid bilayers. A and B. One-dimensional ^{31}P NMR spectra of the phospholipids. C and D. One-dimensional ^{15}N NMR spectra of the polypeptides. E and F. Two-dimensional ^{1}H–^{15}N dipolar coupling/^{15}N chemical shift PISEMA spectra of the polypeptides. G and H. Two-dimensional ^{1}H–^{15}N dipolar coupling/^{15}N chemical shift planes extracted from three-dimensional ^{1}H chemical shift/^{1}H–^{15}N dipolar coupling/^{15}N chemical shift correlation spectra at selected ^{1}H chemical shift frequencies. G. ^{1}H chemical shift = 15.5 ppm. H. ^{1}H chemical shift = 17.0 ppm. In G the arrow identifies the resonance from Val17 of magainin. The ^{15}N and ^{1}H chemical shifts are referenced to 0 ppm for liquid ammonia and tetramethylsilane, respectively. The recombinant polypeptides were derived from fusion proteins expressed in *Escherichia coli.* For uniformly labeled samples, *E. coli* cells were grown in medium containing ^{15}N-enriched ammonium sulfate. For the specifically labeled sample used to obtain the spectrum in Fig. 3A, the growth medium contained all amino acids with only valine ^{15}N labeled. All isotopically labeled materials were from Cambridge Isotope Laboratories (Andover, MA). The preparation of oriented lipid bilayer samples of magainin (*21*) and Vpu_{28-81} (*13*) has been described. The solid-state NMR spectra were obtained at 23°C (magainin) or 0°C (Vpu_{28-81}) using homebuilt double-resonance probes with "square" RF coils wrapped directly around the samples, which consisted of thin glass plates containing the hydrated polypeptide and phospholipids. The spectrometers had magnets with field strengths corresponding to ^{1}H resonance frequencies of 550 and 400 MHz. The NMR data were processed using the program *FELIX* (Biosym Technology, San Diego, CA) on a Silicon Graphics computer workstation (Mountain View, CA).

There are a few resolved resonances in the one-dimensional ^{15}N NMR spectrum of magainin (Fig. 1C); the signals at 166 and 180 ppm most likely correspond to the side chain nitrogens of His7 and Asn22. There are three peaks in the isotropic ^{15}N chemical shift region of the spectrum (115 ppm), but only one with a ^{1}H–^{15}N dipolar coupling frequency of 0 kHz. It is improbable, although possible, that residues at the N- and C-termini of the peptide undergo motions on a time scale intermediate between the ^{15}N chemical shift and ^{1}H–^{15}N dipolar coupling interactions (0.1–1 ms). More likely, a few residues proximate to the termini do not form part of a canonical helix in the lipid bilayers as is the case in lipid micelles (*20*) and hence adopt conformations where the amide NH bonds are not parallel to the membrane surface.

Only a few resonances are genuinely resolved in the in-plane helical region of the two-dimensional PISEMA spectra of magainin (Fig. 1E) or Vpu_{28-81} (Fig. 1F). The favorable resonance linewidths reflect well-oriented samples and a properly tuned spectrometer (250 Hz in the ^{1}H–^{15}N dipolar coupling dimension and 3 ppm in the ^{15}N chemical shift dimension). However, the spectral resolution is limited by the small frequency dispersion in both dimensions, which is characteristic of in-plane α-helices. Since one of the main advantages of uniform ^{15}N labeling of proteins (*23*) is that three different spin interactions are available for interrogation at each amide site, the way to deal with the severe overlap in the in-plane helical region of the two-dimensional PISEMA spectra is to invoke the ^{1}H amide chemical shift interaction and increase the number of frequency dimensions. The three-dimensional ^{1}H shift/^{1}H–^{15}N dipolar coupling/^{15}N shift correlation spectrum of magainin II in oriented bilayers is completely resolved, dramatically illustrating the value of the ^{1}H chemical shift in separating resonances from residues in a helix oriented in the plane of the bilayer. We have identified 22 resonances in the three-dimensional spectrum of magainin, as expected from the amino acid sequence of this 23-residue peptide. The two-dimensional ^{1}H–^{15}N dipolar coupling/^{15}N shift planes extracted from a three-dimensional spectrum of uniformly ^{15}N-labeled magainin at selected ^{1}H chemical shift frequencies are shown in Fig. 1G and in Fig. 2. Similarly, the ^{1}H chemical shift separated two-dimensional spectral plane of uniformly ^{15}N-labeled Vpu_{28-81} in Fig. 1H shows that individual resonances can be resolved in a polypeptide with twice as many residues, despite the severe overlap observed in the two-dimensional PISEMA spectrum. The observation of single resonances from individual backbone amide sites indicates that both magainin and Vpu_{28-81} bind tightly to the membrane surface in unique orientations and are structured and immobilized by their interactions with the lipid bilayers, even though they are soluble and unstructured in the absence of lipids (*13*, *20*, *24*, *25*).

In Fig. 1G, the arrow points to the resonance assigned to Val17 of magainin. This resonance was assigned by comparison with the two-dimensional PISEMA spectrum of specifically ^{15}N Val-labeled magainin in Fig. 3A. The ^{1}H chemical shift, ^{1}H–^{15}N dipolar coupling, and ^{15}N chemical shift frequencies measured from the three-dimensional correlation spectrum are sufficient to determine the orientation of Val17 relative to the direction of the applied magnetic field. The angular restrictions from these experimental frequencies were combined with the solution NMR structure in lipid micelles (*20*) to characterize the three-dimensional orientation of magainin in the lipid bilayers. As shown in Fig. 4A, the magainin helix is parallel to the bilayer surface. It is rotated about its long axis (Fig. 4B) so that the polar–apolar boundary, separating the hydrophilic (white) and hydrophobic (gray) faces of the helix, is parallel to the membrane surface. Residues Phe5, Phe12, and Phe16 are all near this boundary region and, hence, equidistant from the hydrophobic core of the lipid bilayer. This is in agreement with data from fluorescence quenching studies at these sites (*26*). Because of symmetry considerations (*16*), the solid-state NMR data in Figs. 1 and 2 do not provide information about the "sidedness" of the helix–bilayer interaction, i.e., whether

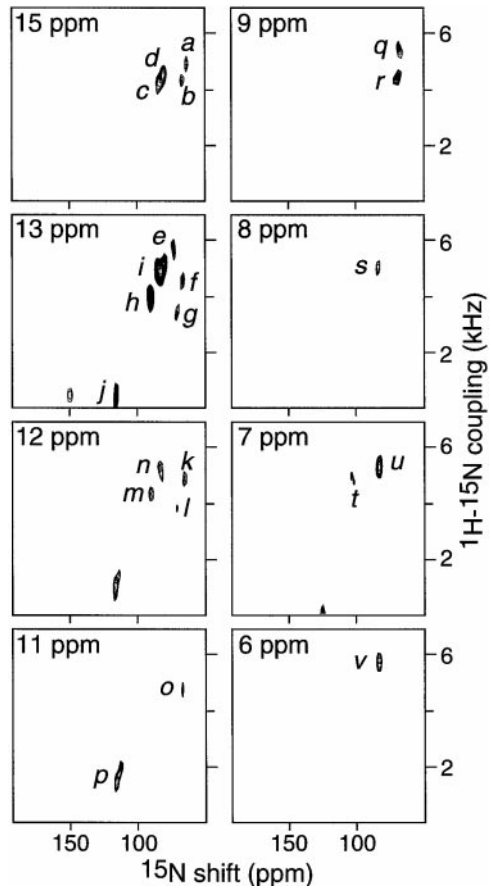

FIG. 2. Two-dimensional ^{1}H–^{15}N dipolar coupling/^{15}N chemical shift planes extracted from three-dimensional ^{1}H chemical shift/^{1}H–^{15}N dipolar coupling/^{15}N chemical shift correlation spectrum of uniformly ^{15}N-labeled magainin in oriented lipid bilayers at selected ^{1}H chemical shift frequencies. A total of 22 resonances labeled a to v were identified.

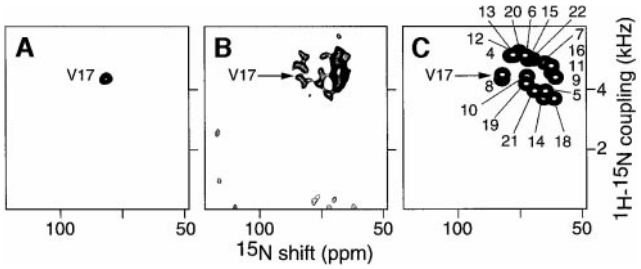

FIG. 3. Experimental (A and B) and calculated (C) two-dimensional PISEMA spectra of magainin in oriented lipid bilayers. In all spectra the arrow identifies the resonance from Val17. A. ^{15}N Val-labeled magainin. B. Uniformly ^{15}N-labeled magainin. C. Spectrum calculated from the coordinates of the solution NMR structure of magainin in lipid micelles (PDB file 2MAG). The spectrum was calculated on a Silicon Graphics O2 computer (Mountain View, CA), using the FORTRAN program *FINGERPRINT*. The input to the program consisted of the orientation of magainin specified by the solid-state NMR angular constraints determined for Val17; the backbone dihedral angles of residues 4 to 22 from the solution NMR structure in lipid micelles; the ^{15}N chemical shift tensor; and the NH bond length. The amide ^{15}N chemical shift tensor used in the chemical shift calculation had principal values of $\sigma_{11} = 64$ ppm, $\sigma_{22} = 77$ ppm, $\sigma_{33} = 217$ ppm, and an orientation relative to the N–H bond axis described by $\sigma_{33} \angle \text{NH} = 17°$. A NH bond length of 1.07 Å was used in the ^{1}H–^{15}N dipolar coupling calculation.

Val17 is on the same or opposite side of the bulk water phase. We present the magainin peptide at the membrane–water interface with Val17 and the hydrophobic face of the amphipathic helix cis to the hydrophobic core of the lipid bilayer, as expected from basic energy considerations.

The three-dimensional structure of magainin in the membrane shown in Fig. 4 was validated by back-calculation of the two-dimensional PISEMA spectrum. It is possible to calculate the solid-state NMR spectra of proteins in oriented samples because the frequencies directly reflect the angles between individual bonds or chemical groups and the direction of the applied magnetic field. The PISEMA spectrum of magainin calculated from the solution NMR structure of the peptide in lipid micelles (PDB file 2MAG) and the orientation of the Val17 peptide plane determined from solid-state NMR (*24*) is shown in Fig. 3C. Many features of the experimental spectrum are reproduced by the calculated spectrum where the resonance frequencies reflect the helix orientation, specified by Val17; the backbone dihedral angles; the amide ^{15}N chemical shift tensor; and the NH bond length. Differences between the experimental spectrum of magainin and the calculated spectrum may be due to differences between the structure in lipid micelles and that in lipid bilayers. Variations in the backbone dihedral angles of $\pm 10°$ between the two conformers lead to excursions as large as ± 15 ppm and ± 1 kHz in the ^{15}N chemical shift and ^{1}H–^{15}N dipolar coupling frequencies, respectively, and are sufficient, by themselves, to account for the differences between the experimental and calculated spectra. Further, variations in the magnitudes of the principal elements of the ^{15}N chemical shift tensor of ± 20 ppm lead to excursions as large as ± 15 ppm in the calculated ^{15}N chemical shift frequencies. This is in keeping with the residue-dependent variability observed for the amide ^{15}N chemical shift tensor; in contrast, little variability has been observed in the orientations of the principal elements in the molecular axis system. Finally, variations in the NH bond length as large as ± 0.02 Å correspond to excursions of only ± 0.6 kHz in the calculated ^{1}H–^{15}N dipolar coupling frequency. In this "in-plane" region of the PISEMA spectrum the NMR resonance frequencies are very sensitive to small changes in molecular orientation, as well as NH bond length, and principal values of the amide ^{15}N tensor.

The similarity of the calculated and experimental PISEMA spectra in Fig. 3 corroborates the conformation and orientation of magainin II shown in Fig. 4. Distance measurements made on unoriented bilayer samples of magainin with magic angle spinning experiments (*27*) were interpreted to show that residues 15 to 19 have one-third β-sheet and two-thirds α-helix secondary structure in this

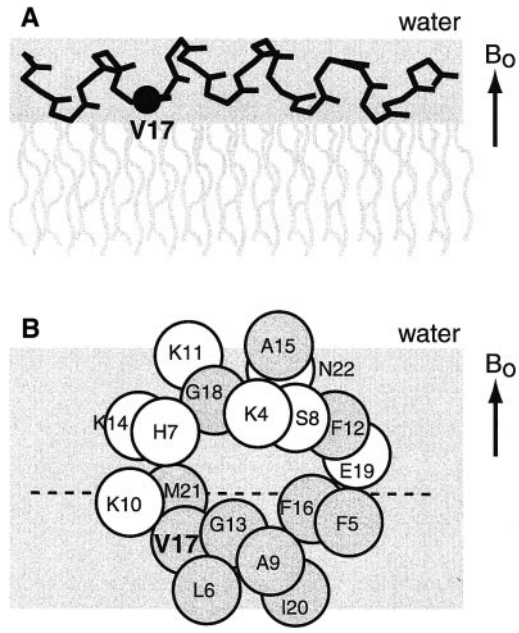

FIG. 4. Schematic drawing of magainin II aligned on the surface of the lipid bilayer membrane. The three-dimensional orientation of the peptide was determined from the angular constraints for Val17 derived from the three-dimensional solid-state NMR spectrum. The solid gray area represents the polar/hydrophobic interfacial region of the lipid bilayer membrane. The arrow specifies the direction of the applied magnetic field (B_0). A. Side view of the magainin II helix backbone. The amide nitrogen of Val17 is shown as the closed circle. The amide NH bonds (pointing to the right of the page) are aligned nearly parallel to the surface of the lipid bilayer and perpendicular to the direction of the applied magnetic field. B. View of the backbone C_α atoms down the long helix axis, with the N-terminus in front. The dashed line marks the boundary between hydrophilic (white) and hydrophobic (gray) residues in the amphipathic helix.

region. PISEMA spectra of β-sheets and β-turns inserted at various orientations in a membrane exhibit characteristic patterns that are distinctly different from those of α-helices (unpublished results). The difference between the results obtained on oriented bilayer samples, which show only α-helix, and those from unoriented samples, which show a mixture of secondary structures, may be explained by differences in the peptide to lipid ratios of the samples.

CONCLUSIONS

Only the sequential assignment of all resonances and the determination of the complete three-dimensional structure can provide the definitive answer about the topology of a polypeptide in the membrane. The first steps in structure determination are the resolution of individual resonances and measurement of orientationally dependent frequencies. The results shown in Figs. 1 and 2 demonstrate that three-dimensional correlation spectroscopy overcomes the severe spectral overlap observed in one- and two-dimensional solid-state NMR spectra of membrane in-plane helices in oriented bilayer samples. With further development of the instrumentation, especially the use of high magnetic fields, and experimental methods, the ability to obtain completely resolved spectra of uniformly isotopically labeled proteins in oriented bilayers should extend far beyond 50 residues to take advantage of solid-state NMR having no fundamental size limitation.

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