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Membrane Protein Structure Determination *in Membrana*

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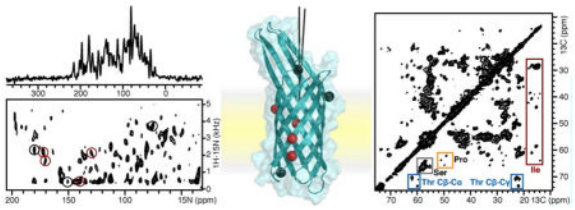
CONSPECTUS



The two principal components of biological membranes, the lipid bilayer and the proteins integrated within it, have coevolved for specific functions that mediate the interactions of cells with their environment. Molecular structures can provide very significant insights about protein function. In the case of membrane proteins, the physical and chemical properties of lipids and proteins are highly interdependent; therefore structure determination should include the membrane environment. Considering the membrane alongside the protein eliminates the possibility that crystal contacts or detergent molecules could distort protein structure, dynamics, and function and enables ligand binding studies to be performed in a natural setting.

Solid-state NMR spectroscopy is compatible with three-dimensional structure determination of membrane proteins in phospholipid bilayer membranes under physiological conditions and has played an important role in elucidating the physical and chemical properties of biological membranes, providing key information about the structure and dynamics of the phospholipid components. Recently, developments in the recombinant expression of membrane proteins, sample preparation, pulse sequences for high-resolution spectroscopy, radio frequency probes, high-field magnets, and computational methods have enabled a number of membrane protein structures to be determined in lipid bilayer membranes.

In this Account, we illustrate solid-state NMR methods with examples from two bacterial outer membrane proteins (OmpX and Ail) that form integral membrane β-barrels. The ability to measure orientation-dependent frequencies in the solid-state NMR spectra of membrane-embedded proteins provides the foundation for a powerful approach to structure determination based primarily on orientation restraints. Orientation restraints are particularly useful for NMR structural studies of membrane proteins because they provide information about both three-dimensional structure and the orientation of the protein within the membrane. When combined with dihedral angle restraints derived from analysis of isotropic chemical shifts, molecular fragment replacement, and *de novo* structure prediction, orientation restraints can yield high-quality three-dimensional structures with few or no distance restraints. Using complementary solid-state NMR methods based on oriented sample (OS) and magic angle spinning (MAS) approaches, one can resolve and assign multiple

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peaks through the use of $^{15}N/^{13}C$ labeled samples and measure precise restraints to determine structures.

Introduction

Biological membranes are essential for cellular life. These ancient structures evolved very early on before the split from the last universal common ancestor that led to the three branches of cellular life: bacteria, archaea, and eukarya. The chemical composition and structural organization of biological membranes indicate that they emerged through a process of coevolution of their two principal components: the lipid bilayer and the proteins integrated within it.[1] The lipid bilayer provides an elastic yet ion-impermeable barrier for the effective compartmentalization of specialized cellular components, while integral membrane proteins mediate all interactions of cells with each other and with the outside world through their specific functions in transmembrane transport, signaling, adhesion, and much more. The coevolution model reflects the intimate relationship between membrane proteins and lipids. The lipid bilayer environment is anisotropic and heterogeneous, with large gradients in fluidity, water concentration, and dielectric constants from the bilayer core to the water–lipid interface. These features have profound effects on membrane protein structure, dynamics, and function,[2] underscoring the importance of determining the structures of membrane proteins in lipid bilayers at or near physiological conditions of temperature, pH, and hydration.

X-ray diffraction and solution NMR structural studies of membrane proteins typically require proteins dissolved in detergents because lipid bilayers are incompatible with the requisite three-dimensional crystallization and isotropic motion. Lipid nanodiscs typically yield broader lines compared with micelles but can be useful solution NMR membrane mimics for some membrane proteins, as shown recently for the bacterial outer membrane protein OmpX.[3] By contrast, solid-state NMR is compatible with structure determination of membrane proteins in a wide variety of phospholipid bilayer membranes under physiological conditions.

From the very beginning, solid-state NMR has played an important role in elucidating the physical and chemical properties of biological membranes. Early solid-state NMR studies provided key information about the structure and dynamics of the phospholipid components.[4–7] More, recently, solid-state NMR studies have shifted attention to integral membrane proteins, facilitated by the use of recombinant DNA technology and automated PCR protocols, which enable a variety of proteins to be prepared biosynthetically and labeled isotopically. Recent developments in the areas of recombinant bacterial expression, sample preparation, pulse sequences for high-resolution spectroscopy, radio frequency probes, high-field magnets, and computational methods have enabled a number of membrane protein structures to be determined in phospholipid membranes (a few recent examples[8–11] are shown in Figure 1). Furthermore, solid-state NMR is providing important structural information for membrane proteins and peptides in native cell membranes and for key cytoskeletal components of cell envelopes.[12–15] These advances are described in recent excellent reviews.[16–22]

Using oriented sample (OS)[23,24] and magic angle spinning (MAS)[16,18] solid-state NMR approaches, it is possible to resolve and assign multiple peaks through the use of $^{15}N/^{13}C$-labeled samples and to measure precise restraints for structure determination. OS solid-state NMR uses samples that are uniaxially aligned in the magnetic field (e.g., planar lipid bilayers) to yield orientation-dependent single line resonances. MAS solid-state NMR uses unaligned samples (e.g., proteoliposomes) and yields single line spectra by averaging the spin interactions to their isotropic values. In both cases, the uniaxial order inherent to

membrane proteins undergoing rotational diffusion around the axis perpendicular to the membrane plane (i.e., the lipid bilayer normal) provides the foundation for a powerful approach to structure determination based primarily on orientation restraints.

In this Account, we illustrate the methods with examples from studies of the outer membrane protein Ail (attachment invasion locus) from *Yersinia pestis*, an extremely pathogenic organism with a long history of precipitating massive human pandemics. Ail belongs to the same protein family (pfam PF06316) as OmpX. However, unlike OmpX, Ail is a virulence factor essential for evading the human host's immune system through its two principal functions: mediating the adhesion of bacteria to human cells and providing resistance to human innate immunity.[25] Outer membrane proteins in this family share amino acid sequence homology in the membrane-spanning segments but vary widely in the sequences of the four extracellular loops, which are critical for function. Both Ail and OmpX adopt a transmembrane eight-stranded β-barrel structure.[26,27] However, key functional loops of Ail are not visible in its crystal structure. Thus, structural studies in membranes will be needed to understand the molecular basis for protein functionality.

Sample Preparation

The efficient production of isotopically labeled, recombinant outer membrane proteins, including Ail and OmpX, can be typically obtained by cloning the genes corresponding to the mature proteins (i.e., without their signal sequence) in *Escherichia coli*, to drive protein expression into inclusion bodies (Figure 2a).[26,28] The resulting inclusion bodies are highly enriched in recombinant protein, as evidenced by their white, fluffy appearance, enabling significant quantities of essentially pure protein to be readily obtained by performing a few centrifugation and detergent wash steps following bacterial cell lysis (Figure 2b) and subsequent purification by chromatography yields highly purified protein (Figure 2c). The purification strategy depends on the specific chemical properties of each protein. For example, the isolelectric points of OmpX (pI = 5.0) and Ail (pI = 7.8) dictate the use of anion and cation exchange chromatography, respectively. Notably, additional purification by size exclusion chromatography is needed to obtain samples of Ail suitable for high-resolution spectroscopy, even though SDS polyacrylamide gel electrophoresis (PAGE) shows no evidence of impurities prior to this step. This is likely due to the presence of lipids or other *E. coli* components that copurify with the protein during ion exchange.

SDS-PAGE analysis is very useful for monitor folding and unfolding of outer membrane proteins: unfolded and folded species migrate at distinctly different apparent molecular weights, and the protein fold, once established, is resistant to SDS as well as significant concentrations of urea and other denaturing conditions. For example, folded Ail is clearly detected at lower molecular weight by SDS-PAGE (Figure 2c). Fold is confirmed by solution NMR spectroscopy, where the $^{1}H/^{15}N$ HSQC spectra obtained for Ail in DPC (decylphosphocholine) micelles have dramatically greater chemical shift dispersion, consistent with β-barrel formation, compared with those obtained in urea where the protein is unfolded (Figure 2d,e).

Samples of folded Ail and OmpX can be obtained in a variety of phospholipid samples suitable for OS and MAS solid-state NMR studies,[28–30] including uniaxially oriented bilayers and unoriented liposomes (Figures 3 and 5). Efficient refolding in relatively short-chain phospholipids, such as DMPC(1,2-dimyristoyl-*sn*-glycero-3-phosphocholine) and DMPG (1,2-dimyristoyl-*sn*-glycero-3-phosphoglycerol), seems to be a common feature shared by many outer membrane proteins,[31] including Ail and OmpX. This observation suggests that the hydrophobic thickness of bacterial outer membranes may be smaller than

that of the inner membranes, and is in agreement with the relatively thin belt of hydrophobic residues surrounding the β-barrel of OmpX (see below).

NMR Restraints for Membrane Protein Structure Determination

Modern NMR methods for protein structure determination rely increasingly on orientation restraints derived from dipolar coupling (DC) and chemical shift anisotropy (CSA) measurements and on dihedral angle (φ,ψ) restraints derived from isotropic chemical shift (CS) analysis. Orientation restraints can be used very effectively both in the early stages of structure determination to guide the generation of structural models and in the final stages of structure refinement, and they are especially powerful when coupled with *de novo* structure prediction and molecular fragment replacement. Examples of this approach include solid-state NMR structures of membrane proteins in lipid bilayers,[8–11] as well as solution NMR structures of membrane proteins in detergent micelles[32] and globular proteins in water.[33,34] Importantly, they help shift the burden away from time-consuming measurements of multiple long-range distances between side chain sites, thus facilitating the determination of high-quality three-dimensional structures with very few or no distance restraints, derived from spin diffusion,[35] rotational resonance,[36] and paramagnetic relaxation enhancement.[37]

The orientation-dependent DC and CSA signals from $^{1}H/^{15}N/^{13}C$-labeled protein sites contain information about molecular structure, orientation, and dynamics. They are particularly useful in solid-state NMR structural studies of membrane proteins incorporated in liquid crystalline lipid bilayers; since the membrane-integrated regions of membrane proteins typically exhibit uniform dynamics dominated by uniaxial diffusion around the membrane normal, their corresponding DC and CSA signals can be readily interpreted in terms of orientation restraints. The extra-membrane, globular regions can exhibit different modes and time scales of internal motions, with significant scaling of the observed DC and CSA values that must be considered in their interpretation as orientation restraints.

Many DC and CS tensors are well characterized for $^{1}H/^{15}N/^{13}C$-labeled protein sites, and CS tensors themselves can be used as effective structural restraints.[38] Their dependence on secondary structure and amino acid type can be significant[39] However, CS tensor variations can be factored into the structure calculation protocol. For example, the ^{15}N CSA restraints of glycine sites are typically computed separately from those of other residues to account for their significantly different tensors.

Isotropic CSs are important sources of structural information in both solid-state NMR and solution NMR. Furthermore, the ability to predict isotropic CS from structural models could be useful for guiding MAS solid-state NMR assignments, which can be complicated by the lack of ^{1}H signals and by broader or overlapped lines. Because proteins are intrinsically dynamic, experimentally measured isotropic CS represent an ensemble and time average over all conformational states explored by the protein up to the millisecond time scale. Thus, improvements in isotropic CS prediction can be obtained by averaging predicted CS values over extended molecular dynamics trajectories, as well as over an ensemble of predicted structural models.[40,41] In two recent examples, the integral membrane protein MerF and the soluble protein MerP, the agreement between predicted and experimentally observed CS was shown to improve significantly by averaging the predicted CS over 10–20 structural models.[41] Since the models were “blind” predictions from Rosetta[42] (i.e., obtained by excluding known structures from the database search), this approach could be used to guide MAS solid-state NMR assignment during *de novo* structure determination and provide confidence in the selection of a starting structural model for structural refinement.

Orientation Restraints from Uniaxially Oriented Lipid Bilayers

Uniaxial alignment of planar lipid bilayers in the magnetic field yields spectra with orientation-dependent, single line resonances, and high-resolution separated local field (SLF) spectroscopy of membrane proteins enables multiple orientation restraints to be measured directly in a single spectrum from uniformly labeled protein.[8,43] For example, the one-and two-dimensional $^{1}H/^{15}N$ spectra of OmpX in magnetically aligned lipid bicelles show very high resolution (Figure 3). Each peak in the SLF spectrum provides a unique set of DC and CSA orientation restraints for protein structure determination.

The OS solid-state NMR spectra of uniaxially oriented samples exhibit distinctive wheel-like patterns of single line resonances, *PISA wheels*, that reflect protein structure and orientation.[44–46] Well-defined PISA wheels are observed in the spectra of many integral membrane protein domains, indicating that the structure-dependent features of the spectra are not overshadowed by any residue-dependent ^{15}N CS tensor variations. PISA wheels are useful for guiding resonance assignment performed with traditional spectroscopic approaches and for obtaining resonance assignments through methods that have been developed for simultaneous assignment and structure refinement (SASR). They also help reduce or eliminate the degeneracy of orientation solutions associated with DC and CSA measurements, allowing structures to be built by linking consecutive peptide planes or fragments through their common CA atom.[8]

The SASR approach is based on minimizing the difference between the experimentally observed spectral frequencies and the frequencies back-calculated from a structural model.[8,30] It relieves the burden of having to obtain near-complete resonance assignments prior to structure determination: assignments are obtained as a side product of fitting a structural model to the NMR data, but it is not a prerequisite for structure determination.

Several recently developed bioinformatics methods enable the generation of structural models of proteins, including membrane proteins, based on their amino acid sequence. Of these, Rosetta[42] has been used extensively in conjunction with NMR structure determination. Rosetta can provide very effective starting structural models for SASR and for the refinement of atomic resolution structures with high precision. Furthermore, even a few DC and CSA measurements can provide effective orientation restraints enabling structural information to be obtained prior to complete resonance assignment.

Typically, a SASR calculation cycle starts with an initial structural model (e.g., an ideal α-helix) and a set of unassigned DC and CSA frequencies for a particular residue type (e.g., from a selectively labeled sample). Each SASR cycle consists of generating optimal residue-specific assignments for the input data consistent with the structural model, and then using the assigned DC and CSA restraints to refine the model, which provides the input for the next SASR cycle where a new set of DC and CSA are assigned. The cycles are continued until all DC and CSA restraints are assigned and the resulting structure is consistent with the entire data set.

The program AssignFit,[47] facilitates SASR by computing and scoring for best fit all of the multiple residue-specific assignment possibilities for a given NMR data set and structural model. This type of analysis can quickly evolve into a complicated problem when the number of assignment permutations to be tested is very large, since for n number of peaks there are $n!$ possible assignment sets. The quality of the final result can be assessed in terms of RMSD between the experimental NMR data and the frequencies that are back-calculated from the refined structure (Figure 4).

Additional assignments of OS solid-state NMR spectra can be made through the implementation of multidimensional triple-resonance experiments.[23] Assignments can also be made through comparisons with isotropic NMR data[48] obtained by MAS for similar, albeit unoriented, samples, or even obtained by solution NMR for micelle samples, provided that the protein structure is maintained in detergent.

Orientation Restraints from Unoriented Proteoliposome Samples

Accurate orientation restraints can also be measured from the solid-state NMR powder patterns that are observed for unoriented samples in which uniaxial order is established by rapid rotational diffusion around a unique axis (Figure 5). This principle was first demonstrated with solid-state ^{31}P NMR spectroscopy for phospholipids assembled in liquid crystalline bilayer membranes: the lipids undergo rapid rotational diffusion around the axis perpendicular to the membrane plane (i.e., the bilayer normal) and, thus, yield narrowed, axially symmetric powder patterns whose frequency edges reflect the orientation of the phosphate group relative to the membrane.[4]

Membrane-integrated proteins also undergo uniaxial rotational diffusion around the bilayer normal and thus yield rotationally narrowed powder patterns that can be used to extract orientation restraints. Solid-state ^{13}C NMR spectroscopy provided the first demonstration of this effect in a protein, by showing that rotationally narrowed ^{13}CO powder patterns of ^{13}CO-Leu-labeled bacteriorhodopsin incorporated in membranes can provide CSA orientation restraints useful for determining protein secondary structure and overall orientation within the membrane.[50] Uniaxial protein rotation was further shown to yield collinear ^{15}N CS and ^{1}H–^{15}N DC tensors, resulting in two-dimensional SLF spectra with distinctive cross-like patterns whose end points provide precise DC and CSA values (Figure 6, black).[49] This property was used to measure a pair of ^{1}H–^{15}N DC and ^{15}N CSA orientation restraints for the single ^{15}N-Trp-labeled site membrane-integrated gramicidin, without the need for physically aligning the sample relative to the magnet.[49] More recently, useful solid-state NMR orientation restraints have been measured from unoriented samples of membrane peptides and proteins labeled with ^{13}C, ^{15}N, or ^{2}H at a single or a few specific sites by chemical synthesis.[19] In these cases, isotopic labeling at one or a few sites was necessary because the observation of powder patterns cannot provide single-site resolution.

To address this issue, a method has been developed for structure determination of uniformly ^{15}N/^{13}C-labeled membrane proteins reconstituted in proteoliposomes.[10,11,51] Multiple peaks are resolved and assigned by MAS through the use of triple-resonance ^{1}H/^{13}C/^{15}N experiments,[16,18–22,52,53] and MAS also provides isotropic CS frequencies that can be interpreted as dihedral angle restraints for structure determination. Rotationally averaged DC and CSA powder patterns, associated with individually resolved protein sites from uniformly labeled protein, are recoupled and measured under MAS; since the frequency measured from the edge of a rotationally averaged powder pattern is equivalent to that measured from OS NMR spectra (Figure 6, red), the same analytical methods developed for data analysis and structure determination are applicable. This approach has been used to determine the structures of two α-helical integral membrane proteins in DMPC liposomes: the bacterial mercury transporter MerF and the G-protein coupled receptor CXCR1.[10,11]

As for OmpX, Ail can also be incorporated in phospholipid bilayers for high-resolution solid-state NMR spectroscopy. As shown in Figure 6, the two-dimensional MAS ^{13}C–^{13}C correlation spectra of uniformly ^{15}N/^{13}C-labeled Ail incorporated in DMPC proteoliposomes show several resolved signals (Figure 6a). Peaks from alanine, isoleucine, serine, and threonine residues populate the regions expected for β-sheet conformation, as

described.[54] Four signals are observed with ^{13}C shifts in the expected region corresponding to the four threonine residues of Ail. Furthermore, seven signals are resolved in the region expected for isoleucine signals, including two signals with significantly greater intensity, as might be expected from residues in the extracellular loops of Ail (Figure 6b). Additional experiments demonstrating rotational diffusion of the β-barrel in the membrane will be needed before the implementation of MAS experiments that recouple DC and CSA similar to those described for helical proteins.

Embedding Proteins in Membranes

OS solid-state NMR provided an early view of the orientation of OmpX within the lipid bilayer membrane (Figure 3c).[30] The seven pairs of DC and CSA restraints in the $^{1}H/^{15}N$ SLF spectrum of ^{15}N-Phe-labeled OmpX were assigned by SASR and ^{2}H exchange experiments, performed by acquiring the data after equilibrating the sample in D_2O, provided information about the depth of membrane integration. Four of the seven phenylalanines (F24, F43, F115, F125) form strong backbone hydrogen bonds that resist water exchange after overnight equilibration in D_2O at pH 7. They are located within a relatively narrow, 16 Å, band of hydrophobic residues on the exterior of the β-barrel. The other three phenylalanines (F90, F107, F148) exchange readily. They are located just outside the 16 Å HD-exchange-resistant band and, therefore, help to define its boundaries, which include about half of the total 32 Å length of the OmpX β-barrel. This is significantly narrower than the approximately 27 Å thickness predicted to form the membrane-spanning region of the barrel solely on the basis of residue hydrophobicity.[26] It is also narrower than the 27 Å hydrophobic thickness estimated on the basis of NOEs observed between backbone amide or side chain methyl groups of OmpX and the hydrocarbon chain of DHPC (dihexanoyl-phosphatidylcholine), which provided the micelle environment for solution NMR studies.[55]

The preference of β-barrels for thinner membranes has been noted previously and has been proposed to provide a mechanism for targeting outer membrane proteins to the outer membranes of Gram-negative bacteria rather than to the inner membranes.[31] Indeed, most outer membrane proteins have relatively short hydrophobic, membrane-spanning regions (~20–24 Å), compared with the longer transmembrane regions of α-helical membrane proteins (~30–32 Å) that are found predominantly in inner membranes.

Conclusions

Structure determination in a membrane environment provides the most biologically relevant view of an integral membrane protein; it eliminates the potential for distorting protein structure, dynamics, and function due to crystal contacts or detergent molecules and enables ligand binding studies to be performed in a setting as close as possible to native. The solid-state NMR spectra of both α-helical and β-barrel membrane proteins have very high-resolution, enabling measurements of isotropic CS, anisotropic DC and CSA and strategic distances that provide restraints for structure determination. A number of solid-state NMR structures have already been determined for membrane proteins in phospholipid bilayers, and many more will be forthcoming with recent progress in sample preparation, instrumentation, NMR experiments, and computational methods.

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Biographies

Yi Ding obtained her B.Sc. degree in Chemistry from the University of Science and Technology of China where her research project focused on understanding the antitumor activity of trans-coordinated platinum compounds. She joined the graduate program at the Sanford-Burnham Medical Research Institute in 2011. Her thesis research focuses on the structure determination and functional characterization of the outer membrane protein Ail, a major virulence factor of *Yersinia pestis*.

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Francesca M. Marassi obtained her Ph.D. degree in Chemistry from the University of Toronto, where she studied NMR of lipids in membranes with Peter MacDonald. From 1993 to 1998, she was a postdoctoral fellow of the Natural Sciences and Engineering Research Council of Canada and of the Medical Research Council of Canada at the University of Pennsylvania. She was subsequently an Assistant Professor at the Wistar Institute in Philadelphia from 1998 to 2000 and is currently on the faculty of the Sanford-Burnham Medical Research Institute in La Jolla, CA. Her research interests are focused on developing methods for the structure determination of membrane proteins within their natural environment of the phospholipid bilayer membrane.

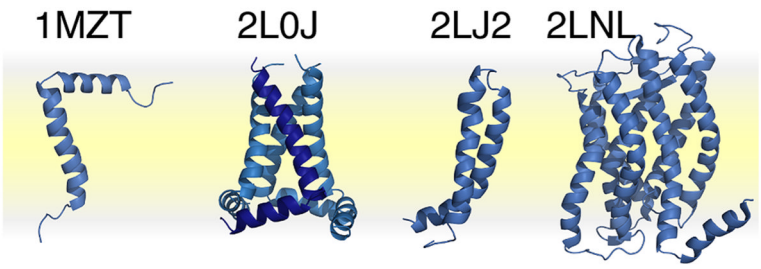

FIGURE 1.

Recent structures of membrane proteins determined in phospholipid bilayers by solid-state NMR. PDB codes: 1MZT, membrane-bound bacteriophage fd coat protein;[8] 2L0J, pore-forming domain of influenza M2;[9] 2LJ2, mercury transporter MerF;[10] 2LNL, human chemokine receptor CXCR1.[11] The phospholipid bilayer membrane is depicted in yellow.

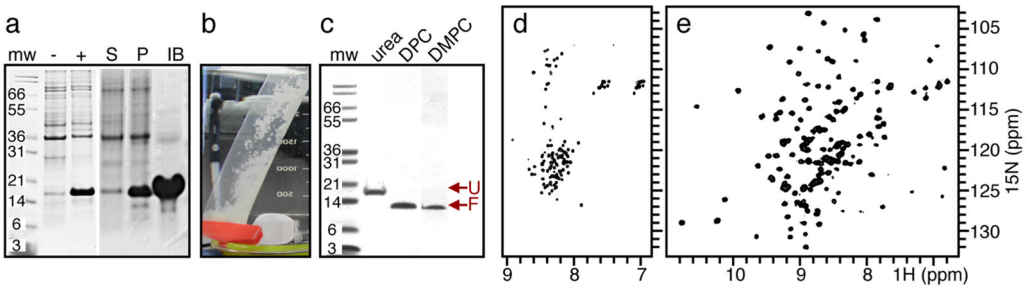

FIGURE 2.

Expression, purification, and refolding of *Y. pestis* Ail. (a) SDS PAGE analysis. Whole cells isolated before (−) or after (+) induction with IPTG show that induction of Ail yields a band near the predicted molecular weight (mw). Cell lysis and separation into supernatant (S), pellet (P), and inclusion bodies (IB) fractions show that inclusion bodies are enriched in Ail. (b) Isolated Ail inclusion bodies are white and fluffy. (c) SDS-PAGE analysis of Ail folded from urea into DPC micelles or DMPC bilayers. Unfolded Ail (U) migrates at an apparent molecular weight (mw) of 18 kDa; folded Ail (F) migrates near 12 kD. (d, e) Solution NMR $^{1}H/^{15}N$ HSQC spectra of Ail unfolded in urea (d) or folded in DPC (e). Tris-tricine gels were stained with Coomassie blue.

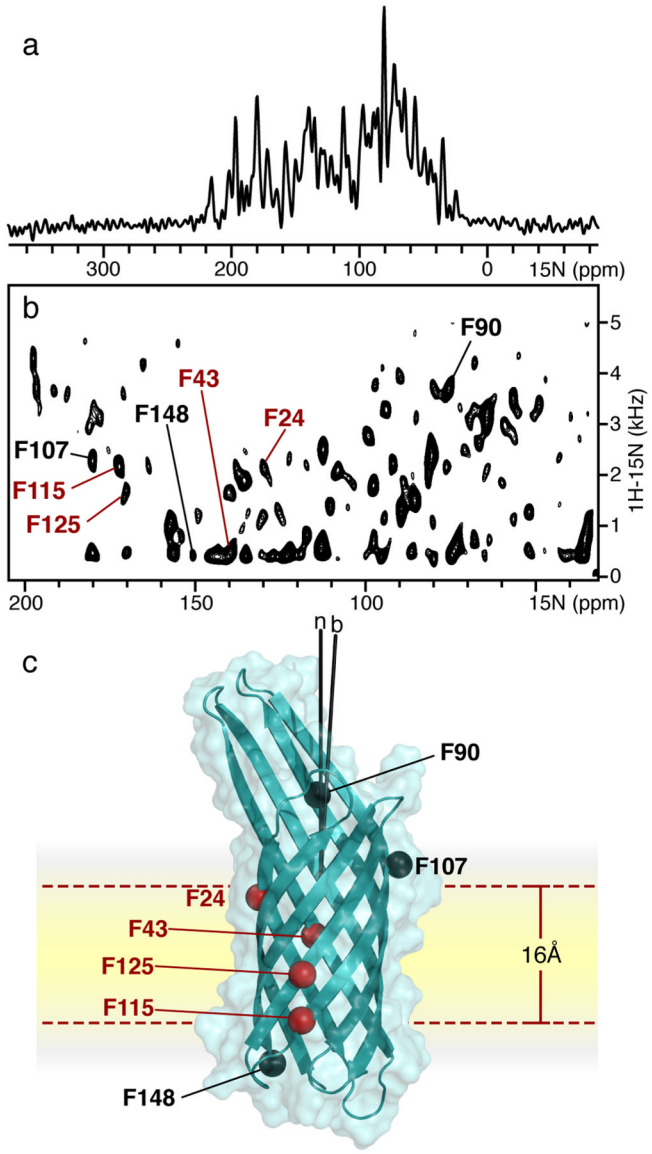

FIGURE 3.

OS solid-state NMR spectra of ^{15}N labeled OmpX in DMPC phospolipid bilayers. (a, b) One- and two-dimensional $^{1}H/^{15}N$ SLF spectra of OmpX in magnetically aligned DMPC/DHPC lipid bilayers with the bilayer normal parallel to the magnetic field. Peaks from phenylalanine sites were assigned as described.[30] Peaks labeled in red resist ^{2}H exchange. Spectra were obtained on a Bruker Avance 700 MHz spectrometer using a home-built radio frequency probe. (c) Crystal structure of OmpX[26] aligned in the membrane on the basis of orientation restraints derived from ^{15}N phenylalanine signals. Amide N atoms from phenylalanine are shown as spheres. Phenylalanine residues labeled in red define a 16 Å band of membrane-integrated, nonexchangeable H bonds.

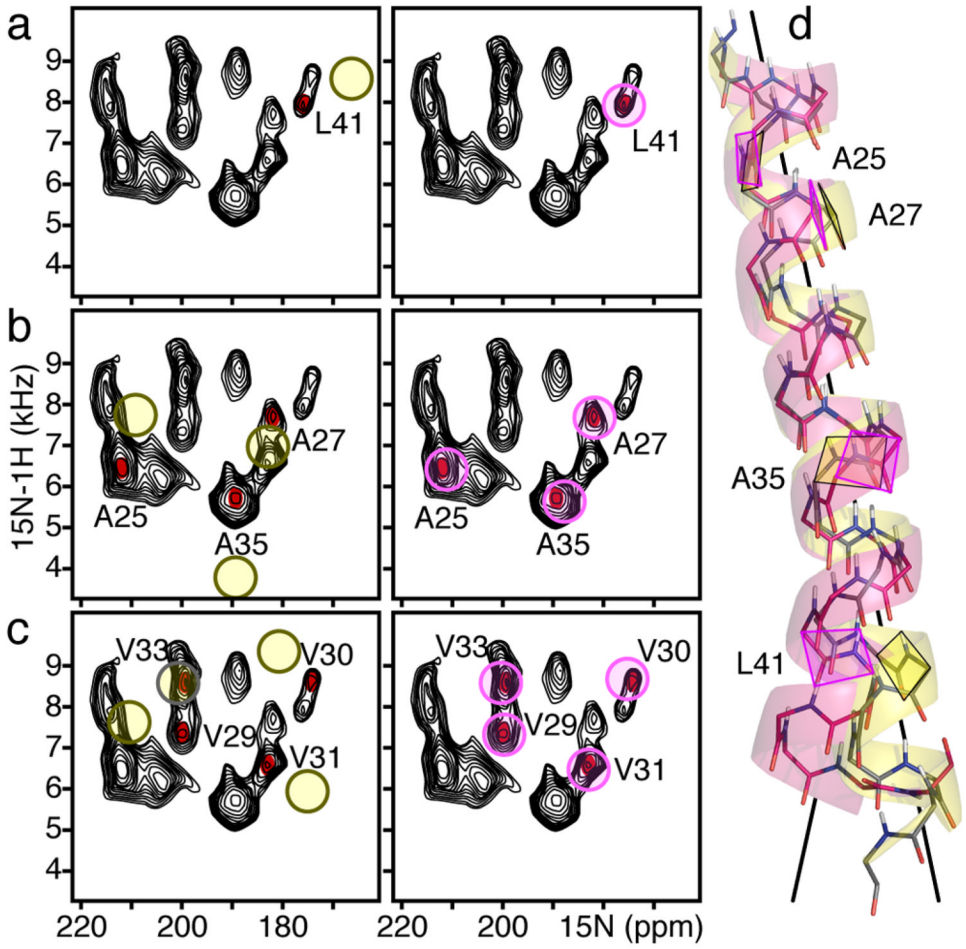

FIGURE 4.

SASR refinement of the transmembrane helix of fd coat protein in lipid bilayers aligned with the bilayer normal (n) parallel to the magnetic field.[8,47] (a) Peaks in the $^{1}H/^{15}N$ SLF spectrum are first assigned to residue types leucine, alanine, and valine (red dots) by comparison with spectra from selectively ^{15}N-Leu-, ^{15}N-Ala-, and ^{15}N-Val-labeled protein. Then AssignFit is used to specifically assign each peak to a residue number based on best fit of the observed signals (black) to the signals back-calculated from an ideal helix starting model (yellow circles). (b) Refinement of the ideal helix with the assigned DC and CSA restraints extracted from the experimental spectrum yields back-calculated signals (pink circles) that agree closely with the observed data (black). (c) The refined helix (pink) differs from the starting ideal model (yellow) and changes direction after Lys40.

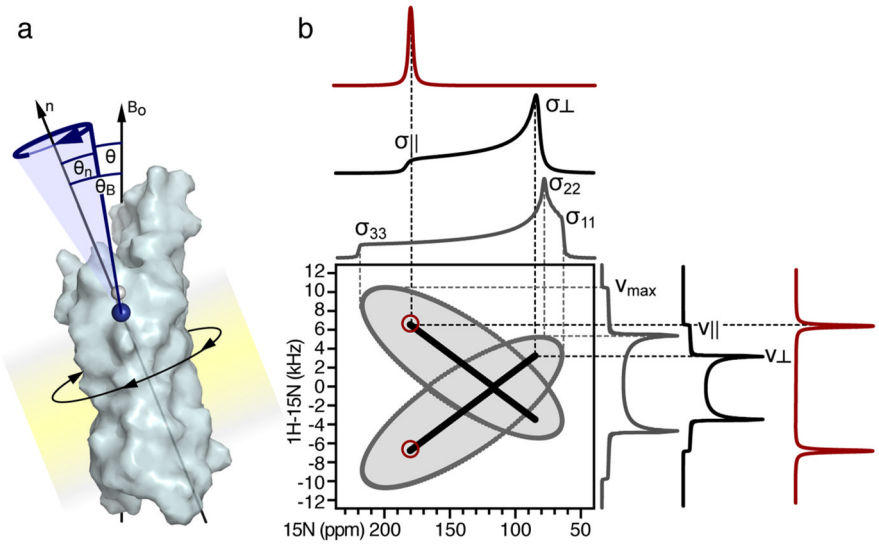

FIGURE 5.

Uniaxially ordered samples yield orientation-dependent solid-state NMR spectra. (a) A membrane protein undergoing rotational diffusion around an axis (n) normal to the membrane plane. The angle θ_B defines the membrane orientation relative to the magnetic field ($\mathbf{B}_o$); the angle θ_n defines the orientation of an amide NH bond (blue) relative to the axis n. Rotational diffusion of the protein around n averages the angle θ between the NH bond and $\mathbf{B}_o$. (b) Predicted solid-state NMR $^1H/^{15}N$ spectra. A static powder sample yields a butterfly-shaped two-dimensional SLF spectrum (gray) whose edges correspond to the maximum values of the ^{15}N CS tensor (σ_{11}, σ_{22}, σ_{33}) and $^1H–^{15}N$ DC (ν_{max}). Rotational diffusion around n produces motionally averaged, axially symmetric powder spectra (black), whose edges correspond to parallel ($\sigma_{||}$, $\nu_{||}$, $\theta_B = 0°$) and perpendicular ($\sigma_\perp$, $\nu_\perp$, $\theta_B = 90°$) orientations of n relative to $\mathbf{B}_o$. The two-dimensional SLF spectrum has a characteristic cross-like pattern, as observed for gramicidin.[49] Uniaxial alignment of the sample (e.g., with n parallel to $\mathbf{B}_o$) yields single line spectra (red) with ^{15}N CS and $^1H–^{15}N$ DC frequencies that correspond to the parallel edges of rotationally averaged powder patterns.

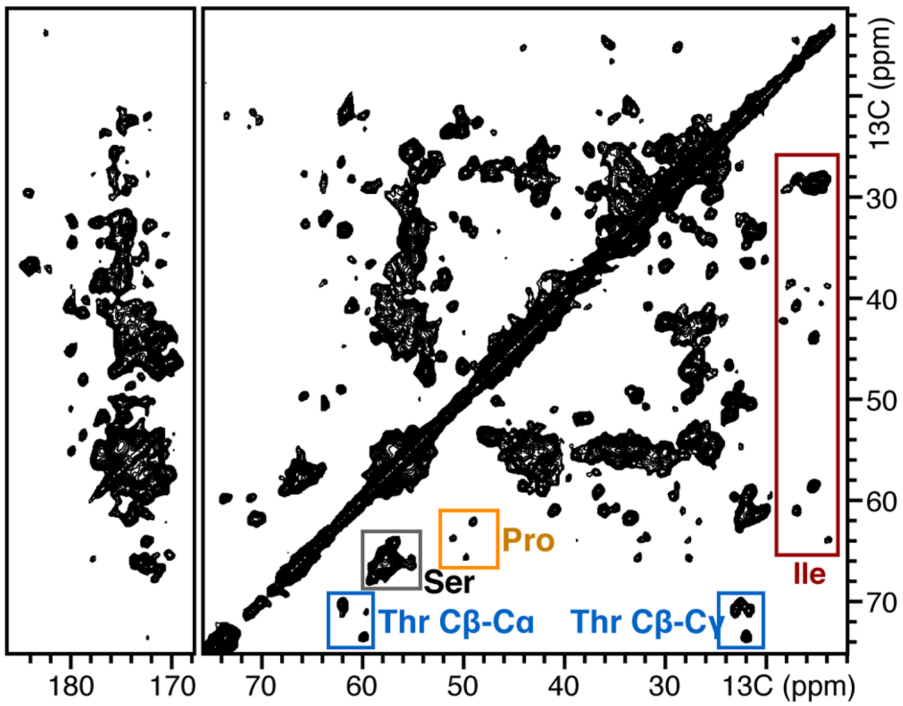

FIGURE 6.

Solid-state NMR spectroscopy of $^{15}N/^{13}C$-labeled Ail in DMPC proteoliposomes. Two-dimensional $^{13}C/^{13}C$ correlation MAS solid-state NMR spectrum of $^{15}N/^{13}C$-labeled Ail in DMPC liposomes. Peaks from 4 threonine (blue), 4 proline (gold), 16 serine (gray), and 7 isoleucine (red), are highlighted. Spectra were obtained on a Bruker Avance 700 MHz spectrometer using a Bruker MAS probe and controller.