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Applications of NMR to membrane proteins

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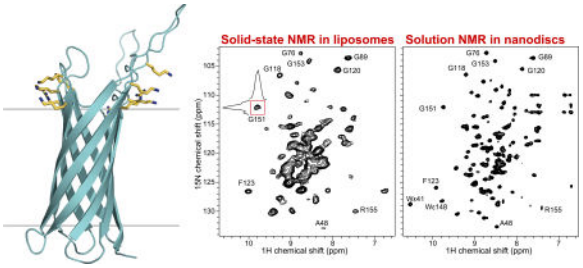
Abstract

Membrane proteins present a challenge for structural biology. In this article, we review some of the recent developments that advance the application of NMR to membrane proteins, with emphasis on structural studies in detergent-free, lipid bilayer samples that resemble the native environment. NMR spectroscopy is not only ideally suited for structure determination of membrane proteins in hydrated lipid bilayer membranes, but also highly complementary to the other principal techniques based on X-ray and electron diffraction. Recent advances in NMR instrumentation, spectroscopic methods, computational methods, and sample preparations are driving exciting new efforts in membrane protein structural biology.

Graphical Abstract



Keywords

NMR; membrane protein; lipid; bilayer; solid-state NMR

Introduction

Membrane proteins are encoded by 30–40% of all expressed genes, and are essential for both cellular life and human health. Due to their importance, membrane proteins are major

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targets of biomedical research and drug discovery efforts aimed at understanding their biological functions and harnessing their therapeutic potential. Molecular structure determination is essential for achieving both goals.

Structural biology of membrane proteins has advanced considerably in recent years. X-ray crystallography, electron microscopy (EM) and nuclear magnetic resonance (NMR) have all contributed important and complementary structural data [1–4], each with distinct advantages and unique challenges. X-ray crystallography has made important contributions to membrane protein structural biology, with notable recent successes in the area of G protein coupled receptors (GPCRs) [5–7]. EM has long been used to determine the structures of membrane proteins in proteolipid two-dimensional (2D) crystals [8], and the recent development of single-particle cryo-EM [9–11] is enabling higher resolution structures of membrane proteins to be obtained without the need to prepare large, well-ordered crystalline samples [2,12]. NMR has a long history as a key technology in advancing our understanding of the structural, chemical, and dynamic properties of lipid bilayer membranes.

Early NMR studies provided fundamental information about the structures and dynamics of phospholipid assemblies, and the effects of membrane proteins and various other membrane components on lipid bilayer membranes [13–16]. NMR, however, also plays a central role in membrane protein structural biology, providing information that is both unique and complementary to that derived from X-ray diffraction and EM. NMR methods are available for studying membrane proteins in a wide variety of samples, including soluble detergent micelles, detergent-free lipid bilayer membranes, and native cell envelope preparations [4,17–20]. The range of sample types reflects the versatility of NMR as a tool for characterizing the structures, dynamics, and functional interactions of biomolecules. NMR is also adept at characterizing intrinsically disordered regions of proteins [21]. Moreover, since NMR signals are highly sensitive to the local environment, they are extremely useful for characterizing even weak ligand binding through chemical shift changes, enabling structure activity correlations to be made for binding events or conformational changes [22].

The principal advantage of NMR as a technique for structural analysis is that is compatible with detergent-free membrane samples that are similar to the physiological protein environment [23]. This is in contrast to X-ray and single particle cryo-EM studies, which typically require non-lipid amphiphiles, such as detergents or amphiphilic polymers, for protein solubilization, and often involve extensive sample engineering, such as antibody stabilization and protein mutations, truncations, insertions and modifications [2,12,24–28]. These factors, combined with the cryogenic temperatures of the samples, all work to stabilize a single molecular conformation, thus dampening the structural plasticity that is often integral to biological function. Furthermore, in the case of EM, three-dimensional (3D) reconstruction remains challenging for proteins smaller than ~100 kDa, and while signals can be enhanced by binding antibody fragments [29], shape asymmetry and relatively small size [30] pose impediments for high-resolution structure characterization.

Recent advances in NMR structural studies of membrane proteins reflect exciting developments in the areas of recombinant protein expression, sample preparation, pulse

sequences for high-resolution spectroscopy, radio-frequency probes, high-field magnets and computational methods, which enable single atomic sites of membrane proteins to be probed with ever greater accuracy. Here we describe recent results with focus on studies in detergent-free lipid bilayers, where NMR is poised to make the most substantial contributions.

Solution NMR of membrane proteins in detergents

Solution NMR methods can be used for structure determination of membrane proteins in detergent micelles or detergent/lipid mixed micelles [17,18]. The protein data bank (PDB) structures of the outer mitochondrial voltage dependent anion channel VDAC-1 [31,32], the archaeal phototaxis receptor sensory rhodopsin II pSRII [33], and the bacterial inner membrane protein DsbB [34], illustrate the range of structural complexity that can be elucidated in atomic detail by solution NMR (Fig. 1). Human mitochondrial VDAC-1 forms a 19-stranded β-barrel held together by a network of hydrogen bonds (Fig. 1A). NMR chemical shift mapping experiments revealed several perturbations in the presence of protein and small molecule ligands, which were interpreted to reflect specific binding sites. Microbial pSRII, from *Natronomonas pharaoni*, forms a seven-transmembrane helix bundle, reminiscent of GPCRs (Fig. 1B). The structure, determined with excellent precision (0.48 Å backbone RMSD), represents the potential of solution NMR structure determination for other membrane proteins with this topology. Bacterial DsbB forms a four-helix bundle and a shorter fifth helix whose position suggests association with the membrane surface (Fig. 1C). The protein functions in disulfide bond formation and NMR analysis of its structure and dynamics helps explain how it mediates the flow of electrons together with its quinone cofactor. All three structures agree with their counterparts determined by x-ray crystallography, with no major conformational differences observed between crystalline and micelle samples.

Solution NMR has also made significant contributions to structure determination of membrane proteins with a single transmembrane helix [35–42]. These single-pass membrane proteins have important functions in biology. They often rely on intra-membrane, helix-helix interactions for establishing their functionalities, and are often too small to form the crystallographic contacts necessary for analysis by x-ray diffraction. NMR spectroscopy, however, is not limited by this constraint and can yield precise structural information, provided that sample conditions can be established to maintain structural integrity, as illustrated by several structures determined in micelles with solution NMR (Fig. 1D–G).

Solution NMR studies of GPCRs in detergents have also provided insights about receptor-ligand binding [43–53]. Detergents, however, can perturb or disrupt receptor-ligand interactions [54,55] and influence conformational exchange between GPCR functional states [47]. Similar effects are also seen for other membrane proteins, where detergents can cause structural distortions, loss of native functionality, or gain of non-native activity due to the unmasking of adventitious ligand binding sites [23].

Amphiphilic polymers (amphipols) have been proposed as a membrane mimetic alternative to detergents [56]. Amphipol-solubilized membrane proteins can yield solution NMR

spectra that appear suitable for structural studies [57]. In one notable case, amphipols have been shown to stabilize the trimeric, Chlamydia native major outer membrane protein, nMOMP, and enhance its protective ability as a vaccine [58].

Solution NMR of membrane proteins in lipid bilayer nanodiscs

The long correlation times of large protein-lipid assemblies pose a limitation on the types of membrane proteins that can be studied by solution NMR. Nevertheless, advances are being made in the development of experimental methods [59] and the preparation of specialized detergent-free lipid bilayer nanodiscs [60–62] suitable for solution NMR studies. Nanodisc samples have been used very effectively for structure/activity correlation studies of a variety of membrane proteins [60–66].

The combined use of small nanodiscs, extensive ^{2}H-labeling of both protein and lipids, TROSY (transverse relaxation-optimized spectroscopy) sequences [67] at high magnetic fields, and non-uniform sampling schemes for signal acquisition [68–71], has enabled solution NMR structure determination of the bacterial outer membrane proteins OmpX, OmpA, and Ail [60,63,72]. These eight-stranded β-barrel proteins fall in a class that is highly amenable to NMR studies since their 3D fold can be established by measuring distance restraints across the extensive, inter-strand network of amide backbone hydrogen bonds. By contrast, the hydrogen bonds of helical proteins are intra-helical, and, while valuable, do not provide information about 3D fold. Thus, structure determination of helical proteins relies much more significantly on distance restraints measured between side chain atoms, a task that requires side chain resonance assignments.

In nanodiscs, the absence of detergent ensures that structural studies are conducted for functional protein states. Interestingly, even though a number of membrane proteins have been shown to adopt the same overall structure in lipid micelles, detergent-free nanodiscs, and 3D crystalline samples [42,60,62–64,73], the presence of detergent can have a dramatic effect on activity. For example, the solution NMR structure of the *Yersinia pestis* outer membrane protein Ail, has been determined in decyl-phosphocholine (DePC) detergent micelles [74] and agrees very well with its crystal structure [75]. Ail forms an eight-stranded β-barrel with four extracellular loops and three intracellular turns (Fig. 2A). The same structure is also observed in nanodiscs [72], and the NMR spectra of the protein in micelles and nanodiscs are very similar (Fig. 2B). Nevertheless, the ligand binding activity of the protein is abolished in detergent (Fig. 2C) [73], precluding structure-activity studies. Interestingly, NMR analysis indicates that the addition of DePC to Ail nanodiscs perturbs signals from the extracellular loops, suggesting that detergent molecules associating with these key sites affect activity.

Notably, while the solution NMR spectra of native GPCRs in detergents are typically dominated by broad, overlapped resonances, except for a few narrow signals from the disordered termini [76,77], selective truncations and mutagenesis based on directed evolution techniques [78,79] have been shown to generate GPCR sequences with conformational and dynamic properties amenable to solution NMR structural studies. For example, high resolution ^{1}H/^{15}N spectra were reported, recently [62], for a direct-evolved

and truncated sequence of the neurotensin receptor NTR1, reconstituted in lipid nanodiscs prepared with a short and covalently circularized version of the membrane scaffold protein. These circularized nanodiscs have greater stability and size homogeneity than the non-circularized versions, and thus help enhance NMR spectral resolution.

Solid-state NMR of membrane proteins in lipids

Solid-state NMR methods are compatible with detergent-free lipid samples and are not limited by molecular size. In recent years, the structures of a number of membrane proteins have been determined in phospholipids using oriented sample (OS) and magic angle spinning (MAS) solid-state NMR approaches. Structural studies have relied primarily on experiments based on detection of dilute nuclei (^{15}N, ^{13}C), which can be readily introduced in a protein's amino acid sequence by growing the expression vector in isotopically defined minimal media. A wide range of experiments [4,80–83] can be performed to obtain site-specific resonance assignments and to measure restraints for structure determination. These are facilitated by the use of high magnetic fields and efficient radiofrequency probes that do not dissipate heat energy [84–86]. Isotropic chemical shifts and spin exchange signals, measured in MAS experiments, can be converted to torsion angles and inter-atomic distances, while dipolar couplings and anisotropic chemical shifts, measured using either MAS or static probes with uniaxially ordered samples, can be converted to bond orientation restraints or used to obtain information about protein dynamics [87,88].

In addition to structures, solid-state NMR studies are providing fundamental insights about the molecular mechanisms underpinning membrane protein functionalities, including: ion channel conduction [89–92], multidrug resistance [93]; antimicrobial peptide activity at membranes [94–96]; bioenergetics [97]; as well as conformation, dynamics and ligand binding of intact GPCRs [76,98–105]. The ability to examine structure, dynamics, and ligand binding in native-like samples similar to those used to assay biological functions is an important advantage of NMR spectroscopy. Solid-state NMR is making particularly exciting advances as a tool for examining the structural and dynamic properties of membrane proteins, peptides and cytoskeletal envelope components in native cell preparations [20,106–110].

Structures of membrane proteins determined by solid-state NMR (Fig. 3) include: sensory rhodopsin from *Anabaena* [111]; the human chemokine receptor, CXCR1 [112]; the M2 ^{1}H channel from influenza virus [113,114]; the bacterial inner membrane protein DsbB [115,116]; the *Mycobacterium tuberculosis* cell division protein, CrgA [117]; the membrane-inserted form of the fd bacteriophage major coat protein [118]; the bacterial mercury transporter, MerF [119]; the bacterial trimeric autotransporter YadA [120]; and the human PLB pentamer [121]. The structures of DsbB and PLB were determined with hybrid refinement approaches based on restraints from solid-state NMR and crystallography (DsbB), or on solid-state NMR and solution NMR (PLB), to enhance structural precision. Such hybrid approaches can be very valuable but should be used with caution as they may be complicated by sample-specific structural differences. Notably, the structures of influenza M2 and PLB illustrate the importance of the membrane environment. The solid-state NMR structures determined in detergent-free lipid samples are distinctly different from those

determined using solution NMR or crystallography, where detergents caused distortions incompatible with membrane insertion and protein functionality [23].

OS Solid-state NMR

At present, the majority of solid-state NMR structures of membrane proteins available in the PDB are based on bond orientation restraints, measured in the NMR spectra of proteins in uniaxially aligned lipid bilayers. Orientation restraints are very valuable because they contain information about protein 3D structure, protein orientation in the membrane, and protein dynamics [122]. Moreover, they provide a powerful, independent method for validating structural accuracy [123]. Experimental approaches are available for obtaining resonance assignments in the NMR spectra of oriented samples, including, methods based on the direct relationship between the global membrane-integrated structure and the frequencies of the NMR signals [118,124–128], and spectroscopic methods based on spin diffusion between atomic sites [83,129–131].

Uniaxially aligned samples of membrane proteins in bilayers can yield high resolution NMR spectra that directly reflect the protein's conformational properties. For example, the $^{1}H/^{15}N$ PISEMA (polarization inversion with spin exchange and the magic angle) spectra of the human Na,K-ATPase regulatory protein FXYD2 in oriented bilayers [132] show signals from amide sites in a wheel-like pattern that reflects the ~20° orientation of the transmembrane helix axis relative to the membrane normal (Fig. 4A, C). Spectra from bilayers aligned perpendicular to magnetic field have ^{15}N frequencies in the range of 150–200 ppm (Fig. 4A, B), while the spectra from parallel bilayers have ^{15}N frequencies in the range of 85–100 ppm (Fig. 4C, D). For both bilayer orientations, ^{15}N signals at 100–130 ppm are from sites with sufficient mobility to cause isotropic averaging of the ^{15}N chemical shift and ^{1}H-^{15}N dipolar coupling, as might be expected for the non-helical regions of the protein. Measureable dipolar couplings from the Arg side chains are also observed, indicating that these sites, positioned at the lipid-water interface, do not undergo rapid isotropic reorientation. Notably, the ^{1}H-^{15}N dipolar couplings and the ^{15}N chemical shift frequencies of the Arg peaks also depend on the alignment of the lipid bilayer membrane in the magnetic field, indicating that these side chains have specific orientations in the context of the lipid bilayer.

Accurate orientation restraints can also be extracted from the powder patterns measured for, unoriented liposome samples [87,112,119]; in this case, rapid rotational diffusion of the protein around the axis perpendicular to the lipid bilayer plane is exploited to generate uniaxial order. Furthermore, chemical shift tensors themselves can provide effective restraints for structure determination and refinement [133].

MAS Solid-state NMR

High resolution MAS solid-state NMR spectra have been reported for membrane proteins in proteolipid 2D crystals, precipitates, and microcrystals [114,134–139]. These samples have the advantage of maximizing protein concentration in the NMR spectrometer, but they also have lipid to protein ratios that are lower than most biological membranes [140,141]. Low

lipid content can compromise protein stability and induce the formation of non-native contacts between protein molecules with opposite transmembrane orientations [114,142,143]. Moreover, 2D crystals can be structurally heterogeneous [143], a factor that poses a unique challenge for NMR, where signals reflect ensemble properties of the bulk sample, and heterogeneity manifests as broader line widths and diminished spectral resolution. Fortunately, the only requirement for reducing inhomogeneous line broadening and obtaining single, narrow NMR lines, is that the protein adopts a homogeneous conformational ensemble state on the timescale determined by the frequency span of spin interactions. For solid-state NMR studies of membrane proteins, this can often be achieved with proteoliposomes prepared with sufficient lipid to prevent the formation of non-native conformations.

The 2D $^{13}C/^{13}C$ correlation spectrum of Ail in liposomes (Fig. 5) illustrates the spectral resolution attainable for homogeneous liposome preparations [144]. In this sample, the protein was uniformly labeled with ^{15}N and ^{13}C, and fractionally (~70%) ^{2}H labeled, with amides back exchanged to ^{1}H during protein purification, to enable NMR detection. Deuteration dilutes the ^{1}H concentration and suppresses the strong homonuclear ^{1}H-^{1}H dipolar couplings, resulting in longer lifetimes of the ^{13}C and ^{15}N coherences, and leading to enhanced sensitivity and resolution.

The $^{13}C/^{13}C$ correlation spectrum together with additional 2D and 3D spectra that correlate N, CA, CO and side chain atomic sites [145–148], enable resonance assignments and the measurement of distance restraints for structure determination. Signals from Thr, Ser, Ile and Ala sites can be readily identified based on their characteristic chemical shifts, which reflect β-strand conformation. Moreover, several cross peaks observed in the ^{13}C-^{13}C spectrum (Fig. 5B) reflect intra-residue (Fig. 5B, blue) or inter-residue (Fig. 5B, green, red) connections, including long-range (Fig. 5B, red) connections between neighboring β-strands (Fig. 5C) that are essential for structure determination.

Recently, NMR experiments based on ^{1}H-detection have become an important technique for enhancing sensitivity and resolution in solid-state NMR spectroscopy. These experiments are facilitated by very fast spinning rates (>40 kHz), high static magnetic fields, and ^{2}H-labeling of the protein, to reduce homogeneous line broadening by suppressing and diluting the large network of strong homonuclear ^{1}H-^{1}H dipolar couplings [114,135,138,149–156]. NMR experiments of ^{2}H-labeled proteins, based on ^{1}H detection, require back exchange of backbone amides from ^{2}H to ^{1}H. Back exchange is very readily obtained for proteins that are refolded from inclusion bodies, like many b-barrels, but can be less efficient for proteins that are purified as folded entities, where strong hydrogen bond networks are established before purification. Thus, extensive deuteration may not always be a feasible approach. Nevertheless, the availability of radiofrequency probes with spinning rates greater than 100 kHz [157] is removing this obstacle by enabling NMR studies of fully protonated samples, including membrane proteins.

Solid-state NMR experiments based on ^{1}H detection with fast spinning rates are enabling high resolution NMR spectroscopy of membrane proteins in lipids. A number of structural studies have been reported for both β-barrel and α-helical membrane proteins in liposomes

and 2D crystal preparations [138,139,144,155,158,159]. For example, the ^{1}H detected $^{1}H/^{15}N$ CP-HSQC (Cross Polarization - Heteronuclear Single Quantum Correlation) spectrum (Fig. 6A) of the *Y. pestis* outer membrane protein Ail, in liposomes, displays high resolution [144]. The spectrum was obtained at a magnetic field of 900 MHz, and a temperature, 30°C, where the lipid bilayer is fully liquid crystalline. Resonance line widths are in the range of 0.11–0.15 ppm (107–138 Hz) for ^{1}H, and 0.46–0.64 ppm (42–58 Hz) for ^{15}N, consistent with a high level of sample homogeneity, and comparable with those observed in the ^{1}H-detected spectra of membrane proteins in 2D crystals [138,155]. Notably, the solid-state NMR spectrum overlaps significantly with the solution NMR $^{1}H/^{15}N$ TROSY spectrum (Fig. 6B) measured for Ail in nanodiscs with the same lipid composition [73], illustrating the potential of performing structural studies on essentially the same, detergent-free sample, over the wider, combined experimental time scale of solution and solid-state NMR spectroscopy.

NMR structure calculations of membrane proteins

The quality of NMR structures depends on both the accuracy and quantity of the experimental data as well as on the computational methods used in the structure calculations. Methods that facilitate NMR-restrained protein structure calculations in a physically realistic energy landscape, significantly improve structure precision, accuracy and quality.

All atom force fields, with complete chemical energy functions and explicit atomic representation of the protein, water, and lipid molecules, can be used in NMR-restrained molecular dynamics (MD) simulations of membrane proteins [113,160–162]. Recent advances are providing optimized force fields [163,164] and are enabling long MD simulations. Nevertheless, because these simulations rely on a reasonably accurate input structure, they are best suited for the final stages of refinement and remain impractical for routine de novo calculations that start from fully extended polypeptides.

Geometric restraining terms [38,165] or empirical models of membrane insertion depth [166] can be used to impose virtual water-membrane boundaries during NMR-restrained simulated annealing calculations of membrane proteins, but do not provide realistic atomic-level representations of the protein in its environment. In addition, implicit solvation models have been developed specifically for NMR-restrained MD [167–170] or simulated annealing [171–173] protocols. Among these, the implicit solvation potential EEFx (effective energy function for Xplor-NIH) is highly effective for NMR-restrained, simulated annealing calculations of both soluble and membrane proteins from unfolded templates [74,171–176]. EEFx is based on the implicit solvation energy functions developed for CHARMM [177,178] and is available with the Xplor-NIH package [179,180]. It includes an implicit membrane model that provides a physically realistic, anisotropic water-lipid environment and supports the native structures of membrane proteins.

EEFx is designed to work with NMR restraints measured in detergent-free lipid bilayer samples, but is also compatible with restraints measured in micelles [74], and with a wide range of experimental restraints measured by techniques other than NMR, to impose

boundaries between hydrophobic and polar environments, establish proper protein topology and prevent water-exposed loops of side chains from folding back against protein regions that are membrane-embedded. EEFx is very effective at guiding structure calculations towards the native state, even in the absence of large numbers of experimental measurements, and yields significant improvements in structural quality, accuracy and precision, as illustrated for the structure of Anabaena sensory rhodopsin (Fig. 7).

Conclusions

Recent technological advances in NMR spectroscopy are opening a new chapter in membrane protein structural biology. The studies reviewed in this article, and the many other exciting efforts in laboratories around the world, reflect the breadth of capabilities of modern NMR. The ability to probe membrane proteins in detergent-free membranes, ever closer in composition to the native environment, is particularly exciting. The high resolution and high sensitivity seen in the NMR spectra reported in recent studies will significantly benefit from the additional enhancements made possible through the use of high magnetic fields, non-uniform sampling schemes that reduce experimental times or increase signal, efficient radiofrequency probes that do not dissipate heat energy, and fast MAS probes that enable ^{1}H detection experiments.

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HIGHLIGHTS

- Membrane proteins are major targets of biomedical research and drug discovery.
- Molecular structure determination is essential for achieving both of these goals.
- NMR is ideally suited for structural studies of membrane proteins in hydrated lipid bilayers.
- Recent advances in NMR are opening a new chapter in membrane protein structural biology.
- Exciting new efforts in this area reflect the breadth of capabilities of modern NMR.

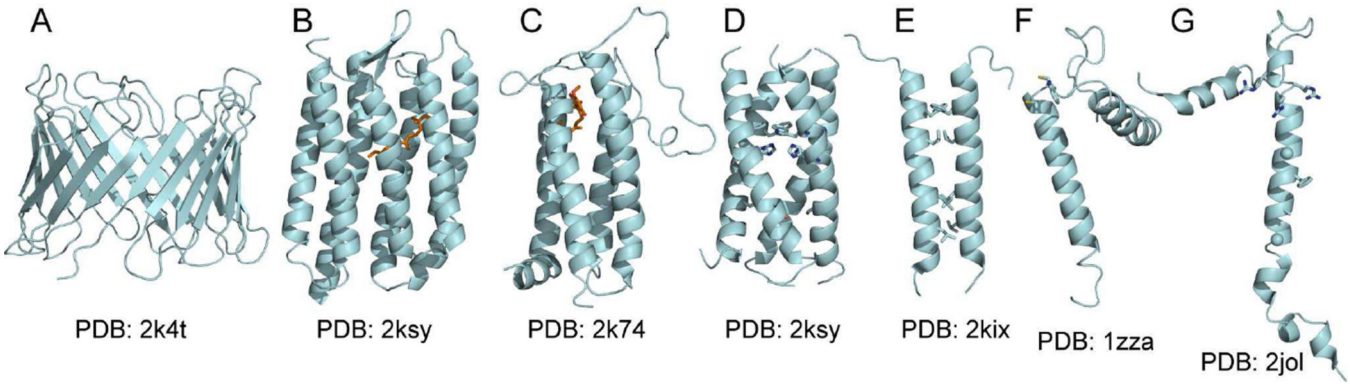

Figure 1. Examples of membrane protein structures determined in detergent micelles by solution NMR

The PDB file numbers are listed below each structure. (A) Mitochondrial voltage dependent anion channel VDAC [31]. (B) Archaeal photo sensory rhodopsin pSRII with bound retinal (orange) [33]. (C) Bacterial inner membrane protein DsbB with bound quinone (orange) [34]. (D) Channel domain of influenza BM2, showing His, Trp and Ser side chains (sticks) lining the pore [40]. (E) Transmembrane domain ζ-ζ dimer of the T cell receptor CD3, showing side chains (sticks) that mediate dimerization [37]. (F) Mitochondrial stannin showing the metal-binding Cys-Trp-Cys motif (sticks) at the membrane surface [36] (G) Human Na,K-ATPase regulator FXYD1 showing side chains (sticks) and Gly CA atoms (spheres) that mediate intra-membrane association with the Na,K-ATPase α subunit [38].

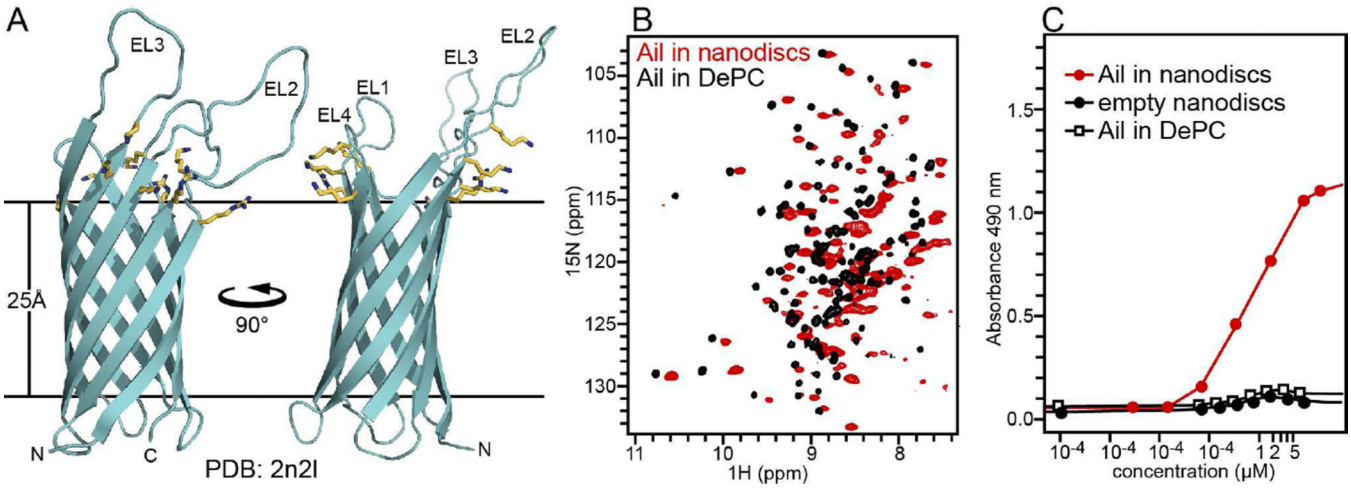

Figure 2. Structure of *Y. pestis* outer membrane protein Ail and effect of detergent on its ligand binding activity

(A) Solution NMR structure of Ail, determined in DePC micelles, showing Arg and Lys side chains (yellow) at the membrane surface [74]. (B) Solution NMR $^{1}H/^{15}N$ TROSY spectra of Ail in 170 mM DePC (black) or nanodiscs (red). (C) Fibronectin binding activity of Ail analyzed by enzyme linked immunosorbent assay, where increasing concentrations of Ail are added to fibronectin-coated plates. Adapted from [73].

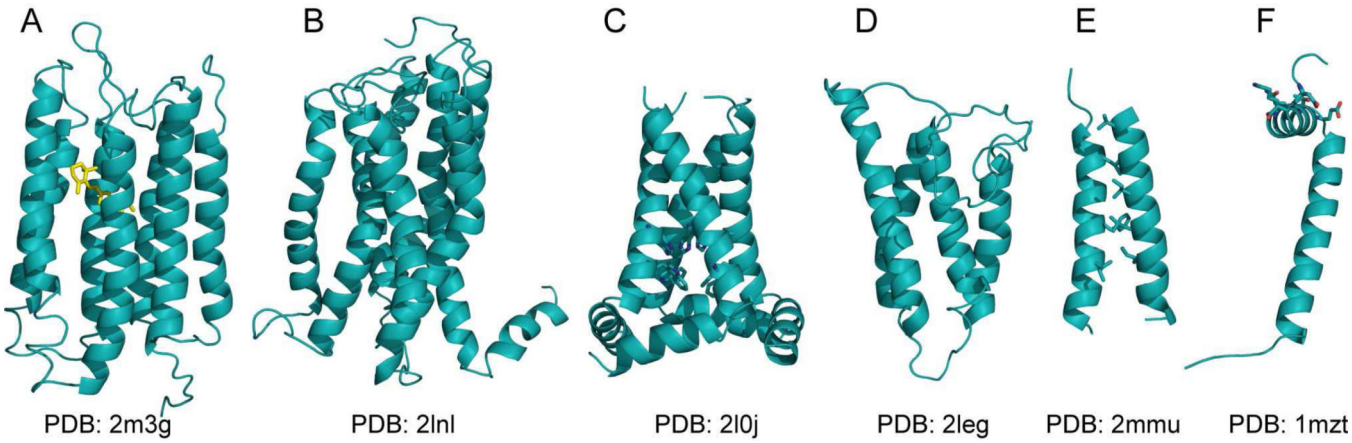

Figure 3. Examples of membrane protein structures determined in detergent-free phospholipids by solid-state NMR

The PDB file numbers are listed below each structure. (A) Anabaena sensory rhodopsin with bound retinal (yellow) [111]. (B) human chemokine receptor, CXCR1 [112]. (C) M2 ^{1}H channel from influenza virus showing His and Trp side chains (sticks) lining the channel pore [113]. (D) Bacterial inner membrane protein DsbB [115,116]. (E) *Mycobacterium* cell division protein, CrgA showing side chains (sticks) that mediate intra-membrane helix-helix association [117]. (F) Membrane-inserted form of the fd bacteriophage coat protein showing polar side chains (sticks) in the N-terminal helix exposed to bulk water [118].

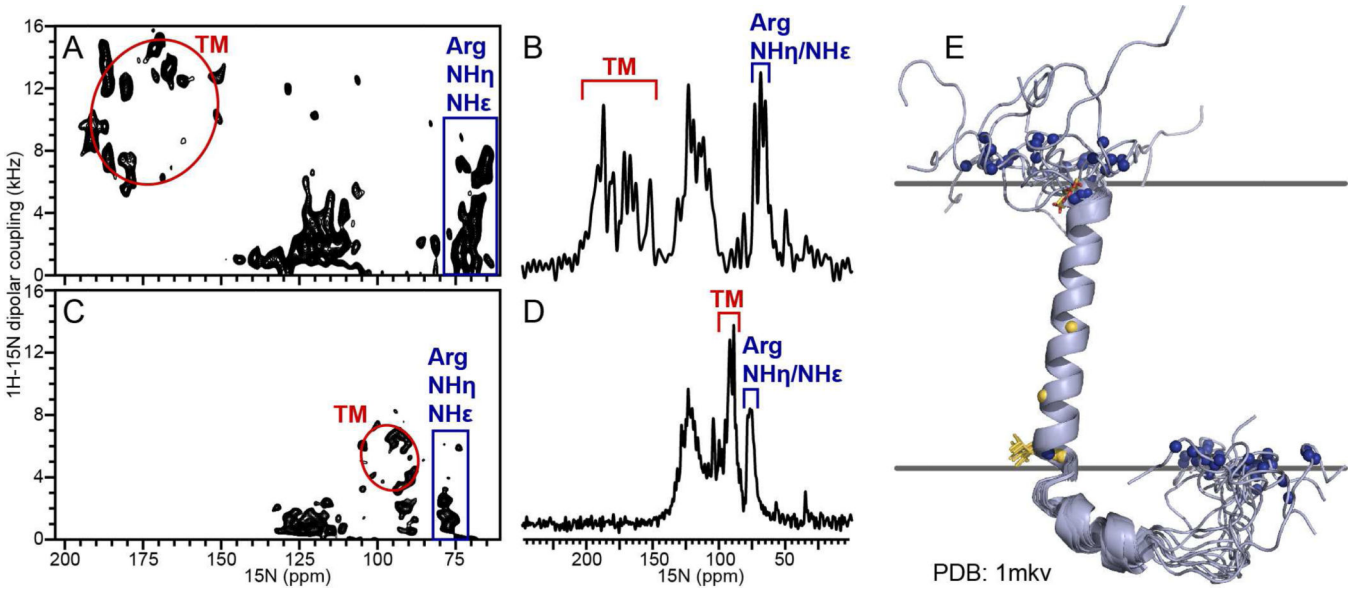

Figure 4. Solid-state NMR spectra of uniformly ^{15}N labeled FXYD2 in magnetically oriented phospholipid bilayers

(A–D) 2D ^{1}H/^{15}N PISEMA) and 1D ^{15}N OS solid-state NMR spectra of FXYD2 in lipid bilayers aligned with the membrane perpendicular (A, B) or parallel (C, D) to the magnetic field. Peaks from the transmembrane helix (TM) trace wheel-like patterns (red circles). Peaks assigned to Arg guanidinium NH groups are prominent (blue boxes). **(E)** Solution NMR structure of FXYD2 determined in micelles, showing the bundle of ten lowest energy structures. The positions of amide N atoms (blue spheres) were restricted by experimental plane distance restraints as described [132]. Adapted from [132].

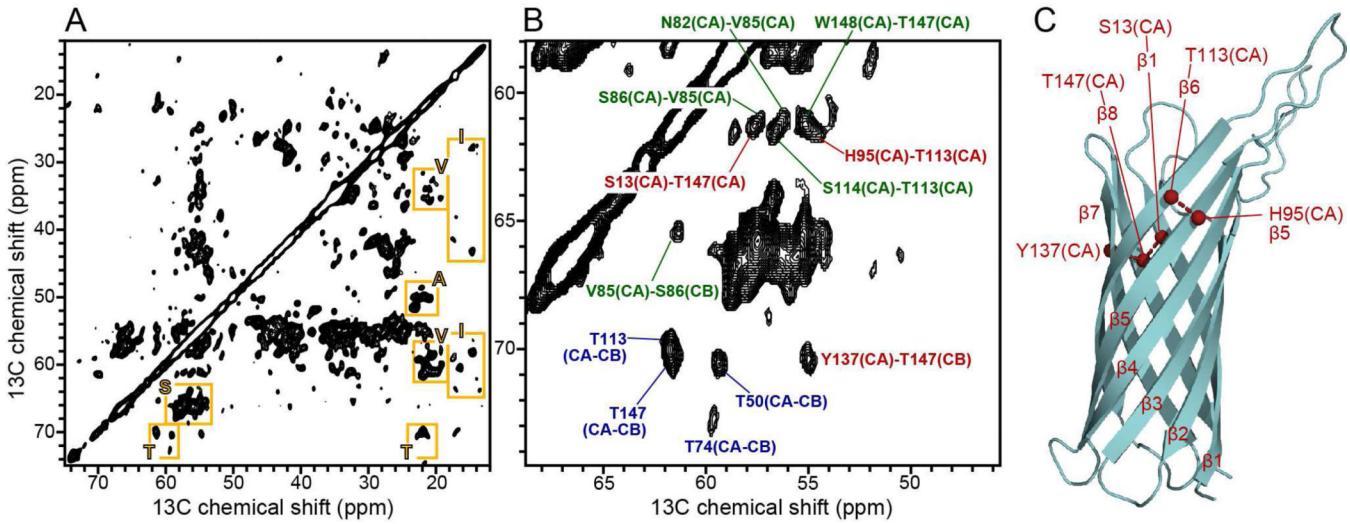

Figure 5. 2D ^{13}C-^{13}C spectrum of (^{15}N, ^{13}C, ^{2}H) Ail in liposomes

(A) Fingerprint region of the DARR (Dipolar Assisted Rotational Resonance) spectrum showing examples of resolved signals from Ala, Ile, Ser, Thr, Val residues (yellow boxes). (B) Expanded spectral region showing examples of short-range intra-residue correlations (blue), short-range inter-residue correlations (green), and long-range inter-residue correlations (red). (C) Structure of Ail in micelles (PDB: 2N2L) showing inter-strand connections (red) assigned in the DARR spectrum. The spectrum was obtained at 900 MHz, at 10°C, with 200 ms DARR mixing time, and 144 scans per increment. Adapted from [144].

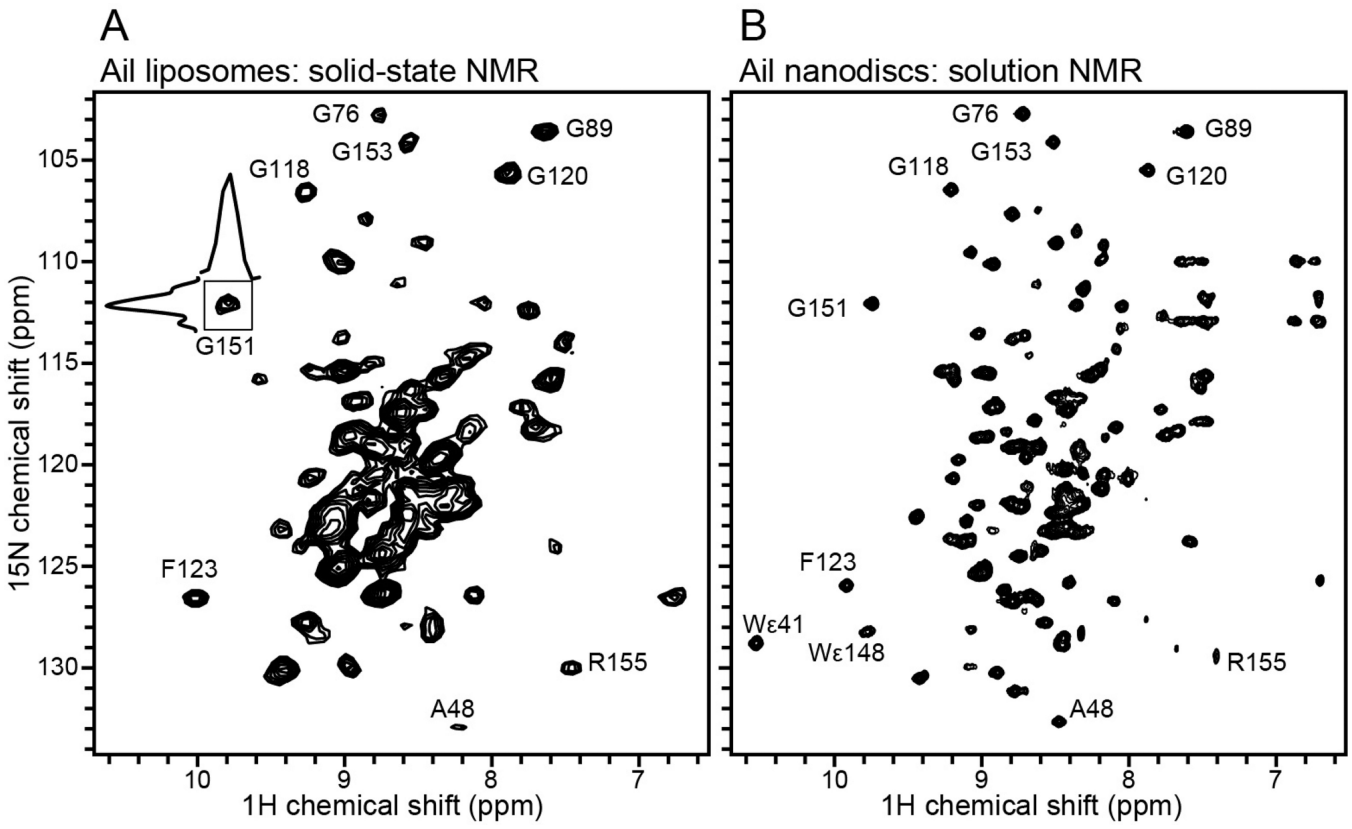

Figure 6. 2D ^{1}H-^{15}N correlation spectra of Ail in phospholipid bilayers

(A) Solid-state NMR ^{1}H-detected CP-HSQC spectrum of (^{15}N, ^{13}C, ^{2}H) labeled Ail in liposomes, recorded at 900 MHz, 30°C, with 160 scans and a MAS rate of 60 kHz. (B) Solution NMR ^{1}H-detected TROSY spectrum of (^{15}N,^{13}C,^{2}H) labeled Ail in nanodiscs prepared with ^{2}H labeled lipids, recorded at 800 MHz, 45°C, with 128 scans. Adapted from [144].

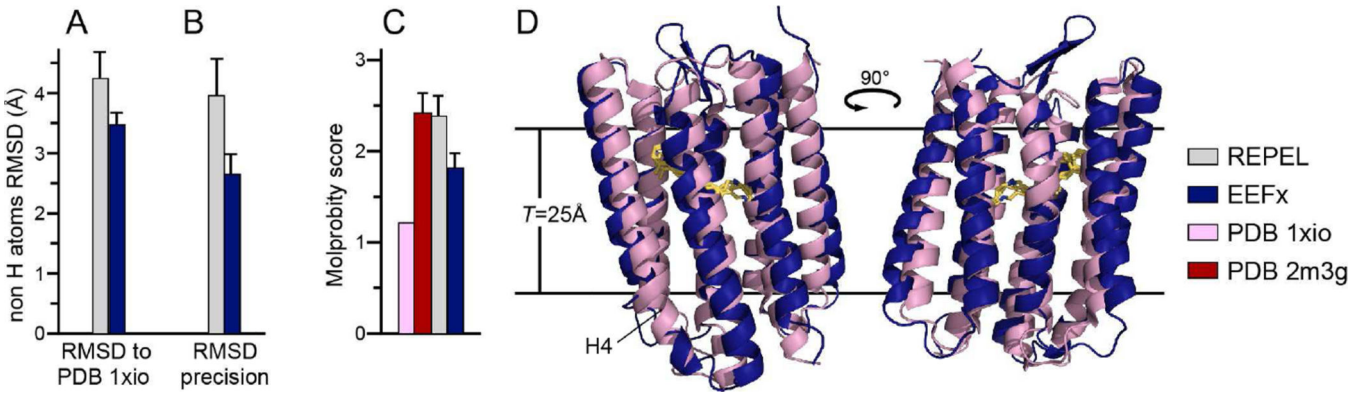

Figure 7. The EEFx potential improves structure calculations of Anabaena sensory rhodopsin in lipid bilayers

Structures of monomeric ASR were calculated using solid-state NMR distance and dihedral angle restraints. Bar plots represent results for: the crystal structure (PDB 1xio; pink) [181]; the average for ten models in the ensembles of the deposited solid-state NMR structure (PDB 2m3g; red) [111]; the ensemble calculated with the standard simple repulsive potential of Xplor-NIH (gray); or the ensemble calculated with the EEFx potential of Xplor-NIH (blue) [173]. (A) Agreement with the PDB crystal structure (PDB 1xio) evaluated as average pairwise RMSD of atomic coordinates. (B) Precision evaluated as average pairwise RMSD of atomic coordinates in each ensemble. (C) MolProbity score evaluation of the four structures; note this is a cost: the lower the better. (D) Comparisons of the crystal structure (PDB 1zxio, pink) with the lowest energy structure generated with EEFx (blue). The horizontal lines depict the boundaries of the 25 Å thick EEFx membrane. Adapted from [173].