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Structure Determination of Membrane Proteins in Five Easy Pieces

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Abstract

A general method for determining the structures of membrane proteins in phospholipid bilayers under physiological conditions is described. Membrane proteins are high priority targets for structure determination, and are challenging for the existing experimental methods. Because membrane proteins reside in a liquid crystalline phospholipid bilayer membranes it is important to study them in this type of environment. The approach we have developed can be summarized in five steps, and incorporates methods of molecular biology, biochemistry, sample preparation, construction and modification of NMR instrumentation, the development and execution of NMR experiments, and structure calculations. It relies on solid-state NMR spectroscopy to obtain high-resolution spectra and residue-specific structural restraints for membrane proteins which undergo rotational diffusion around the membrane normal, but whose mobility is otherwise restricted by interactions with the membrane phospholipids. The spectra of membrane proteins alone and in complex with other proteins and ligands set the stage for structure determination and functional studies.

Keywords

solid-state NMR; oriented sample; magic angle spinning; CXCR1; MerF; proteoliposomes; protein structure

1. INTRODUCTION

1.1 Membrane Proteins and Structure Determination

The three-dimensional structures of proteins provide the keys to understanding their biological functions. Although protein structure determination has played a prominent role in biomedical research for sixty years (1, 2), significant limitations and gaps remain in the experimental methods, and this is nowhere more evident than for membrane proteins. There has been recent progress in determining the structures of some membrane proteins by X-ray

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crystallography, mainly in the lipid cubic phase (3). However, the ultimate goal is to determine the structures of membrane proteins in their native phospholipid bilayer environment under physiological conditions.

Membrane proteins are very high-priority targets for structure determination. They are prevalent in nature where one-third of the genes in organisms ranging in complexity from bacteria to humans are translated into helical membrane proteins (4). They are responsible for essential physical, chemical, and structural properties of membranes. They have many unique biological functions as receptors and enzymes, transporters of ions and organic molecules. Finally, they have roles in the assembly, fusion, and maintenance of cells, organelles, and viruses. Moreover, human diseases result from mutations in membrane proteins (5), and most drugs act by binding to membrane protein receptors.

The structures and dynamics of membrane proteins are strongly affected by the properties of the surrounding phospholipids. Therefore, one of the most important requirements of a general method for structure determination is that the proteins reside in phospholipid bilayers so that potential perturbations resulting from the presence of detergents or non-natural lipid phases can be avoided. It is equally important that the bilayers are in the liquid crystalline phase where the proteins undergo the motions, including rotational and translational diffusion, necessary for their biological functions. The presence of fast (on the NMR timescale of $\sim 10^5$ Hz) rotational diffusion around the bilayer normal is essential to provide rotational alignment of the proteins for the newly developed method of structure determination by solid-state NMR spectroscopy (6).

In addition, a general method for determining the structures of membrane proteins must be applicable to proteins with a wide range of sizes, secondary structures, and levels of structural complexity, even though most or all of the polypeptides reside in the same phospholipid bilayer environment. Figure 1 illustrates the panel of membrane proteins that we are studying because of their biomedical interest and because they provide a range of sizes and other properties for the development of new methods of structure determination. All of them have been expressed, isotopically labeled and purified from *E. coli* in the multi-milligram amounts required for NMR studies. These proteins range in size from 35 residues to 350 residues, have between one and seven transmembrane α -helices, and include β -barrels. They include MerF, a membrane protein involved in mercury transport with two transmembrane helices, and the G-protein coupled receptor (GPCR)* CXCR1 with seven transmembrane helices. Here we illustrate the spectroscopic and structure determination methods with examples from our studies of an N- and C- terminal truncated construct of MerF, MerFt (7, 8), and full-length CXCR1 (9, 10) that are high-lighted in red in Figure 1. We have also obtained spectra and calculated structures from a variety of other membrane proteins with both α -helical and β -barrel architectures (8, 11-15).

1.2 Mercury Transport Proteins

Although organomercury compounds are among the oldest known antibacterial agents, some bacteria thrive in the presence of high concentrations of the same mercury-containing compounds that are toxic to humans and most other bacteria. In particular, bacteria isolated from the mercury-polluted sediment around Minamata Bay in Japan contain an operon for the structural proteins that detoxify mercury (16). The highly toxic Hg(II) is transported into the cytoplasm by a membrane protein where it is enzymatically reduced to Hg(0), which is less toxic and volatile. Various isolates of these bacteria contain one or more mercury transport membrane proteins, which all have high degrees of sequence homology. Determining the three-dimensional structures of these proteins is an essential first step towards understanding their mechanisms of metal transport and interactions with periplasmic and cellular proteins. They also provide an opportunity to compare the

structures of homologous membrane proteins with between two and four transmembrane helices. For example, MerF has 81 residues and two hydrophobic transmembrane helices (17) and MerT has 116 residues and three hydrophobic transmembrane helices; they have about 20% sequence identity when all of MerF is compared to the first two-thirds of MerT, but significantly MerF has two and MerT has one pair of vicinal Cys residues that are thought to be key parts of their Hg(II) transport mechanism

1.3 G-protein coupled receptors (GPCRs)

GPCRs are the largest class of membrane proteins. Not only are approximately 4% of all proteins in a cell GPCRs, there are about 800 different GPCR sequences encoded in the human genome. Half of the GPCRs are responsible for sensory functions, such as smell, taste, and vision, and half are potential drug targets since they are involved in various aspects of metabolic signaling. At present, about one-third of all drugs have GPCRs as their receptors. Yet, only about sixty are receptors for small molecules and about twenty-five for bio-therapeutic agents including antibody-based therapeutics. There is a great deal of room for the discovery of new drugs that interact with the unused GPCRs (18). CXCR1 is a chemokine receptor that interacts with interleukin-8 (IL-8) as part of the inflammatory response, and it has other roles including in cancer metastasis that make it an important target for structure determination (19, 20). All membrane proteins are challenging because of their liquid crystalline bilayer environment. With 350 residues and seven trans-membrane helices, CXCR1, like other GPCRs, presents a substantially greater level of structural complexity than the smaller mercury transport proteins discussed in section 1.2, which have as a few as two transmembrane helices.

2. MATERIALS AND METHODS

2.1 Biochemical Materials and Methods

The general approach to sample preparation is outlined in section 3. The most important features are that the membrane protein sequence of interest is expressed as a fusion protein to ensure that it is sequestered in inclusion bodies, which prevents overloading the cell membranes and assists in protein purification. The specific details of the expression, isotopic labeling, purification, and sample preparation of both MerFt (7, 8) and CXCR1 (9, 21) have been described previously.

2.2 NMR Methods and Instrumentation

Seminal advances in MAS and OS solid-state NMR have enabled the structure of complex membrane proteins to be characterized or determined or characterized in recent years (8, 11-15, 22-33).

Solid-state NMR experiments were performed on a Bruker Avance 750 MHz NMR spectrometer equipped with a Bruker 3.2 mm low-E triple resonance $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ probe. The OS solid-state NMR experiments were performed on a Bruker Avance 700 MHz spectrometer equipped with a home-built double-resonance $^1\text{H}/^{15}\text{N}$ probe with a single solenoid coil that included a strip shield (34) to minimize sample heating due to radiofrequency irradiations.

3. RESULTS AND DISCUSSION

3.1 Rotational alignment (RA) solid-state NMR

The characteristic effects of motional averaging on static powder patterns enable the methods of oriented sample (OS) solid-state NMR and magic angle spinning (MAS) solid-state NMR to be merged. When the motion is about a single axis it is particularly

straightforward to analyze, as demonstrated in some of the earliest solid-state NMR studies (35, 36). Rotational alignment RA solid-state NMR results from fast rotational diffusion about the bilayer normal. It was originally demonstrated by ^{31}P NMR on phospholipid head groups in bilayers (37), and by ^{13}C NMR on proteins in bilayers (38). We and others have shown that the same orientation-dependent frequency can be measured from the parallel edge of a rotationally averaged powder pattern and from a single-line resonance from samples of membrane proteins oriented mechanically on glass plates (39, 40) or in magnetically aligned phospholipid bilayers (6, 41).

OS solid-state NMR has the principal advantage of providing angular constraints directly from the measurement of frequencies of single line resonances. These constraints are used for both structure determination and for orienting the protein within the framework of the membrane bilayer (42). There is no uncertainty about the alignment of the protein in the membrane. Two and three-dimensional separated local field (SLF) spectra (43-45) of isotopically labeled proteins are resolved and assigned based on the frequencies from the anisotropic chemical shift and heteronuclear dipolar interactions. These frequencies can be directly translated into the angular constraints that provide input for the calculations of proteins structures. MAS solid-state NMR has the advantages of resolving resonances based on their isotropic chemical shifts, higher sensitivity due to ^{13}C detection, and facile backbone assignment methods from the use of uniformly ^{13}C , ^{15}N labeled proteins. Briefly, the isotropic resonances are resolved and assigned using correlation methods similar to those applied to polycrystalline proteins (25, 46, 47), and the rotationally averaged chemical shift and heteronuclear dipolar coupling powder patterns are recoupled using established techniques (48, 49).

The dramatic effects of fast rotational diffusion on the shape and breadth of the ^{13}C chemical shift anisotropy powder pattern from a carbonyl group in a peptide bond are illustrated in Figure 2 with simulated spectra (10). The static powder pattern for a carbonyl group is highly asymmetric with a large frequency span (~150 ppm), as illustrated in Figure 2B, which is observed in spectra from samples of a polycrystalline peptide or a membrane protein in lipid bilayers at very low temperatures. When the same carbonyl group of a peptide bond is found in the backbone of an α -helix aligned with its long axis approximately parallel to the bilayer normal (e.g. in a transmembrane helix) and undergoing rotational diffusion about the bilayer normal along with the rest of the protein, the resulting powder pattern is substantially reduced in breadth as shown in Figure 2A. All rotationally averaged powder patterns are axially symmetric (35, 36). The sign of the powder pattern and the frequency span between its parallel and perpendicular edges are determined by the angle between the principal axis of the chemical shift tensor and the axis of rotational diffusion. Thus, individual powder patterns can be used to define the orientation of a peptide plane relative to the bilayer normal.

The simulated solid-state NMR spectra in Figure 2A (motionally averaged) and 2B (static) are representative of experimental data obtained for a protein with a single ^{13}C -labeled carbonyl group or with a group of ^{13}C labeled carbonyl groups with similar orientations (e.g. for residues in transmembrane helices). Figure 2D shows that in a slow spinning MAS experiment the powder pattern is mapped out by the spinning sidebands and can be calculated from their intensities (50). In this case, the family of sidebands span the frequency breadth of the entire powder pattern, which is much greater than the relatively slow spinning rate (5 kHz). In contrast, for a protein undergoing rotational diffusion about the bilayer normal, no sidebands are observed because of the limited frequency span of the rotationally averaged powder pattern (Figure 2C). In a stationary sample, a labeled protein undergoing rotational diffusion yields a single broadened line; by comparison, it can be deduced that the underlying powder pattern is similar to that in Figure 2A. When MAS is

applied, a single sharper line is observed. The large differences between both the stationary and MAS spectra in Figure 2C and 2D make it easy to differentiate between proteins that are static or are undergoing fast rotational diffusion in lipid bilayers (10).

Although it is possible to characterize the presence or absence of rotational diffusion in a uniformly labeled membrane protein based on the properties of the overlapping, similar ^{13}C chemical shift powder patterns, in a uniformly $^{13}\text{C}/^{15}\text{N}$ labeled sample it is essential to resolve the resonances of individual ^{13}C , $^{13}\text{C}\alpha$, and ^{15}N amide sites to obtain high resolution NMR spectra and structural restraints. With relatively fast magic angle spinning (~ 11 kHz) individual center bands can be resolved using a variety of two- or three-dimensional MAS solid-state NMR experiments. The third frequency dimension can also be used to observe the powder pattern line shapes in experiments that recouple the chemical shift anisotropy, as illustrated in Figure 2 and Figure 4, or the ^1H - $^{13}\text{C}\alpha$ or ^1H - ^{15}N amide heteronuclear dipole-dipole interactions, which appear as static or rotationally averaged axially symmetric Pake powder patterns (51) in Figures 4 and 5.

3.2 Structure Determination of Membrane Proteins in Five Easy Pieces

Our most recently developed approach to structure determination of membrane proteins (6) can be summarized with five steps: (1) prepare a sample of a uniformly $^{13}\text{C}/^{15}\text{N}$ labeled membrane protein in proteoliposomes; (2) resolve individual signals with MAS solid-state NMR experiments; (3) assign each signal to a specific residue; (4) measure two or more orientation-dependent frequencies for each residue and a few long-range distance restraints; (5) calculate de novo the three-dimensional structure of the membrane protein. These five steps are described in sections 3.2.1 – 3.2.5 below. They are all “Easy Pieces” (52) that can be performed with commercially available materials and solid-state NMR hardware, and with publically available software – riding the fast lane on the road to membrane protein structure, as in the movie.

3.2.1 Piece number 1: prepare a sample of a uniformly $^{13}\text{C}/^{15}\text{N}$ labeled membrane protein in proteoliposomes—Sample preparation for a typical membrane protein starts with synthesis of the gene encoding the protein sequence and ends with the sample for NMR experiments. If the protein is not from bacteria, then its gene is codon-optimized for heterologous expression in *E. coli*. For many membrane proteins, it is possible to obtain four to six milligrams of purified protein from each one-liter culture grown on minimal M9 media for uniform $^{13}\text{C}/^{15}\text{N}$ isotopic labeling. The use of a fusion protein ensures that the overexpressed polypeptide is sequestered in inclusion bodies, protecting the cell membranes from protein overload, and aiding protein purification (21, 53-55). A variety of fusion proteins can be used. After inclusion body isolation, the solubilized polypeptide is subjected to nickel affinity chromatography. Smaller membrane proteins are generally cleaved by treatment with cyanogen bromide at a strategically placed methionine residue after the fusion protein is washed from the column. Larger proteins are generally subjected to enzymatic cleavage on the column. Final purification is accomplished by chromatography, with either HPLC or FPLC, depending on the properties of the individual protein, and the associated polypeptide impurities that need to be removed. The purification procedures undergo extensive optimization so that the final product incorporated in proteoliposomes yields a single band on polyacrylamide gel electrophoresis (PAGE) within the limits of detection ($> 98\%$). For GPCRs it is essential to avoid the presence of even very small amounts of oligomers in order to prepare stable samples.

A benefit of using unoriented proteoliposomesamples for NMR experiments is the increased flexibility in the choice of lipids, temperatures, and other conditions. For example, it is feasible with proteoliposomes to describe the effects on the protein structure of: the length

and unsaturation of the phospholipid hydrocarbon chains, the phospholipid head group chemistry, various phospholipid mixtures, the addition of other membrane components, such as cholesterol, and the addition of protein or small molecule ligands for specific experiments. The proteoliposomes used in the MAS solid-state NMR experiments typically contain ~2 mg of isotopically labeled protein; they are ultracentrifuged to a hydrated pellet, and then transferred and sealed inside an MAS rotor.

3.2.2 Piece number 2: resolve individual signals with MAS solid-state NMR experiments—The proteoliposome-containing rotor is placed in the stator of a commercial or home-built MAS $^1\text{H}/^{15}\text{N}/^{13}\text{C}$ triple-resonance probe equipped with a low-E resonator to avoid sample heating. An essential first step is to verify that the protein in the sample undergoes fast rotational diffusion at relatively high temperatures ($>25^\circ\text{C}$) and is static at lower temperatures ($<10^\circ\text{C}$) by monitoring the line shapes or spinning sideband intensities as a function of temperature. As illustrated in Figure 2, the $^{13}\text{C}'$ chemical shift anisotropy (CSA) powder pattern is a particularly convenient spectral parameter for this purpose; the static and rotationally averaged powder patterns can be readily differentiated by their frequency spans and shapes in stationary samples, spinning sideband patterns with slow (5 kHz) magic angle spinning, and by recoupling in n -multidimensional MAS experiments (49).

Once the sample and experimental conditions are optimized, the first experiments are focused on resolution of signals from the protein. MerFt is a 60-residue N- and C- terminal truncated version of MerF, containing the central helix-turn-helix motif of the protein. We have used this polypeptide in samples for many of our spectroscopic developments (7, 8). Figure 3A displays a two-dimensional $^{13}\text{C}/^{15}\text{N}$ chemical shift heteronuclear correlation spectrum of a uniformly $^{13}\text{C}/^{15}\text{N}$ labeled sample of MerFt in DMPC bilayers. While the spectrum appears quite crowded in this presentation, when it is expanded all of the expected resonances can be accounted for as completely or partially resolved signals. The partially resolved signals from L31 and D69 are marked in the two-dimensional spectra in Figure 3A and 3B. The two-dimensional planes from three-dimensional experiment show complete resolution of the $^1\text{H}-^{15}\text{N}$ heteronuclear dipolar couplings for individual $^{13}\text{C}\alpha$ resonance frequencies in Figure 3C and 3D. The two-dimensional spectra of substantially larger proteins provide very limited resolution and three-dimensional spectra are essential in order to identify individual resonances and to measure the recoupled chemical shift and heteronuclear dipolar coupling powder patterns.

3.2.3 Piece number 3: assign each signal to a specific residue—In addition to enabling the resolution of individual signals in two- and three-dimensional spectra, the use of uniformly $^{13}\text{C}/^{15}\text{N}$ labeled samples enables magnetization to be transferred through dipole-dipole couplings among proximate ^{13}C and ^{15}N nuclei. This provides a mechanism for correlating inter- and intra-residue chemical shift frequencies enabling backbone walks from residue to residue for assignment. The $^{13}\text{C}\alpha$ and ^{15}N amide resonances of CXCR1 were assigned in this way following two principal types of experiments, NCACX and NCOCA, which are routinely used in studies of polycrystalline proteins. This approach to making assignments is not currently feasible for OS solid-state NMR experiments on aligned, stationary samples, and requires the development of new classes of triple-resonance experiments (56); some assignment schemes, such as flipping the lipid bilayer normal between perpendicular and parallel alignments, and pairing signals according to their heteronuclear couplings (57), are unique to aligned, stationary samples and are complementary to the methods used with magic angle spinning.

3.2.4 Piece number 4: measure two or more orientation-dependent frequencies for each residue and some distances—The measurement of resolved rotationally averaged powder patterns by recoupling the $^{13}\text{C}\alpha$ and ^{15}N CSA (49) and

the ^1H - ^{13}C and ^1H - ^{15}N heteronuclear dipolar coupling (DC) in three-dimensional MAS solid-state NMR experiments provides the orientation-dependent frequencies used in structure determination. Figure 3 shows two-dimensional ^1H - ^{15}N DC / $^{13}\text{C}\alpha$ shift SLF planes at selected ^{15}N shift frequencies from a three-dimensional spectrum of MerFt in proteoliposomes. Each plane, which is representative of those in the full data set, has only a few signals; thus, examination of all the planes provides complete resolution of the MerFt spectrum and measurement of the rotationally averaged ^1H - ^{15}N DC frequencies. These data are clearly shown in one-dimensional slices from the two-dimensional planes in Figure 4. Figure 4A-4D represent the ^{15}N amide chemical shift anisotropy. The full breadth of a powder pattern from a static sample is shown in Figure 4A (experimental) and Figure 4B (simulated). The parallel and perpendicular edges of the static powder pattern are shown with dashed vertical lines for comparison to the rotationally averaged powder patterns from D69 and L31 of MerFt. The ^1H - ^{15}N amide and ^1H - $^{13}\text{C}\alpha$ heteronuclear dipolar coupling powder patterns in Figure 3E-3J directly provide angular restraints between the ^1H - ^{15}N bond and the bilayer normal due to the well-characterized global dynamics of the protein in the bilayers.

Significantly, it is possible to obtain data of similar quality from much larger membrane proteins. This is illustrated for the ^1H - ^{15}N heteronuclear dipolar couplings at two different $^{13}\text{C}\alpha$ resonance frequencies of CXCR1. Figure 5A shows a simulated ^1H - ^{15}N DC static powder pattern (Pake doublet (51)). The experimental data in Figure 5 are ^1H - ^{15}N DC slices at selected $^{13}\text{C}\alpha$ shifts. Two resonances (bottom) have the same $^{13}\text{C}\alpha$ shift of 54.6 ppm but quite different ^1H - ^{15}N DC values of 1.0 kHz and 8.2 kHz. This demonstrates the utility of heteronuclear DCs in resolving resonances, in addition to providing angular restraints for structure calculations. The signal with a $^{13}\text{C}\alpha$ shift of 58.9 ppm has a single ^1H - ^{15}N DC frequency of 9.7 kHz.

Since the frequencies measured from the parallel edges of the rotationally averaged CSA and DC powder patterns are equivalent to those measured from the single-line resonances of aligned samples (6), the methods of structural analysis developed for OS solid-state NMR can be applied. For α -helices, these include PISA wheels (58-60), which are plots of heteronuclear DC as a function of CSA, and dipolar waves (61), which are plots of heteronuclear DC as a function of residue number. Quantitative sinusoidal fitting of dipolar waves reveals the length, membrane tilt angle and rotation angle of a helix. By contrast, inter-helical loops and structured terminal regions yield complex PISA wheel and dipolar wave patterns, consistent with irregular tertiary folds in these regions.

Structure calculations, especially those aimed at placing helices within the tertiary structure, are facilitated when a few long-range distance restraints are available from paramagnetic relaxation enhancement (PRE) (62) or rotational echo double resonance (REDOR) experiments as described (22, 63).

3.3.5 Piece number 5: calculate de novo three-dimensional structure—We have participated in the development of many of the computational methods used for determining protein structures from orientation restraints obtained with OS solid-state NMR experiments on stationary, aligned samples (12, 14, 58, 60, 61, 64-72). These methods are equally applicable to RA solid-state NMR, using the frequencies measured from the parallel edges of rotationally averaged powder patterns. The ^1H - ^{15}N DC and ^{15}N CSA are sufficient to determine the orientation of the associated peptide plane relative to the axis of alignment. Triple-resonance experiments (73-76) provide orientation-dependent frequencies for the out-of-plane $^{13}\text{C}\alpha$ sites, as well as the majority of side chain sites. The assigned experimental frequencies and distances can be used as restraints in simulated annealing calculations to

obtain refined structures that are accurately aligned relative to the bilayer normal with RMSDs <1.5 Å.

The structure of MerFt in phospholipid bilayers (Fig. 6A) demonstrates the feasibility of determining the three-dimensional structures of membrane proteins in native-like membrane environments and conditions. The structure was obtained starting with a Rosetta (77-80) model of the protein refined against the experimental data by simulated annealing in XPLOR-NIH (81). The correlation plot in Figure 6B compares the measured ^1H - ^{15}N amide heteronuclear DCs to those back calculated from the refined structure in Figure 6A. The correlation coefficient is 0.94, which provides assurance that the structure is consistent with the experimental data. Since data of similar quality can be obtained from MerFt and CXCR1, this demonstrates the potential of the method for determining the structures of the membrane proteins represented in Figure 1, as well as others, including those dominated by β -sheet rather than α -helix.

4. CONCLUSIONS

The benefits of merging the two previously disparate branches of high-resolution solid-state NMR spectroscopy are described. The end result, RA solid-state NMR, is a robust method that takes advantage of the systematic methods for resonance assignments inherent with the use of uniformly ^{13}C and ^{15}N labeled proteins in MAS solid-state experiments and the strong orientation-dependent restraints obtained from OS solid-state experiments.

The RA solid-state NMR method described here has four principal advantages over competitive methods of structure determination of membrane proteins. First and foremost, the proteins are examined in the native-like environment of the phospholipid bilayer membrane under physiological conditions. Second, the proteins do not need to be modified with mutations, truncations, insertions of other proteins, or the presence of stabilizing antibody fragments. Third, ligands, (e.g. other proteins, small molecules, drugs, antibody fragments) can be easily added to the samples so that direct comparisons can be made. Fourth, NMR is adept at describing both local and global dynamics of proteins, and this will contribute to understanding the motions and multiple conformations that membrane proteins appear to utilize in their mechanisms of action.

Atomic-resolution descriptions of the structure and dynamics of membrane proteins in their native phospholipid environment has the potential to contribute to many areas of biomedical research, ranging from fundamental studies of protein structure and mechanisms of action to drug discovery.

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Figure 1. Membrane proteins that have been expressed, purified, and demonstrated to give high-resolution solid-state NMR spectra in lipid bilayers

This panel provides a series of targets for the development of NMR spectroscopy and structure determination methods. From left to right they have increasing size and complexity, ranging from a 35-residue, single transmembrane, α -helical domain, to a full-length 350-residue protein with seven transmembrane helices, and an outer membrane protein with β -barrel architecture. Solid-state NMR can be used to determine the structures of these membrane proteins in native-like membrane environments and conditions. This is demonstrated here with MerFt, a truncated construct of MerF with 60 residues and two transmembrane helices, and with CXCR1, a G-protein coupled receptor with 350 residues and seven transmembrane helices.

Figure 2. The effects of rotational diffusion on the chemical shift anisotropy powder pattern from $^{13}\text{C}'$ labeled membrane proteins in proteoliposomes using simulated spectra

(A, B) $^{13}\text{C}'$ chemical shift powder patterns for a residue in a transmembrane helix approximately parallel to the membrane normal, rotationally averaged by rotational diffusion (A) or static (B). (C, D) Same as (A, B), respectively, except that the sample is undergoing slow (5 kHz) magic angle spinning. No sideband intensity is observed in (C) because of the narrow frequency breadth of the motionally averaged powder pattern. (D) A family of sidebands is observed because of the broad frequency breadth of the static powder pattern. The sideband intensities can be used to calculate the powder pattern line shape.

Figure 3. Multi-dimensional MAS solid-state NMR spectra of uniformly $^{13}\text{C}/^{15}\text{N}$ labeled MerFt in proteoliposomes

(A) Two-dimensional $^{13}\text{C}/^{15}\text{N}$ heteronuclear correlation spectrum of MerFt in proteoliposomes. (B) Two-dimensional $^1\text{H}-^{15}\text{N}$ dipolar coupling/ ^{13}C chemical shift SLF spectrum of MerFt in proteoliposomes. The signals for L31 and D69 can be identified in these crowded two-dimensional spectra, which have had all resonances assigned. (C, D) Two-dimensional $^1\text{H}-^{15}\text{N}$ dipolar coupling/ ^{13}C chemical shift SLF planes from a three-dimensional spectrum. The few signals in each plane demonstrate that complete resolution is achieved in the three-dimensional spectrum. The same dipolar coupling frequencies are

measured in panels B, C, D for both L31 and D69. Similar results are obtained for all other residues and provide the input for the structure calculations.

Figure 4. One-dimensional slices from multi-dimensional MAS solid-state NMR spectra of uniformly $^{13}\text{C}/^{15}\text{N}$ labeled MerFt in proteoliposomes

One dimensional ^{15}N solid-state NMR spectra of uniformly $^{15}\text{N}/^{13}\text{C}$ labeled MerFt in DMPC bilayers. A. Experimental ^{15}N amide chemical shift powder pattern spectrum of L31 (essentially identical to that for D69) obtained at low temperature (10°C). B. Simulated static ^{15}N amide chemical shift powder pattern spectrum based on the data in Figure 4A. C. Experimental rotationally averaged ^{15}N amide chemical shift powder pattern spectrum of D69 at 25°C. D. Same as C. except for L31. E. Simulated static Pake powder pattern for ^1H - ^{15}N amide heteronuclear dipolar coupling. F. Experimental rotationally averaged ^1H - ^{15}N heteronuclear dipolar coupling for D69 at 25°C. G. Same as F. except for L31. H. Simulated static Pake powder pattern for ^1H - ^{13}C heteronuclear dipolar coupling. I. Experimental rotationally averaged ^1H - ^{13}C heteronuclear dipolar coupling powder pattern spectrum for D69 at 25°C. J. same as I. except for L31. The experimental data in the Figure were extracted from high resolution multidimensional experiments correlating ^{13}C , ^{15}N isotropic chemical shifts and either ^1H - ^{15}N / ^1H - ^{13}C dipolar coupling or ^{15}N chemical shift anisotropy in the third dimension (82).

Figure 5. One-dimensional slices from multi-dimensional MAS solid-state NMR spectra of uniformly $^{13}\text{C}/^{15}\text{N}$ labeled CXCR1 in proteoliposomes

(A) Simulated static Pake powder pattern spectrum for a ^1H - ^{15}N heteronuclear dipolar coupling of an amide group in a peptide bond. (B, C) One-dimensional rotationally averaged ^1H - ^{15}N heteronuclear powder patterns extracted from a three-dimensional ^{13}C -detected ^1H - ^{15}N dipolar coupling / ^{15}N chemical shift SLF spectrum. ^1H - ^{15}N heteronuclear dipolar coupling frequencies (noted on the left side of the spectra) are associated with $^{13}\text{C}\alpha$ isotropic chemical shifts (noted on the right side of the spectra).

Figure 6. Three-dimensional structure of MerFt in phospholipid bilayers

(A) Structure of MerFt determined by the RA solid-state NMR method described here. (B) Correlation plot of back-calculated ^1H - ^{15}N heteronuclear dipolar couplings from the refined structure with experimentally measured couplings. The correlation coefficient is 0.94.