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NMR Structural Studies of Membrane Proteins

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INTRODUCTION

More than one-third of the proteins expressed by the genomes of simple and complex organisms are hydrophobic membrane proteins. As a result, determining the structures of this class of proteins is important for understanding many biological functions, including those performed by drug receptors, ion channels, and molecular transporters. High resolution structural information, such as that obtained by x-ray crystallography and multidimensional solution NMR spectroscopy on globular proteins is essential for membrane proteins. Knowledge of the secondary structure or even the global fold of a protein is unlikely to be sufficient for understanding its functions regardless of whether it resides in the cytoplasm or a membrane. However, very few of the more than 9000 protein structures in the Protein Data Bank are of membrane proteins. Membrane proteins are difficult to crystallize for x-ray diffraction, and only rarely is it possible to prepare samples suitable for solution NMR experiments. Fortunately, there are no properties associated with membrane proteins, other than the effects of lipids on their reorientation rates, that make them difficult to study by NMR spectroscopy.[1] Proteins immobilized in phospholipid bilayers can be studied with solid-state NMR experiments where radiofrequency irradiations, supplemented with sample orientation or magic angle sample spinning, replace molecular motions as the principal line narrowing mechanism.[2-4] Not only do solid-state NMR methods give spectra with high resolution and sensitivity; they enable

measurements of distances and angles that can be used to determine the three-dimensional structures of proteins. Our primary approach to studying membrane proteins involves the use of oriented samples and the measurement of spectral parameters that reflect the orientations of individual bonds with respect to the direction of the applied magnetic field.[2]

In the initial examples, membrane peptides and proteins studied by solid-state NMR spectroscopy of oriented samples required that the polypeptides be labeled with stable isotopes in only one or a few selected sites.[5-7] Not only did this make determinations of complete structures laborious, it limited applications to peptides prepared by solid-phase synthesis, small proteins with favorable distributions of amino acids, or selected regions of larger proteins. Nonetheless, this approach enabled the determination of the orientations of helical peptides and segments of proteins in phospholipid bilayers, as illustrated in Figure 1, as well as the three-dimensional structure of gramicidin in phospholipid bilayers.[8] These findings led to models for the assembly of filamentous bacteriophages,[7] the formation of ion channels,[5] and the mechanism of action of antibiotic peptides.[6]

Specific and Selective Isotopic Labeling of Helical Membrane Peptides and Proteins

It is possible to determine the orientations of ^{15}N labeled helical peptides within bilayer membranes from their one-dimensional solid-state ^{15}N chemical shift NMR spectra in oriented phospholipid bilayers. Each ^{15}N resonance arises from a single amide site in the protein, and has a frequency that reflects the orientation of the corresponding NH bond, and in turn the peptide plane, in the bilayer.

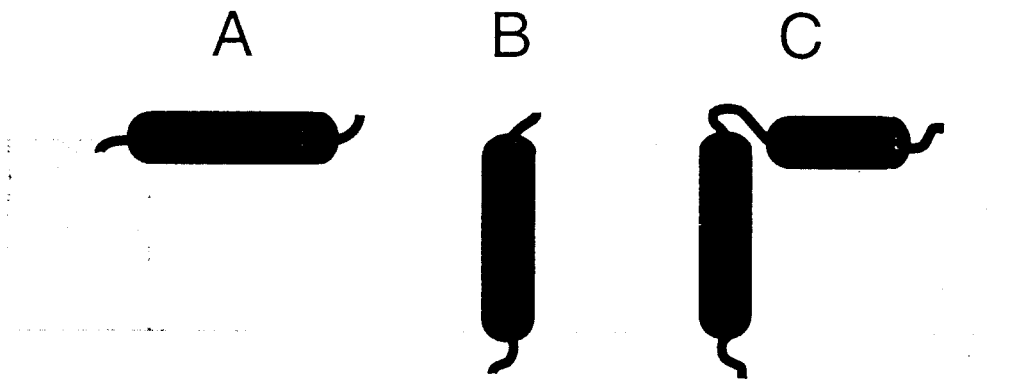

Fig. 1. Representations of proteins in oriented phospholipid bilayers.
A) magainin, B) M2 channel peptide, C) fd coat protein.

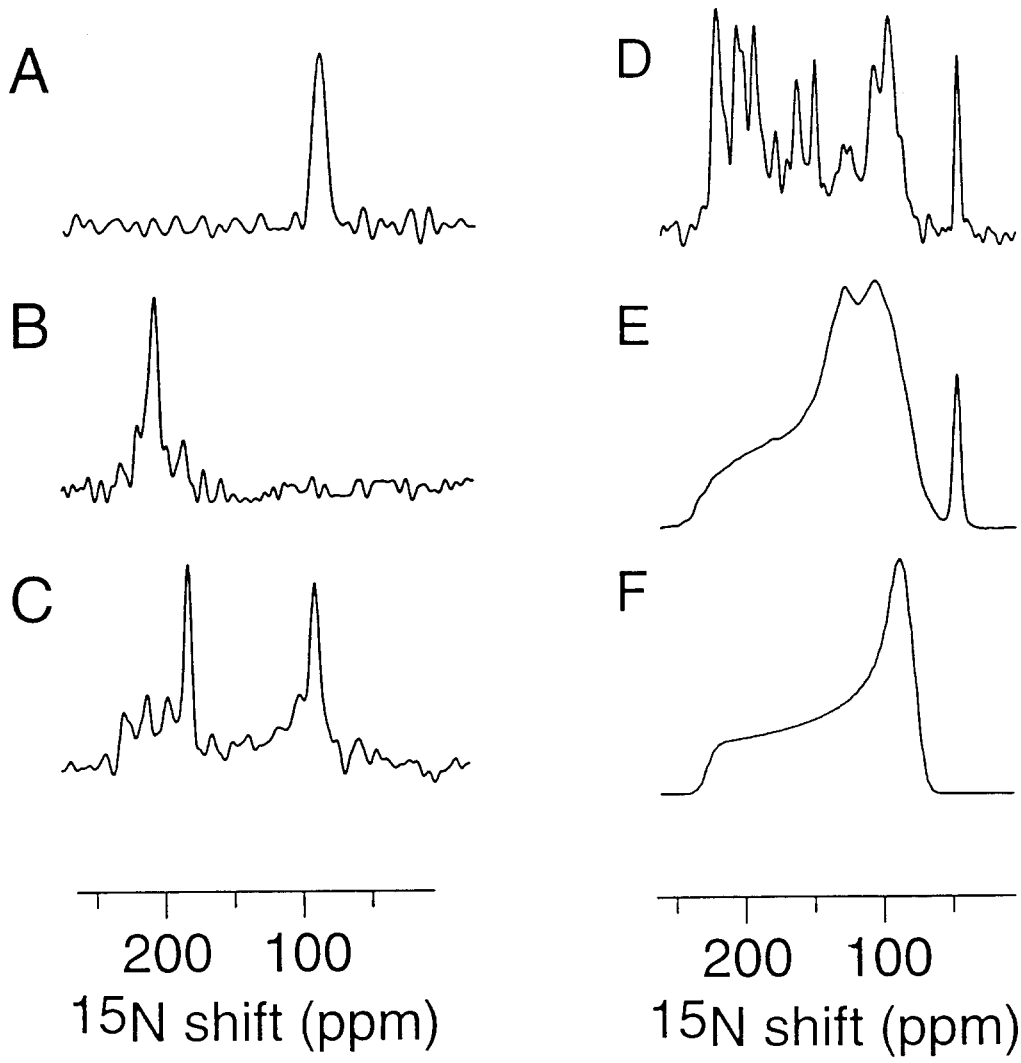

Fig. 2. One-dimensional solid-state ^{15}N NMR spectra of peptides and proteins in oriented bilayer samples. A) Specifically ^{15}N labeled magainin. B) Specifically ^{15}N labeled M2 channel peptide. C) ^{15}N Leu labeled fd coat protein. D) Uniformly ^{15}N labeled fd coat protein. E) Unoriented uniformly ^{15}N labeled fd coat protein. F) Calculated ^{15}N chemical shift powder pattern for an immobile amide site.

The one-dimensional ^{15}N NMR spectra of three different polypeptides in oriented bilayers are compared in Figure 2. Magainin is an antibiotic peptide found originally in frog skins [9] and the M2 peptide has the sequence of the corresponding segment of the acetylcholine receptor,[10] significantly both of these 25 residue peptides exhibit biological activity. fd coat protein has 50 residues in its mature form. It is stored in the bacterial membrane prior to assembly into new virus particles as they are extruded through the membrane.[11] All three of these polypeptides have been shown to have predominantly helical secondary structure by independent methods, including multidimensional solution NMR spectroscopy in micelle samples. The magainin and M2 channel peptides were synthesized with a single ^{15}N labeled amide site and purified by HPLC. The fd coat protein was purified from bacteriophage particles obtained from infection of bacteria grown on defined media for selective and uniform isotopic labeling. The polypeptides were oriented in lipid bilayers between glass plates. The experimental solid-state NMR spectra were obtained with ^{1}H decoupling and have a narrow single-line resonances from each of the ^{15}N-labeled amide sites in the peptides.

The spectra of the magainin and M2 peptides have single resonance lines because there is only one labeled site in each peptide. Significantly, the resonance frequencies observed from the two samples are quite different, indicating that the peptide planes containing the labeled NH groups have very different orientations relative to the direction of the applied magnetic field. In order to determine the orientation of a labeled peptide plane, at least one other spectral parameter associated with that site, such as the heteronuclear dipole-dipole splitting or the ^{1}H chemical shift, has to be measured. However, because the secondary structures of these peptides have been previously determined to be helical, we can take advantage of the structural regularity of the α-helix to provide constraints on the orientations that are consistent with the data and to extend the result from the specifically labeled site to the entire polypeptide. Both qualitative and quantitative analyses of the ^{15}N resonance frequencies are in agreement in determining the orientations of these two helical peptides in lipid bilayers. The magainin peptide is oriented approximately parallel to the plane of the bilayer and the M2 peptide is a trans-membrane peptide oriented perpendicular to the plane of the bilayer as illustrated in Figure 1. Similar results have been found for other labeled sites in magainin and other antibiotic peptides, and for M2 peptides from the glycine and NMDA receptors. These results show a very strong similarity between the structural and functional properties of the acetylcholine, glycine, and NMDA receptor M2 peptides. It is likely that these structural findings reflect a similar organization of the receptor proteins from which the peptide sequences were determined.

The 50 residue fd coat protein has a more complex structure than the 25 residue magainin or M2 peptides. Multidimensional solution NMR experiments have shown that fd coat protein has a two separate helical segments separated by a flexible loop region in micelle samples.[12]

The membrane bound form of fd coat protein has long hydrophobic helix near the N-terminus and a shorter amphipathic helix near the C-terminus. Significantly, there are two distinct resonances in the spectrum (Figure 2C) of selectively ^{15}N-Leu labeled fd coat protein in oriented phospholipid bilayers. The large 80 ppm frequency difference between the resonances from the two labeled Leu residues demonstrates the effect of protein structure on these spectra. Leu-41, in the trans-membrane helix, has its N-H bond approximately parallel to the field because its resonance occurs near the downfield end of the spectrum as seen for the M2 peptide, while Leu-14, in the amphipathic in-plane helix, has its N-H bond perpendicular to the field because its resonance occurs near the upfield end of the spectrum as seen for the magainin peptide. The use of specifically and selectively labeled samples not only gives the opportunity to measure the chemical shift frequencies on assigned resonances, but also to characterize their linewidths. We have observed ^{15}N resonance linewidths narrower than 3 ppm from these samples, consistent with mosaic spreads of sample orientation of less than about 2°. This demonstrates that the samples are very highly oriented.

Uniform Isotopic Labeling

There are many reasons why it is desirable to utilize uniformly isotopically labeled protein samples. Not only does this enable the use of expressed rather than synthesized polypeptides, uniform labeling shifts the burden from sample preparation to spectroscopy. Complete spectral resolution is the essential starting point for structure determination. As illustrated with specifically labeled samples in Figures 2A – 2C, when the protein in the sample is immobile and oriented parallel to the direction of the applied magnetic field, the resulting spectra are characterized by single line resonances from the ^{15}N labeled sites.

The spectrum in Figure 2E was obtained from an unoriented sample of uniformly ^{15}N labeled coat protein in phospholipid bilayers. The narrow peak near 30 ppm results from the amino groups of the lysine sidechains and the N terminus, and is not of concern here. Solid-state NMR spectra are strongly affected by molecular motion. Most of the backbone sites are structured and immobile on the time scale of the ^{15}N chemical shift interaction (10 kHz) and contribute to the characteristic amide powder pattern between about 220 and 60 ppm. The protein ^{15}N amide resonances have powder pattern lineshapes very similar that simulated for an immobile site in Figure 2F. However, several backbone sites of the membrane bound form of fd coat protein including those near the N and C termini and some in the loop region between the two helices are mobile on the 10 kHz timescale, resulting in the narrow resonance band centered at the isotropic resonance frequency near 120 ppm.

The solid-state NMR spectrum of an oriented sample is strikingly different from that of an unoriented sample of the same uniformly ^{15}N labeled fd coat protein. The spectrum in Figure

2D from an oriented sample displays significant resolution with identifiable peaks throughout the frequency range of the ^{15}N amide chemical shift anisotropy powder pattern.

Two- and three-dimensional spectroscopy of uniformly ^{15}N labeled proteins in bilayers

Limited structural information is available from one-dimensional spectra of specifically labeled polypeptides in oriented bilayers. In the context of well characterized systems with secondary structure established by other methods this provided valuable insights into structure and function, for example the arrangements of the helices in the bilayers. However, with more than a few labeled sites, overlap becomes a significant problem, as shown with the spectrum in Figure 2E. Even though the lines are relatively narrow compared to the spectral range (3 ppm vs 170 ppm), the resolution of the one-dimensional spectrum is inadequate for detailed spectroscopic investigations. A family of multidimensional solid-state NMR experiments has been developed to deal with this situation.[13-15]

The two-dimensional PISEMA (polarization inversion spin exchange at the magic angle) experiment [13] is a high resolution version of separated local field spectroscopy, with the oriented ^{15}N chemical shift in one-dimension, as in the spectra in Figure 2, and the heteronuclear dipolar coupling associated with each nitrogen site in the other. This is illustrated for uniformly ^{15}N labeled fd coat protein in Figure 3A. In three dimensional correlation spectra, each amide resonance is characterized by the frequencies from the ^{1}H chemical shift, ^{1}H-^{15}N dipolar coupling, and ^{15}N chemical shift interactions that provide both high spectral resolution and the angular restrictions used for structure determination.[14,16] The two-dimensional planes extracted from a three-dimensional data set in Figures 3B and 3C show that it is possible to measure three frequencies for each resolved resonance. The orientational dependence of the three operative spin-interactions serves as a means for both obtaining spectral resolution and as a source of structural information.

As illustrated in Figure 3, it is now possible to obtain completely resolved solid-state NMR spectra of uniformly ^{15}N labeled membrane proteins in oriented bilayers.[16] Although this example is a relatively small protein, by all criteria it is completely immobilized in the bilayer sample on the relevant NMR timescales. This points to a crucial difference between solution and solid-state NMR experiments. For larger polypeptides, there will be additional resonances in both types of spectra. However, in solid-state NMR spectra the increase in molecular weight is not accompanied by increased linewidths. This is in contrast to solution NMR experiments, where the slower reorientation rates that accompany increases in molecular mass result in broader linewidths.

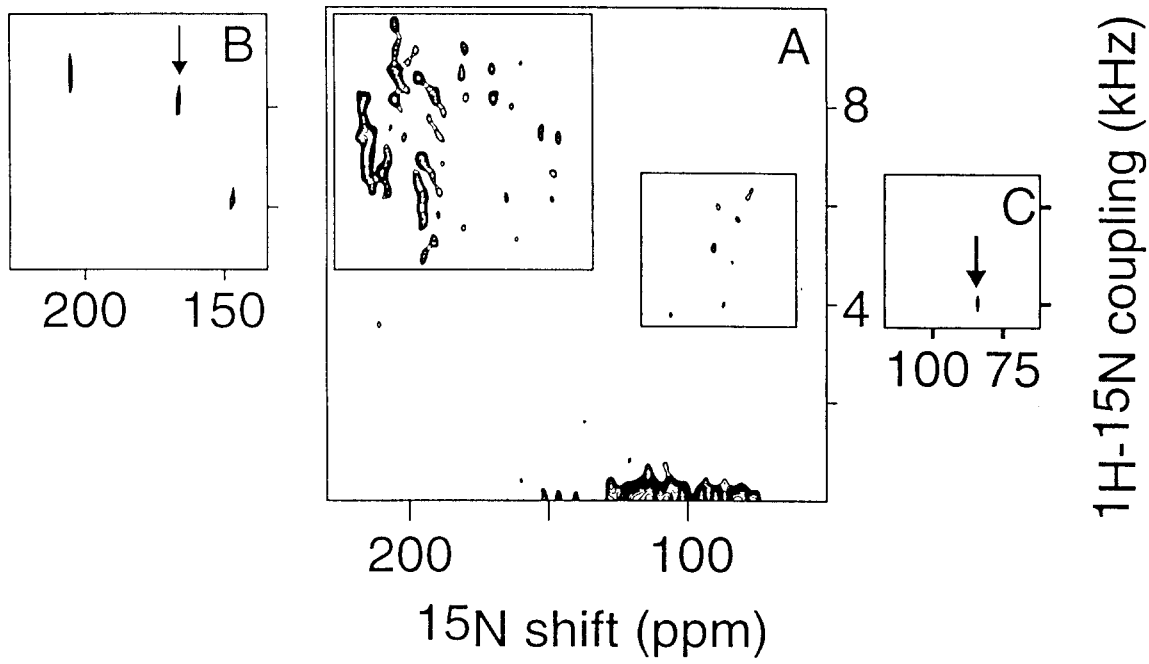

Fig. 3. Two-dimensional ^{1}H-^{15}N heteronuclear dipolar / chemical shift planes from spectra of an oriented sample of uniformly ^{15}N-labeled fd coat protein in lipid bilayers.[16] A) displays the frequencies of the two-dimensional PISEMA spectrum, specifically, those occupied by resonances from residues in the trans-membrane (left box) and in-plane (right box) helices. B) Plane extracted from the three-dimensional correlation spectrum at ^{1}H chemical shift 11.0 ppm. C) Plane extracted at ^{1}H chemical shift11.6 ppm. The resonances assigned to Leu-41 in the hydrophobic trans-membrane helix and Leu-14 in the amphipathic in-plan helix are marked. Three orientationally dependent frequencies can be directly measured for each of the resonances in the protein.

Colicin E1

Colicin E1 is a 522-residue protein that forms ion channels and kills bacterial cells.[17] The channel activity is localized in the 190-residue C-terminal domain. The soluble forms of the colicin channel domain as well as the entire molecule have been determined by x-ray crystallography. However, it is the membrane bound form of the protein that is responsible for its biological effects, and this structure has not been determined.

As discussed above, solid-state NMR studies of oriented samples have been successfully used to distinguish between in-plane and trans-membrane orientations of helical peptides and segments of proteins. Even without complete resolution and structure determination, this capability can be used to answer some of the most important structural and mechanistic questions about the membrane bound form of the colicin channel. Following the basic protocols developed for smaller polypeptides, solid-state NMR experiments were performed on samples of uniformly ^{15}N labeled 190-residue C-terminal colicin E1 polypeptide bound to oriented phospholipid bilayers. The portion of the two-dimensional PISEMA spectrum associated with trans-membrane helices is shown in Figure 4B. In this spectral region the chemical shift values are around 200 ppm and the heteronuclear dipolar splittings are near maximal, 10 kHz, as expected for N-H bonds approximately parallel to the direction of the applied magnetic field. Remarkably there are many resolved individual amide resonances in this region of the two-dimensional spectrum of the 190 residue uniformly ^{15}N labeled polypeptide.[18]

In the complete two-dimensional PISEMA spectrum, there is some intensity at the isotropic chemical shift position, which has no or very small dipolar couplings, as expected for mobile residues. The majority of resonance intensity, however, has chemical shift frequencies near 80 ppm, where the dipolar splitting is about one-half the maximal value (5 kHz). This indicates that most of the N-H bond vectors are oriented perpendicular to the applied magnetic field. For residues in helices, this means that the helices are arranged in the plane of the membrane. This is illustrated with the schematic model of colicin E1 channel domain in Figure 4A, which takes into account the distribution of resonance intensities in the full spectrum with about 20% of the residues in trans-membrane helices, a small percentage motionally averaged, and 70% in helices in the plane of the bilayers.

It is possible to distinguish approximately thirty resonances in that portion of the spectrum shown in Figure 4. Each correlation peak represents a single amide site. This is a dramatic spectroscopic result, since this demonstrates that it is possible to resolve resonances from individual sites in a 190 residue membrane protein. The properties of these resonances differ little from those observed from 25–50 residue polypeptides, demonstrating the insensitivity of the solid-state NMR experiment to molecular weight of the polypeptide. In our experience, it is generally possible to count 15–16 resolved resonances for each transmembrane

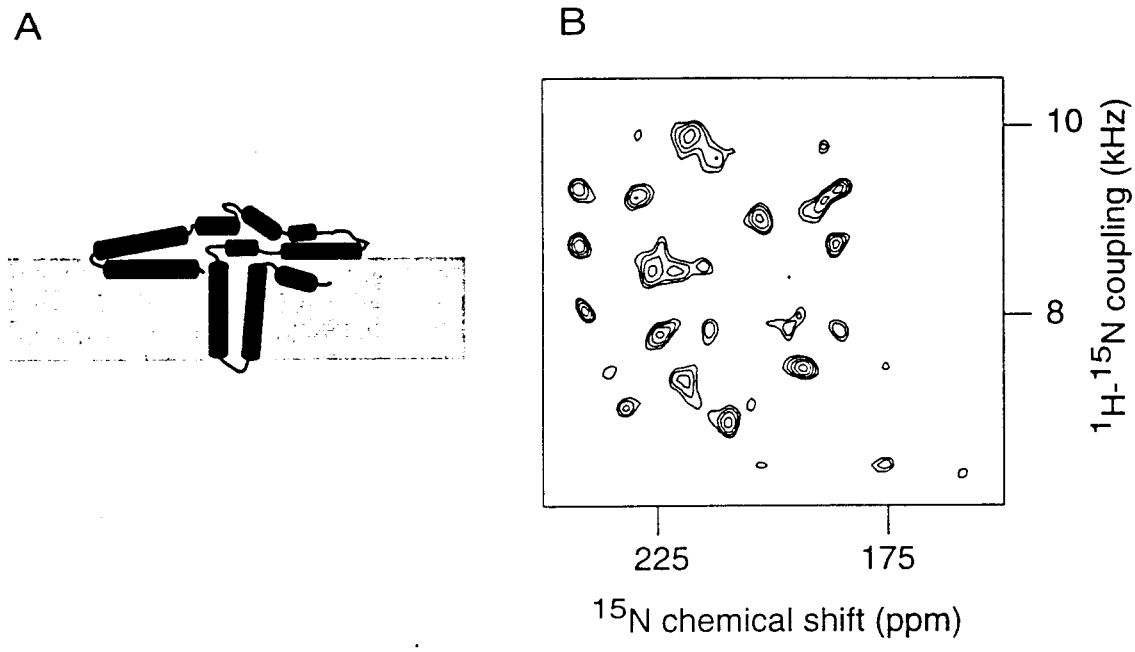

Fig. 4. A) Representation of the "umbrella" model of colicin E1 channel domain in bilayers. B) Transmembrane portion of the two-dimensional PISEMA spectrum of uniformly ^{15}N labeled colicin E1 channel domain in oriented bilayers.

helix from a uniformly ^{15}N labeled polypeptide. Thus, the data in Figure 4 are strongly indicative of the colicin E1 channel domain having two trans-membrane helices. This finding is consistent with the "umbrella" model for binding and insertion of the colicin channel polypeptide to membrane bilayers. In this model, the hydrophobic hairpin spontaneously inserts into the membrane bilayer and serves as an anchor for the insertion of the colicin E1 channel domain in phospholipid bilayers.

CONCLUSIONS

It is very important to be able to determine the structures of membrane proteins in phospholipid bilayer samples. Not only do the functions of these proteins reflect the interplay among structure, dynamics, and the asymmetric bilayer environment, there are always going to be outstanding questions about the influence of a micelle or other model membrane environments on protein structure. Further, there are severe limitations on the ability of solution NMR methods to cope with the slow reorientation rates of larger polypeptides in micelles. Proteins in bilayers are immobilized by their interactions with lipids, and highly oriented samples can be prepared for the most powerful multidimensional experiments. The completely resolved spectra of uniformly ^{15}N labeled membrane proteins in bilayers demonstrate that there is no fundamental size limitation to NMR spectroscopy.

Acknowledgements

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