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Membrane Protein Structure Determination: Back to the Membrane

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Abstract

NMR spectroscopy enables the structures of membrane proteins to be determined in the native-like environment of the phospholipid bilayer membrane. This chapter outlines the methods for membrane protein structural studies using solid-state NMR spectroscopy with samples of membrane proteins incorporated in proteoliposomes or planar lipid bilayers. The methods for protein expression and purification, sample preparation, and NMR experiments are described and illustrated with examples from OmpX and Ail, two bacterial outer membrane proteins that function in bacterial virulence.

Keywords

Membrane protein; NMR; Lipid; Bilayer; Membrane; Protein; Expression; Structure; Barrel

1 Introduction

Membrane proteins mediate all interactions of a cell or organism with the outside world and, as such, are responsible for the basic human experiences (taste, smell, touch, sight, thought, etc.) that constitute life. They are encoded by ~30 % of all known pro- or eukaryotic genes and perform essential biological functions that include cellular transport, signaling, and programmed cell death. Dysfunctions of human membrane proteins are linked with devastating diseases, and the membrane proteins encoded by viruses and bacteria are major players in infection, virulence, or antibiotic resistance. It is, therefore, not surprising that membrane proteins are the principal targets of all drugs on the market today and that understanding their biological functions is a major goal of biomedical research in academic, medical, biotech, and pharmaceutical settings.

Despite their importance, very little structural data exist for them compared to the wealth of information available for their soluble counterparts. This reflects the special amphiphilic properties of membrane proteins and their surrounding membrane environment, which complicate biophysical studies. Membrane proteins differ fundamentally from water-soluble proteins. While the latter exist in an isotropic aqueous environment, the lipid bilayer membrane is anisotropic and heterogeneous, with large gradients in fluidity, water concentration, and dielectric constants from the bilayer core to the water-lipid interface [1–4]. These features lead to phenomena (e.g., stronger hydrogen bonds, membrane thinning, hydrophobic mismatch, curvature frustration, charge polarization, lateral force gradients) that significantly influence membrane protein structure, dynamics, and function and argue very strongly in favor of determining the structures of membrane proteins in lipid bilayers at

or near physiological conditions of temperature, pH, and hydration [reviewed in ref. 5–7]. As noted by Cross [7], this is in line with Anfinsen’s hypothesis, which states that a protein conformation “is determined by the totality of inter-atomic interactions and hence by the amino acid sequence in a given environment” [8]. For membrane proteins, the “given environment” of the lipid bilayer is essential for preserving native structure and function.

Membrane protein structure determination by X-ray diffraction and solution NMR requires proteins dissolved in detergents because lipid bilayers are incompatible with crystallization and solubilization. For some membrane proteins, lipid nanodiscs can be useful membrane mimics for solution NMR [9–11], but typically they yield broader lines and significant sample polydispersity compared to micelles or bicelles. In contrast, solid-state NMR is compatible with structure determination of membrane proteins in membranes, under physiological conditions, and recent developments in sample preparation, recombinant bacterial expression, pulse sequences for high-resolution NMR spectroscopy, and computational methods have enabled a number of membrane protein structures to be determined in lipid bilayer membranes (Fig. 1; [12, 13]).

In this chapter, these methods are illustrated with examples from two homologous bacterial outer membrane proteins, OmpX (outer membrane protein X) from *E. coli* and Ail (attachment invasion locus) from *Yersinia pestis*, an extremely pathogenic organism with a long history of precipitating massive human pandemics of plague [14]. Although the specific function of OmpX is not known, Ail is a *Y. pestis* virulence factor essential for evading the human host’s immune system by mediating the adhesion of *Y. pestis* to human host cells and providing resistance to human innate immunity [15, 16]. Both OmpX and Ail belong to a family (pfam PF06316) of outer membrane proteins that 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 the function of Ail. Both proteins adopt a transmembrane 8-stranded β -barrel structure [17–19]. However, while all extracellular loops of OmpX are fully structured, key functional loops of Ail are not visible in the crystal structure, indicating that the structure of Ail determined within the phospholipid bilayer membrane will be needed to understand the molecular basis of its biological function.

2 Materials

Specialized materials used for the experiments described in this chapter are listed in Table 1. They include *E. coli* cells for recombinant expression, lipids for protein reconstitution, and isotopically labeled salts, sugars, and amino acids used to produce ^{15}N - and ^{13}C -labeled proteins for NMR studies.

3 Methods

3.1 Protein Expression and Purification

Cloning—Expression, purification, and refolding of OmpX and Ail were performed as described previously [17, 20]. The genes encoding mature Ail and OmpX (without the signal sequence) were cloned between the NdeI and XhoI restriction sites of the pET-30b. For both

OmpX and Ail, deletion of the signal sequence directs protein expression into inclusion bodies.

Protein Expression—The Ail- and OmpX-encoding plasmids were transformed in *E. coli* BL21 (DE3) cells. Positive clones were grown in 5 mL of LB medium at 37 °C for 8 h, then 100 μ L of this culture was used to inoculate 50 mL of M9 minimal medium for overnight growth at 37 °C. The next morning, 50 mL of overnight culture was transferred to 1 L of fresh M9 medium, and the cells were grown at 37 °C, to a density of OD₆₀₀ = 0.6, before induction with 1 mM IPTG for 3–6 h. Cells were harvested by centrifugation (6,500 rpm, 15 min, 4 °C) and stored at –80 °C overnight. For ¹⁵N and ¹³C isotopic labeling, the M9 growth medium was prepared with (¹⁵NH₄)₂SO₄ and ¹³C-glucose (Cambridge Isotope Laboratories) as the sole nitrogen and carbon sources.

Inclusion Bodies Isolation—Ail expression and purification were monitored by SDS-PAGE (Fig. 2). Cells from a 1 L culture were suspended in 30 mL of buffer A (Table 2) and lysed by two passes through a French press. The soluble cell fraction was removed by centrifugation (19,000 rpm, 30 min, 4 °C) and the remaining pellet, which is enriched in inclusion bodies, was suspended in 30 mL buffer A_T and gently mixed for 1 h at 37 °C. The soluble fraction was removed by centrifugation (19,000 rpm, 30 min, 4 °C), then the resulting pellet was washed with 30 mL of water to remove residual detergent and again isolated by centrifugation (19,000 rpm, 30 min, 4 °C). The isolated inclusion bodies are white in appearance and contain very pure protein. Finally, the inclusion bodies pellet was dissolved in 30 mL of buffer B by gently mixing for 1 h at 37 °C, and the remaining insoluble fraction was removed by centrifugation (19,000 rpm, 30 min, 4 °C) and discarded (Fig. 2a).

Protein Purification—The purification strategy depends on each protein's properties. The isoelectric points of OmpX (pI = 5.0) and Ail (pI = 7.8) dictated the use of anion and cation exchange chromatography, respectively. For example, Ail was purified by cation exchange chromatography (HiTrap SP HP 5 mL column, GE Healthcare) in buffer B with a NaCl gradient (Fig. 2b, d), followed by size exclusion chromatography (Sephacryl S-200 HR HiPrep 16/60 column, GE Healthcare) in buffer B_s (Fig. 2c, e). Purified protein was concentrated by dialysis (10 kDa cutoff) against water, lyophilized, and stored at –20 °C. Typically, 25–30 mg of purified protein is obtained from a 2 L culture in ¹⁵N, ¹³C isotopically labeled M9 medium.

3.2 Reconstitution in Phospholipid Bilayers

Pure Ail or OmpX (8 mg of lyophilized powder) was dissolved in 1 mL of 100 mM SDS in water, and added dropwise, at 40 °C, to a suspension of small unilamellar vesicles prepared by probe sonication with 50 mg of DMPC (Avanti Polar Lipids), or its ether-linked analog 14-O-PC, in 20 mL of buffer C. Although the chemical structure of these lipids is very similar, the ether link of 14-O-PC prevents lipid hydrolysis and is better suited for long-term sample stability. The protein/lipid mixture was incubated at 30 °C for 24 h and refolding was monitored by SDS-PAGE (Fig. 3). After complete refolding, SDS was removed by dialysis (10 kDa cutoff) against two 4 L changes of buffer C, followed by four 4 L changes

of buffer C supplemented with 30 mM KCl. The resulting proteoliposomes were dialyzed against 4 L of buffer A and then concentrated by ultracentrifugation (41,000 rpm, 4 h, 4 °C). To prepare magnetically aligned planar bilayer samples, 10 mg of DHPC, or its ether-linked analog 6-O-PC, was dissolved in 50 μ L of water, added to the proteoliposomes and thoroughly mixed by repeated freezing and thawing, as described previously [21, 22]. Alignment of the lipid bilayer normal parallel to the magnetic field was induced by adding 5 μ L of 100 mM YbCl₃ directly to the NMR tube precooled at 4 °C, mixing thoroughly, and resealing the tube.

3.3 Solid-State NMR Studies in Lipid Bilayer Membranes

Approach for NMR Structural Studies—Modern NMR methods for protein structure determination increasingly rely 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 [23–28]. For both solution NMR and solid-state NMR, these restraints can be used to guide both the generation of structural models and structure refinement and are especially powerful when coupled with molecular fragment replacement (MFR) and de novo structure prediction using programs such as ROSETTA [29, 30]. By shifting the burden away from time-consuming measurements of multiple long-range distances between side chain sites, these approaches significantly facilitate protein structure determination and yield reliable three-dimensional structures, with very few or no distance restraints, for soluble proteins in water [27, 31], membrane proteins in micelles [32], and membrane proteins in lipid bilayers [25, 33, 34].

By combining features of magic-angle spinning (MAS) [35–37] and oriented-sample (OS) [38–40] solid-state NMR approaches, it is possible to resolve and assign multiple peaks through the use of ¹⁵N/¹³C-labeled samples and to measure DC, CSA, and isotropic CS to obtain precise orientation and dihedral angle restraints for structure determination [33, 41]. OS solid-state NMR uses samples that are uniaxially aligned relative to the magnetic field (e.g., planar lipid bilayers) to yield orientation-dependent single-line resonances. MAS solid-state NMR uses nonaligned samples (e.g., proteoliposomes) and yields single-line spectra due to averaging of the spin interactions to their isotropic values.

In both cases, the uniaxial order inherent to membrane proteins undergoing rotational diffusion around the lipid bilayer normal [42–46] provides the foundation for a powerful approach to structure determination based on orientation restraints [41, 47–49]. The orientation-dependent DC and CSA signals correlate directly with molecular structure and enable both protein structure and global orientation (i.e., supramolecular structure) to be determined in the membrane [50–52]. Their frequencies can be read directly from the single-line resonances of OS solid-state NMR spectra, or MAS can be used to recouple and measure rotationally averaged powder patterns: since the frequency measured from the edge of a rotationally averaged powder pattern is equivalent to that measured from OS NMR spectra, the same analytical methods developed for data analysis and structure determination are applicable.

The DC and CSA interactions are well characterized for ¹H/¹⁵N/¹³C-labeled protein sites [39, 40]. Their interpretation in terms of orientation restraints is greatly facilitated by the

fact that solid-state NMR spectra display them at full, or near full, magnitudes, enabling a priori knowledge of the order tensor. Thus, a set of CSA and DC frequencies can provide sufficient restraints for high-precision structure determination, requiring few or no distances (e.g., [25, 33, 34, 53, 54]).

The spectra of uniaxially ordered samples exhibit distinctive wheel-like patterns [50–52] that reflect protein structure and orientation (Fig. 4). These patterns are observed for both α -helices [25, 50, 51, 55] and β -strands [21, 22, 52]. They stem from the direct relationship that exists between orientation-dependent solid-state NMR data and molecular structure and are useful both for guiding resonance assignment performed with traditional spectroscopic approaches (e.g., [56]) and for obtaining resonance assignments through methods that we have developed for simultaneous assignment and structure refinement (SASR) [25]. They also help reduce or eliminate the degeneracy of orientation solutions associated with DC and CSA measurements [25, 57], allowing structures to be built by linking consecutive peptide planes or fragments through their common CA atom.

Simultaneous Assignment and Structure Refinement—The direct relationship between NMR data and structure facilitates a method for SASR, based on minimizing the difference between the experimentally observed spectral frequencies and the frequencies back-calculated from a structural model [22, 25]. The SASR approach relieves the burden of having to obtain near complete resonance assignments prior to structure determination: resonance assignments are obtained as a side product of fitting a structural model to the NMR data, but is not a prerequisite for structure determination. To automate the SASR process, we have developed AssignFit, a Python-based program that is available as part of XPLOR-NIH release 2.29 [58]. AssignFit generates all assignment permutations and calculates the corresponding molecular alignment, the atomic coordinates reoriented in the alignment frame, and the associated set of NMR frequencies, which are then compared with the experimental data for best fit. For example, using AssignFit, the seven Phe peaks in the separated local field (SLF) spectrum of OmpX could be assigned easily and quickly [22, 58] (Fig. 5).

Solid-State NMR Experiments—For OmpX and Ail, MAS and OS solid-state NMR experiments were performed, on 500 or 700 MHz Bruker Avance spectrometers equipped with a Bruker low-E $^1\text{H}/^{13}\text{C}/^{15}\text{N}$ triple-resonance 3.2 mm MAS solid-state NMR probe (MAS experiments) or a static low-E $^1\text{H}/^{15}\text{N}$ double-resonance solid-state NMR probe (OS experiments). During MAS experiments, the sample temperature was maintained at 25 ± 2 °C and the spinning rate was controlled to 11.11 ± 0.002 kHz using a Bruker MAS controller. During OS experiments the sample temperature was maintained at 43 ± 2 °C. Chemical shifts were externally referenced to DSS, by setting the adamantane methylene carbons to a ^{13}C chemical shift frequency of 40.48 ppm, or to liquid ammonia, by setting the ammonium sulfate nitrogen ^{15}N chemical shift frequency to 26.8 ppm [59, 60]. The NMR data were processed and analyzed using NMRPipe [61] and Sparky [62].

Solid-State NMR Studies of Ail and OmpX in Proteoliposomes—MAS solid-state NMR studies benefit from the rapid progress made by numerous laboratories around the world [35–37, 63–68]. Resonances in these spectra can be assigned using NCACX and

NCOCA experiments [69–74], complemented by ^{13}C - ^{13}C [75, 76] and ^{13}C - ^{15}N correlation experiments [77]. Two-dimensional ^{13}C - ^{13}C correlation MAS spectra of Ail and OmpX in proteoliposomes (Fig. 6a, b) show several resolved peaks, and we anticipate substantial improvements with optimization of sample and experimental conditions. In both spectra, peaks from Ala, Ile, Ser, and Thr populate the regions expected for β -sheet conformation. For example, Ail has four Thr, and four signals are observed with ^{13}C shifts in the expected region (Fig. 6a).

Solid-State NMR Studies of Ail and OmpX in Planar Lipid Bilayers—OS solid-state NMR spectra yield single-line resonances that directly reflect the orientations of molecular sites relative to the membrane (Fig. 6d–f). Multidimensional SLF and heteronuclear correlation experiments [78–83] with uniformly and selectively labeled samples can be used to resolve the spectra and measure orientation-dependent DC and CSA frequencies for structure determination [39, 40]. Both the one-dimensional ^{15}N spectra and the two-dimensional $^1\text{H}/^{15}\text{N}$ SLF spectra of OmpX in magnetically aligned DMPC/DHPC bilayers show very high resolution (Fig. 6d–f). They benefit significantly from the sensitivity and resolution enhancements possible through the use of high magnetic fields and newly developed radiofrequency probes [84]. These spectra were assigned using SASR/AssignFit methods (Fig. 5). Additional assignments can be obtained using a combination of selective isotope labeling schemes with structural model fitting [25, 50–52, 55, 85–87], comparisons with isotropic NMR data [88], and multidimensional triple-resonance experiments [89–93], similar to the multidimensional ^{15}N spin-exchange experiments that we developed for resonance assignments in a helical membrane protein [56]; we anticipate that the latter will be even more useful for β -barrel proteins because of the wider chemical shift dispersion from neighboring amide sites available in their spectra.

4 Conclusions

Recent progress in sample optimization, instrumentation, and NMR experiments enables very high-resolution solid-state NMR spectra to be obtained for membrane proteins in lipid bilayers. For example, two bacterial outer membrane proteins, OmpX and Ail, refolded in lipid bilayers yield very high-quality MAS and OS solid-state NMR spectra, where several individual peaks can be resolved and assigned (Fig. 6). The resolution observed even in one-dimensional spectra is remarkable, indicating that structure determination of these β -barrel membrane proteins in natural lipid bilayer environments is within reach.

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Fig. 1.

Solid-state NMR structures of membrane proteins in lipid bilayers. PDB codes correspond to (1MAG) gramicidin [94], (1EQ8) M2 pore-forming domain of acetylcholine receptor [95], (1MZT) membrane-bound bacteriophage fd coat protein [25], (2LOJ) channel-forming domain of influenza M2 [34], (2LJ2) mercury transporter MerF [33], and (2LNL) human chemokine receptor CXCR1 [54]

Fig. 2.

Expression and purification of Ail. **(a–c)** SDS-PAGE and **(d, e)** chromatograms showing Ail purification steps. **(a)** Inclusion bodies isolation; the supernatant (s) and wash (w) fractions are free of Ail, while inclusion bodies (IB) are enriched in Ail. **(b, d)** Ion exchange chromatography yields a fraction free of Ail (I) and another containing Ail (II). **(c, e)** Size exclusion chromatography yields a fraction free of Ail (III) and a fraction containing purified Ail (IV)

Fig. 3.

Refolding of Ail and OmpX in lipids monitored by SDS-PAGE. Unfolded proteins (*lane u*) migrate at higher apparent molecular weights (~21 kDa) than folded proteins (~14 kDa). **(a)** Refolding of Ail in DMPC (*lane 1*), DHPC (*lane 2*), or DHPC/DMPC (*lane 3*). **(b)** Refolding of OmpX in DMPC monitored at 0 h (*lane 1*), 2 h (*lane 2*), and 12 h (*lane 3*) after addition from SDS solution. All samples were loaded without boiling

Fig. 4.

Solid-state NMR *PISA wheels* observed in the OS solid-state NMR of α -helical and β -barrel membrane proteins. Theoretical *PISA wheels* are shown for ideal α -helices or β -strands with different tilts (0° , 30° , 45° , 60° , 90°) relative to the phospholipid bilayer membrane normal

Fig. 5.

AssignFit SASR of ^{15}N -Phe-labeled OmpX in oriented bilayers. NMR peaks were assigned by minimizing the difference between experimental (*top*) and back-calculated (*bottom*) spectra after refinement of the OmpX crystal structure [17] with the assigned orientation restraints. The data provide the orientation of OmpX in the membrane (b: barrel axis; n: bilayer normal).

Peaks in *red* persist in D_2O

Fig. 6.

Solid-state NMR spectra of $^{15}\text{N}/^{13}\text{C}$ -labeled OmpX and Ail in lipid bilayers. (a, b) Two-dimensional $^{13}\text{C}/^{13}\text{C}$ correlation MAS solid-state NMR spectra of Ail and OmpX in DMPC proteoliposomes. (c) One-dimensional ^{15}N MAS solid-state NMR spectrum of OmpX in DMPC proteoliposomes. (d) One-dimensional ^{15}N OS solid-state NMR spectrum of OmpX in parallel (bilayer normal || magnetic field) magnetically aligned DMPC/DHPC lipid bilayers. (e, f) Two-dimensional $^1\text{H}/^{15}\text{N}$ SLF OS solid-state NMR spectra of OmpX in parallel magnetically aligned DMPC/DHPC bilayers. Proteins are uniformly $^{15}\text{N}/^{13}\text{C}$ labeled (a–c), uniformly ^{15}N labeled (d), ^{15}N Val labeled (e), or ^{15}N Phe labeled (f)

Table 1

Specialized materials and computer programs

Material	Source
<i>Reagents</i>	
<i>E. coli</i> BL21(DE3) cells	Novagen (www.emdmillipore.com)
IPTG (isopropyl 1-thio- β -D-galactopyranoside)	Sigma (www.sigmaaldrich.com)
DHPC (dihexanoyl-phosphocholine)	Avanti Polar Lipids (www.avantilipids.com)
6-O-PC (di- <i>O</i> -hexyl-phosphocholine)	Avanti Polar Lipids (www.avantilipids.com)
DMPC (di-myristoyl-phosphocholine)	Avanti Polar Lipids (www.avantilipids.com)
14-O-PC (di- <i>O</i> -tetradecyl-phosphocholine)	Avanti Polar Lipids (www.avantilipids.com)
DMPG (di-myristoyl-phosphoglycerol)	Avanti Polar Lipids (www.avantilipids.com)
SDS (sodium-dodecyl-sulfate)	Sigma (www.sigmaaldrich.com)
(^{15}N) $_4$ SO $_4$	Cambridge Isotopes laboratories (www.isotope.com)
^{13}C -glucose	Cambridge Isotopes laboratories (www.isotope.com)
^{15}N -Val	Cambridge Isotopes laboratories (www.isotope.com)
^{15}N -Phe	Cambridge Isotopes laboratories (www.isotope.com)
YbCl $_3$	Sigma Aldrich (www.sigma.com)
HiTrap SP HP 5 mL column	GE Healthcare Life Sciences (www.gelifesciences.com)
Sephacryl S-200 HR HiPrep 16/60 column	GE Healthcare Life Sciences (www.gelifesciences.com)
<i>Computer programs</i>	
NMRPipe	(spin.niddk.nih.gov/bax/software)
Sparky	(www.cgl.ucsf.edu/home/sparky/)
XPLOR-NIH/AssignFit	(nmr.cit.nih.gov)
ROSETTA	(www.rosettacommons.org)

Table 2

Buffers used for protein purification and sample preparation

Buffer A	20 mM Tris-HCl, pH 8.0
Buffer A _T	20 mM Tris-HCl, pH 8.0, 2 % Triton-X
Buffer B	20 mM Na acetate, pH 5.0, 8 M urea
Buffer B _s	20 mM Na acetate, pH 5.0, 8 M urea, 150 mM NaCl
Buffer C	20 mM Tris-HCl, pH 9.5, 150 mM NaCl
Buffer E	20 mM Tris-HCl, pH 8.0, 8 M urea
Buffer F	20 mM glycine, pH 10.2, 200 mM KCl