NMR of Membrane Proteins: Beyond Crystals

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Abstract

Membrane proteins are essential for the flow of signals, nutrients and energy between cells and between compartments of the cell. Their mechanisms can only be fully understood once the precise structures, dynamics and interactions involved are defined at atomic resolution. Through advances in solution and solid state NMR spectroscopy, this information is now available, as demonstrated by recent studies of stable peripheral and transmembrane proteins. Here we highlight recent cases of G-protein coupled receptors, outer membrane proteins, such as VDAC, phosphoinositide sensors, such as the FAPP-1 pleckstrin homology domain, and enzymes including the metalloproteinase MMP-12. The studies highlighted have resulted in the determination of the 3D structures, dynamical properties and interaction surfaces for membrane-associated proteins using advanced isotope labelling strategies, solubilisation systems and NMR experiments designed for very high field magnets. Solid state NMR offers further insights into the structure and multimeric assembly of membrane proteins in lipid bilayers, as well as into interactions with ligands and targets. Remaining challenges for wider application of NMR to membrane structural biology include the need for overexpression and purification systems for the production of isotope-labelled proteins with fragile folds, and the availability of only a few expensive perdeuterated detergents.

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Step changes that may transform the field include polymers, such as styrene maleic acid, which obviate the need for detergent altogether, and allow direct high yield purification from cells or membranes. Broader demand for NMR may be facilitated by MODA software, which instantly predicts membrane interactive residues that can subsequently be validated by NMR. In addition, recent developments in dynamic nuclear polarization NMR instrumentation offer a remarkable sensitivity enhancement from low molarity samples and cell surfaces. These advances illustrate the current capabilities and future potential of NMR for membrane protein structural biology and ligand discovery.

Keywords
High resolution NMR • Solid state NMR • Protein structure • Protein interactions • Membrane targets

3.1 Introduction

Membranes of cells, organelles and some viral envelopes are asymmetric topologically planar bilayer assemblies composed at approximately equal weight fractions of proteins and lipids with diverse structure and function. They present permeability barriers, which support electrical potentials and chemical gradients, and are vital to cells. Membranes contain a significant fraction of specialised transmembrane proteins, which actively maintain an asymmetric electrical and solute environment. Other proteins may be loosely associated with the membrane or persistently anchored to the bilayer, which permits localised functionality with substrates at or near the membrane surface. A complex and dynamic lateral organisation exists within the proteolipid membrane, where sphingolipids and cholesterol-rich domains play an important role in membrane transport and signalling (Simons and Ikonen 1997). A structural variation exists between proteins within the plasma membranes of cells, which are largely made of helical transmembrane segments, and proteins from bacterial outer membranes, which predominantly follow β-barrel folds in contact with outer leaflet lipopolysaccharides.

The challenges in high resolution structural analysis of proteins are greater for membrane proteins, which require a hydrophobic membrane to support their folded state. Model membranes are often used and mimetic systems have been developed, which provide hydrophobic support to maintain the protein fold in the absence of a bilayer, while allowing for the 3D arrangement of isotropically mobile protein suspensions. Mimetics include detergent micelles, detergent/lipid bicelles, lipidic cubic phases and protein/lipid nanodisks (Warschawski et al. 2011) which can accommodate biologically important lipids alongside the proteins of interest. While this approach has provided the opportunity for structural characterisation of membrane peptides and proteins, studying the interactions of membrane proteins with each other or with lipids or looking at the regulation by ligands requires, in most cases, the presence of an intact membrane or a liposome membrane model. This is also required for functional characterisation in cases where an asymmetric solute environment is required, such as in transport or voltage-dependent ionic conductance.

Membrane protein studies by crystallography have yielded a set of key structures, which are bringing our understanding of their fundamental architecture towards a basis of high resolution templates for building a more comprehensive picture of membrane protein folding and function. Yet, studying membrane protein structure under unrestricted, hydrated and functional conditions remains possible by nuclear magnetic resonance

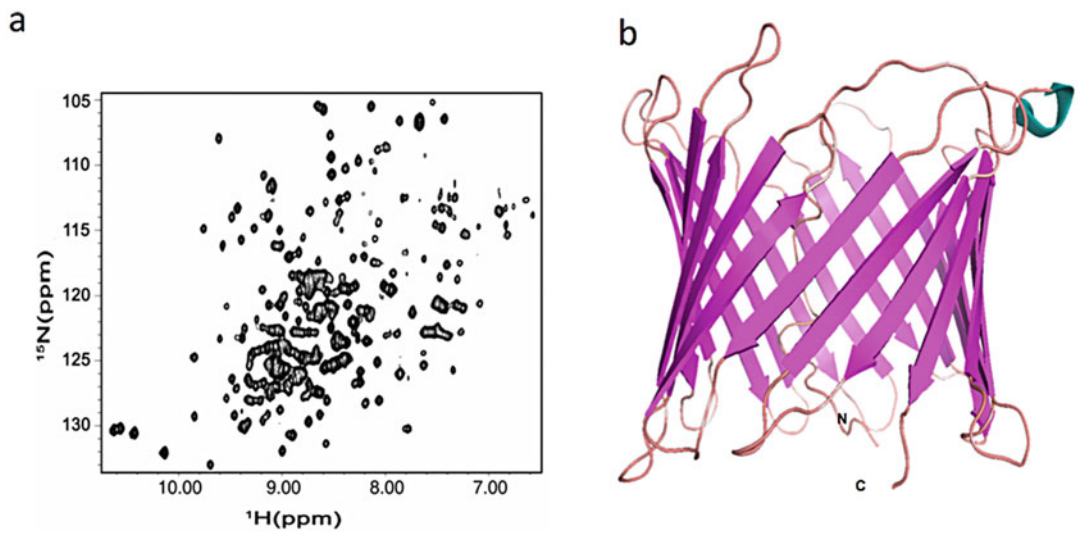

Fig. 3.1 (**a**) Two-dimensional ^{15}N-^{1}H- TROSYNMR spectrum of 0.5 mM [u-^{2}H,^{15}N] VDAC-1 in 300 mM detergentLDAO micelles at 30 °C. (**b**) Cartoon representation of the three-dimensional solution-NMR structure of VDAC-1 in LDAO micelles with the intracellular N- and C-terminus, as labelled (Adapted from (Hiller et al. 2008))

(NMR) alone. Here, we discuss representative applications of NMR to structural studies of membrane proteins and peptides, as well as their interactions with lipids, targets and ligands.

3.2 Barrel Folds

The mitochondrial outer membrane contains a protein known as the voltage-dependent anion channel (VDAC), which exchanges ions and molecules between mitochondria and cytosol. This is the largest β-barrel structure solved by NMR to date, and hence represents the state-of-the-art and also illustrates the technical challenges involved (Fig. 3.1). The VDAC fold consists of 19 β-strands that cross the membrane at a tilted angle and form a central pore that becomes occluded by the flexible N-terminus to mediate channel gating. The assignment involved a suite of 3D TROSY experiments performed at 900 MHz. The structure of the 283 residue VDAC-1 protein in micelles composed of perdeuterated LDAO was solved using 4D NOESY spectra collected with non-uniform sampling (Hiller et al. 2008). The binding sites are evident by ligand-induced perturbations in amide chemical shifts, which map the surfaces that interact with cholesterol, the metabolite β-NADH, and its anti-apoptotic partner Bcl-X_L, respectively. The experimental strategy benefitted from very high expression, purification and refolding yields of isotope labelled protein from *E. coli*, which were essential for cost-effective perdeuteration, selective ^{13}C-methyl labelling and amide proton exchange. Unfortunately, these requirements represent insurmountable barriers for the determination of the majority of eukaryotic membrane proteins, and point to the need for alternative means of producing pure, stable and labelled proteins that are functionally intact. Nonetheless, there is now a growing number of solution structures of bacterial outer membrane proteins, the stable β-barrel folds of which have been determined in detergent micelles composed of phosphocholine (PC)-based detergents and octyl glucoside. This demonstrates that modern solution NMR methods can be used to elucidate novel solution structures of 75–90 kDa complexes of stable β-barrel proteins in micelles if a suitable expression systems can be found.

The challenges of studying membrane proteins within lipid bilayers can be addressed

through the applications of solid state NMR, which is often used in conjunction with solution NMR. Spectral resolution in membrane samples can be enhanced by magic angle sample spinning (MAS) (Haeberlen and Waugh 1968), which permits the determination of distance and torsion angle constraints, similar to those obtained in solution. Using a CP-HSQC approach, resonance assignment under fast MAS has been shown for the monomeric outer membrane poring OmpG from *E. coli* (Barbet-Massin et al. 2014).

3.3 Helical Targets

The diverse array of α-helical membrane proteins is challenging to study using solution NMR and this is reflected in the low number of structures resulved using this technique. Complete structure determination of larger seven-helical transmembrane GPCR proteins are yet to be attempted using solution NMR methods. However solution structures of proteins with multiple transmembrane regions have recently been solved, indicating that helical bundles in micelles can now be characterized if they are sufficiently stable. The successes include the bacterial enzymes DsbB and DAGK (Zhou et al. 2008; Van Horn et al. 2009), proteorhodopsin (Reckel et al. 2011), sensory rhodopsin II (Gautier et al. 2010), mitochondrial uncoupling protein 2 (Berardi et al. 2011), six human proteins with 2 or 3 transmembrane helices (Klammt et al. 2012), YgaP (Eichmann et al. 2014), LAMP2A (Rout et al. 2014), the Cl^- channel of human Glycine receptor α1 (Mowrey et al. 2013), and the voltage sensor-domain of voltage-dependent K^+ channel KvAP (Butterwick and MacKinnon 2010). The typical difficulties in solution NMR studies of helical membrane proteins include insufficient sample production, choice of membrane mimics, spectral overlap, limited spectral dispersion, shortage of long-range distance information and increased NMR data acquisition time.

Most membrane protein structures that have been determined by solution NMR methods were obtained from recombinant proteins expressed in *E.coli*. However not all proteins (e.g. GPCRs) readily express in prokaryotes, and some lack activity due to altered folding pathways and the absence of post-translational modifications. In such cases, eukaryotic expression hosts such as *Pichia pastoris*, *Drosophila* S2 and Sf9 cells serve as alternatives to producing uniformly ^{13}C,^{15}N-labeled membrane proteins for NMR studies (Fan et al. 2011). Recently, cell free expression systems using *E.coli* (Klammt et al. 2012), wheat-germ (Madono et al. 2011) or insect-cell extracts (Sobhanifar et al. 2010) have been successfully developed to produce membrane proteins for NMR. These cell free systems offer the advantage of amino acid selective (^{13}C methyl groups of isoleucine, leucine and valine) or targeted labelling, as well as the combinatorial dual-labelling scheme that help to address problems in NMR assignment, such as spectral overlap and fast relaxation in helical transmembrane proteins. Suitable membrane mimetics are screened for their ability to solubilise proteins from native membranes, mimic the native lipid environment, confer stability to proteins over a period of time at temperatures up to 50 °C and are sufficiently small for NMR studies. Further optimization by varying protein-detergent ratio to improve spectral quality can be done by monitoring the 2D ^{1}H-^{15}N TROSY spectra or by measuring rotational diffusion (Gautier and Nietlispach 2012; Horst et al. 2012). The use of smaller nanodisks can improve the relaxation properties of reconstituted membrane proteins and aided in the high resolution NMR structure calculation of bacteriorhodopsin (Hagn et al. 2013). The spectral overlap, due to limited dispersion of helical membrane proteins, is addressed by measurements at high temperatures, use of selectively methyl-labeled amino acids (ILV) and combinatorial-dual labelling. Unlike β-barrel proteins, helical transmembrane domain (TMD) proteins lack sufficient long range NOEs and even when available, the problem is compounded by the difficulty in obtaining complete side chain assignment. Paramagnetic relaxation enhancement (PRE) allows distance restraints to be measured using spin label attached to selected

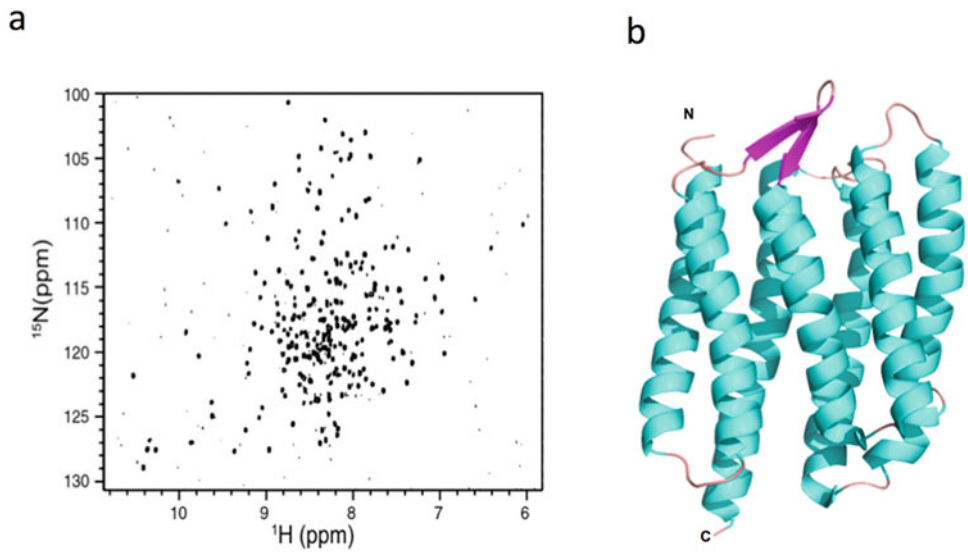

Fig. 3.2 (**a**) Two-dimensional ^{15}N-^{1}H- TROSYNMR spectrum of refolded 0.5 mM pSRII in 60 mM detergent c7-DHPC micelles at 50 °C. (**b**) Cartoon representation of the three-dimensional solution-NMR structure of pSRII in DHPC micelles with the extracellular N- and intracellular C-terminus, as labelled (Adapted from (Gautier et al. 2010))

protein sites. The PRE restraints provide distance information between 12 and 25 Å, and have contributed significantly to a number of large membrane protein structures obtained by NMR (Van Horn et al. 2008). The advantages of a non-uniform sampling scheme in reducing NMR acquisition times while increasing sensitivity of higher dimensionality experiments has been beautifully exemplified in the high resolution structure calculation of pSRII and holds promise for solution NMR studies of membrane proteins in general (Fig. 3.2).

Selective isotope labelling of GPCRs using ^{19}F probes (on cysteines), ^{13}C-methylated methionine (methyl-^{13}C-Met) or dimethylated lysines [Methyl-^{13}C-Lys] was successfully used to define population states and to probe conformational changes in transmembrane helices during receptor activation of β2 adrenergic and μ-opiod class of GPCRs (Kofuku et al. 2012; Liu et al. 2012; Sounier et al. 2015). These studies highlight the versatility of NMR methods in determining conformational dynamics and population states in the protein energy landscape, complementing the static X-ray structures. Understanding multiple conformations during receptor activation is important for designing small molecule inhibitors or monoclonal antibodies targeting these GPCRs.

In some cases, preparations for crystallographic studies can yield low-diffracting crystals, which can be investigated by solid state Magic Angle Spinning (MAS) NMR to obtain protein structures of significantly enhanced resolution. In one example, a combination of solid state MAS NMR and X-ray crystallography has provided structures of the bacterial disulfide-processing proteins DsbB/A (Tang et al. 2011; Shahid et al. 2012) by combining intermediate resolution crystallographic data with solid state NMR from the protein in the microcrystalline state. This was achieved using carefully calculated sets of dihedral angles in combination with selective ^{13}C-labelleing. Fast MAS solid state NMR has been used to obtain spectral assignment of the M2 ion channel from influenza (Barbet-Massin et al. 2014). An alternative to MAS permits angular constraints that can be obtained from proteins in macroscopically aligned membranes, which relies on correlations between heteronuclear dipolar couplings with ^{15}N chemical shifts (Wu et al. 1994). A study of pentameric phos-

phorylated phospholamban in lipid membranes revealed an L-shaped conformation including a TMD and a surface-associated helix within each monomer (Vostrikov et al. 2013).

3.4 Lipid Recognition Domains

A significant proportion of the proteome binds membranes using small, often terminal domains that recognize specific sets of lipids. With each biological membrane in the cell offering unique sets of lipids, this provides a basis for selective recruitment of thousands of proteins to particular organelle surfaces (Overduin and Cheever 2001). The largest superfamily of membrane interactive proteins is defined by the presence of pleckstrin homology (PH) domains, which is the 11th most common domain in the proteome (Lenoir et al. 2015a). Of these, those of the COF subfamily are distinguished by their ability to traffic ceramide, sterols and glycosphingolipids at the trans-Golgi network, and by recognizing phosphatidylinositol 4-phosphate (PI4P), which is the most abundant monophosphorylated inositol lipid. The human FAPP1 protein is the paradigm of this subfamily, and its mechanism has been most intensely studied by solution NMR methods. The complexes of its 108 residue PH domain with micelles and bicelles in the presence and absence of PI4P have been characterized (Lenoir et al. 2015b) (Fig. 3.3a). This revealed one of the largest intracellular membrane binding sites (~1220 Å), as well as convergent PI4P-specific and nonspecific bilayer insertion angles. The contacts with the phosphoinositide as well as several neighboring phospholipid molecules are evident, as are the residues that insert into the bilayer, from NOE and PRE patterns. The resulting distance restraints, along with the associated chemical shift changes that occur during interactions, allow the structures of assemblies to be calculated using high ambiguity-driven biomolecular docking (HADDOCK) (Dominguez et al. 2003) A novel aspect was the recognition of disordered bilayer regions mediated by an extensive and predominantly ordered region of the protein, and the exclusion of ordered bilayer binding. NMR afforded detailed insights not only due to the high expression and stability of the protein, even in detergents, but also to the development of optimized bicelle and mixed micelle formulations. These advances allowed collection of NMR restraints from large

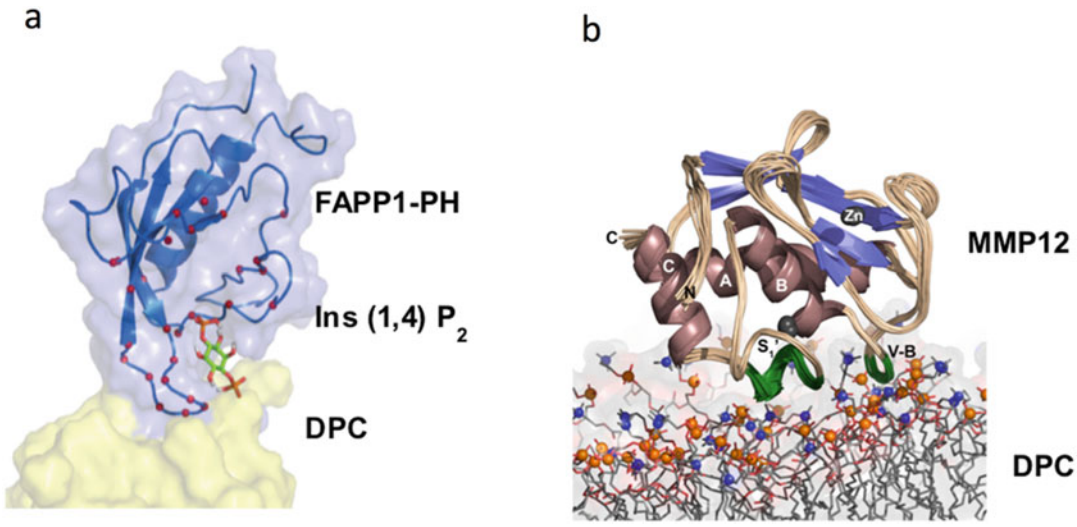

Fig. 3.3 (**a**) Solution-NMR structure of the FAPP1-PH domain (in *blue*) in complex with DPC (*yellow*) micelles in the presence of Inositol(1,4) phosphate (sticks). (**b**) Ensemble of solution-NMR structures of MMP12 (*cartoon*) bound to DPC micelles (*sticks*) with the membrane insertion loop (V-B) and helix (S_1') in *green* (adapted from (Koppisetti et al. 2014))

complexes, as did the use of soluble and lipidic spin labels, ^{13}C-resolved PREs and ultrahigh field magnets.

The membrane interactions of soluble enzymes and receptor domains can be similarly characterized by solution NMR methods, as can those of a variety of lipid binding domains such as C1b, C2, ENTH, FYVE and PX domains (Lemmon 2008). The metalloproteinase MMP-12 is one of the largest such peripheral membrane protein to be studied by solution NMR methods. The addition of bicelles loaded with PC spin labels revealed two opposite surfaces which interact reversibly with the membrane surface (Koppisetti et al. 2014) (Fig. 3.3b). This pair of interfaces is responsible for localising and concentrating the enzyme near the exterior membrane surface of the cell, they also control access to the proximal active site and hinder binding of protein inhibitors such as TIMP2, thus controlling activity and specificity. The alternating membrane-bound orientations can be simulated by molecular dynamics, showing that they differ by 137° and bury 2070 and 2530 Å^2 of surface area, respectively. Despite the large size of the bicelle complex (144 kDa), the PRE values induced by PC spin labels could be measured from relaxation rates to model the complexed structure. Together, this indicates that the reversible membrane interactions of a diversity of folded protein structures that recognize lipids, catalyse reactions, and tubulate and vesiculate membranes can now be characterized by NMR, illuminating fundamental events not readily visible by any other method.

3.5 Protein Structure in Membranes

A further step in developing structural understanding of membrane proteins towards more realistic, *in vivo* conditions is the use of solid state NMR to study proteins reconstituted into lipid membranes. Protein structure in membranes is often sought through a combination of NMR and Small Angle X-ray Scattering (SAXS) in solution or in membrane mimetics, in conjunction with crystallography, or with solid state NMR in membranes (Sanghera et al. 2011). Structural analysis of a microbial retinal-binding photoreceptor ASR from *Anabaena* has been carried out by solid state MAS NMR using PDSD and CHHC from sparsely ^{13}C-labelled protein along with relaxation enhancement (Opella 2013) and in conjunction with existing crystallographic and biochemical data. A hybrid solution/solid state NMR approach has been used in combination with molecular dynamics (MD) simulations to obtain the structure and topology of phospholamban in membranes (Zech et al. 2004). Mechanically oriented samples were used with Polarization Inversion Spin Exchange at the Magic Angle (PISEMA) (Wu et al. 1994) in this study and the approach has since been extended to the analysis of the biologically relevant, pentameric state of phospholamban in conjunction with HSQC in detergent micelles and Dipolar Assisted Rotational Resonance (DARR) MAS NMR from ^{13}C-labelled systems in DOPC (Anderson et al. 2014). The structure and molecular mechanism of a helix-loop-helix 2TMD bacterial mercury transporter, MerF, have been investigated using orientationally restricted sample geometry and in conjunction with multidimensional heteronuclear correlation NMR (Lu et al. 2013) (Fig. 3.4).

Following the successful crystallisation of modified and conformationally stabilised human chemokine receptor CXCR1 from lipidic cubic phases, separated local field solid state NMR has been used to obtain the structure of this GPCR in lipid membranes and to highlight the IL8 binding site near the membrane surface (Park et al. 2012). More challenging proteins amenable to solid state NMR investigation include an 82 kDa bacterial beta-barrel assembly protein called BamA (Renault et al. 2011). Although complete structural characterisation remains outside the capabilities of NMR technologies due to the large size and varied segmental mobility, homology modelling template on crystal structure along with molecular dynamics offer comprehensive insights into its domain dynamics and function. In some cases, sample preparation for membrane protein crystallography can yield

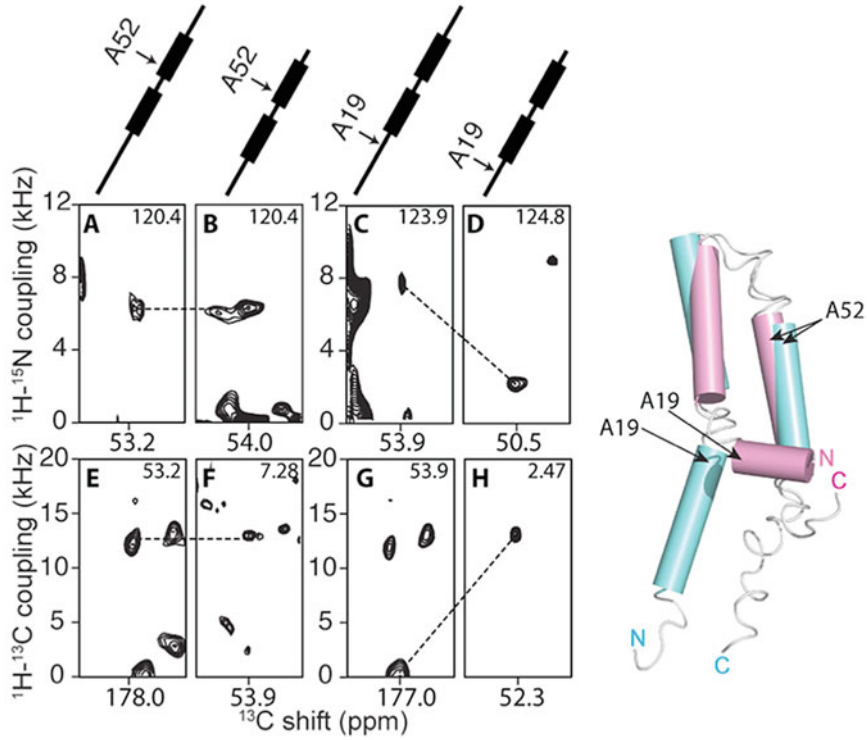

Fig. 3.4 (*Left*) Observed changes in orientationally dependent frequencies demonstrate drastic changes in the structure of MerF (PDB ref. 2 M67) caused by truncation of residues at the N-terminus. All slices are extracted from three-dimensional spectra at the noted values of the third dimension. (*Right*) The structure of the truncated 60-residue protein (magenta) is superimposed on the structure of the full-length 81-residue protein (aqua) (Lu et al. 2013)

crystals, in which segmental dynamics and insufficiently restrictive packing do not permit high resolution crystallographic characterisation. Such weakly diffracting crystals are amenable to characterisation by solid state NMR and offer superior resolution to membrane preparations due to conformational monodispersity, imposed by the crystalline order. In one example, solid state MAS NMR analysis has revealed the structure of the TMD form a Yersinial adhesin, YadA (Tang et al. 2011). Conformational changes in membrane protein ligands can drive changes in the protein functional state and solid state NMR offers high resolution insights. The effects of point mutations in proteorhodopsin on retinal binding and protein conformation have been examined by solid state MAS NMR and the associated retinal shape change, responsible for the green to blue colour shift, has been assessed by Dynamic Nuclear Polarisation (DNP) MAS NMR methods (Mao et al. 2014). The conformation of acetylcholine within the nicotinic acetylcholine receptor has been characterised using ^{13}C-labelled ligand and ligand-receptor contacts have been highlighted (Williamson et al. 2007). Using symmetry-based recoupling (Carravetta et al. 2000) the mature bacterial peptidoglycan intermediate lipid II has been investigated in its membrane complex with antibiotic nisin (Bonev 2013).

Lateral compartmentalisation in membranes allows co-localisation of proteins/targets. In studies of such laterally separated membranes, solid state MAS NMR offers insights into molecular interactions within membrane domains and lipid rafts, such as the specific recognition of raft-embedded ganglioside GM1 by the prion protein (Sanghera et al. 2011) (Fig. 3.5).

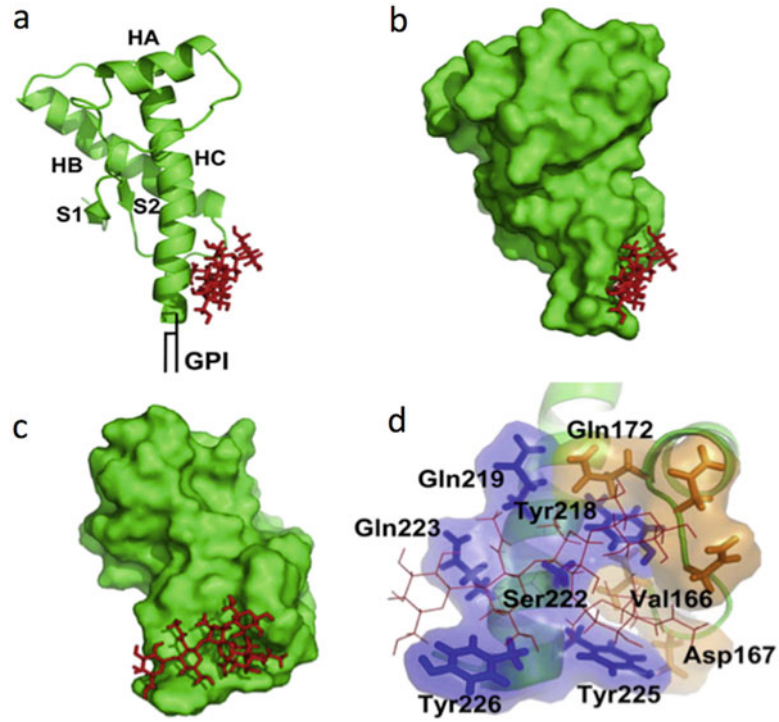

Fig. 3.5 Model of the PrP/GM1os complex: (**a**) Ribbon diagram of the structure of the PrP globular domain (*green*) showing α helices HA, HB, and HC and β sheet strands S1 and S2. The location of the glycosylphosphatidylinositol lipid anchor is highlighted in black; C-terminal end of helix C and the loop between strand S2 and helix B. (**b**) Same as (**a**) but PrP is drawn in surface representation; (**c**) Same as (**b**) but the orientation of this panel is a 90° rotation about the vertical axis of (**b**); (**d**) The putative binding site of GM1os on PrP. The region coloured in orange represents the loop region between strand S2 and helix B, and the region in blue represents the C-terminal end of helix C. The *thin red line* represents GM1os (Sanghera et al. 2011)

3.6 Technologies

Although *E. coli* expression systems have been used to produce proteins, most membrane protein samples for NMR studies have used cell-free expression systems, which can also deliver sufficient yields of protein for structural analysis. These systems rely on yeast or bacterial cell extracts that allow the target mRNA to be transcribed and translated within a reaction chamber. The cell-free approach allows specific amino acid labelling by, for example, the cell-free stereo-array isotope labeling (SAIL) method, and simplifies NMR resonance assignments and enhances resolution (Kainosho et al. 2006). The resulting stereo-specific assignments also improve the quality of the structures determined, including those of eukaryotic membrane proteins (Vinarov et al. 2006). The application of cell-free expression and selective isotope labelling to six helical transmembrane proteins yielded backbone structures using PRE restraints and chemical shift information (Klammt et al. 2012). Elucidating topologies of transmembrane helical bundles can benefit from optimal placement of a minimum number of spin labels (Chen et al. 2011).

In order to avoid the inherent problems associated with detergents, a novel method has been developed to solubilise membrane proteins directly from whole cells or native membranes using an amphipathic polymer. The addition of styrene maleic acid (SMA) results in spontaneous formation of stable bilayer discs that contain the intact protein and complexed lipids. The approach was

first demonstrated with bacteriorhodopsin and the PagP β-barrel, which retain activity and stability within the resulting 11 nm diameter discs (Knowles et al. 2009). The method has been successfully applied to a variety of mammalian ABC transporters, yielding thermally stabilized pure protein with native ligand binding properties and structures which can be rapidly characterized by cryo-EM analysis (Gulati et al. 2014). More recently, GPCRs, including the human adenosine A2A receptor, have been purified directly from Pichia pastoris and mammalian cells into SMA lipid particles (SMALPs), resulting in thermally stable proteins with physiologically relevant binding and structural properties (Jamshad et al. 2015). While the polydispersity of the synthetic polymer limits the resolution of the contained protein signals, the observation of native-like binding and stability indicates the value of these nanoparticles for studying the physiological states and interactions of membrane-associated proteins.

The utility of NMR for validating novel sites of membrane interaction discovered within protein structures was demonstrated recently (Kufareva et al. 2014). A novel software tool, MODA (for "Membrane Optimal Docking Area") accurately detects such sites, having been trained on a set of established membrane interactive proteins. This approach was validated with five proteins: the Alix Bro1 domain, Arf1 GTPase, ATF2 acetyltransferase, the von Willebrand factor A3 domain, and the Neisseria gonorrhoeae MsrB protein, which all exhibited potential sites of lipid interaction. The predictions were borne out by generating point mutations and monitoring amide chemical shift changes when micelles were added, showing that the experimentally defined and computationally predicted sites converged. This technique could significantly expand the universe of protein/lipid interaction sites, with NMR being used as the most reliable and data-rich method for confirming such lipid interactions on tractable proteins, as well as for profiling their specificities and induced structural changes. Already the MODA method has been successfully applied to MMP-12, to map its multiple membrane binding surfaces, as confirmed by NMR analysis (Koppisetti et al. 2014). Together, this indicates the general utility of NMR for identifying novel sites, structures and ligands involved in membrane interaction for even challenging systems, underscoring its importance for membrane structural biology.

The Collaborative Computing Project for NMR (CCPN) has emerged to link high throughput data and existing NMR software via common standards and has provided a forum within the community for the discussion of new methods (Fogh et al. 2002). It aims to offer a pipeline for integration of data processing from solution and solid state NMR of proteins with computational structure analysis.

Structural analysis by solid state NMR has been applied commonly to membrane-incorporated peptides and small proteins. Challenges arise from polydispersity in the sample, which leads to inhomogeneously broadening of the spectral lines, strong dipolar coupling networks leading to homogeneous broadening with both effects hindering the assignment of larger and more complex systems (Bonev 2013). In addition, larger proteins within proteoliposomal preparations are present at low molarity, which leads to comparatively low sensitivity even in isotope-enriched samples. Orientational constraints can be obtained from non-rotating aligned samples (Park et al. 2012). The use of aligned samples and microcrystalline preparations improves monodispersity, while ultrafast MAS can be used to reduce dipolar couplings. Significant sensitivity enhancement in the order of 10–100 s can be achieved by combining dynamic nuclear polarization with MAS NMR (Bajaj et al. 2007).

3.7 DNP Sensitivity Enhancement

Solid state NMR delivers high information content at atomic detail on chemically distinct molecular components but at comparatively moderate sensitivity. The application of DNP in conjunc-

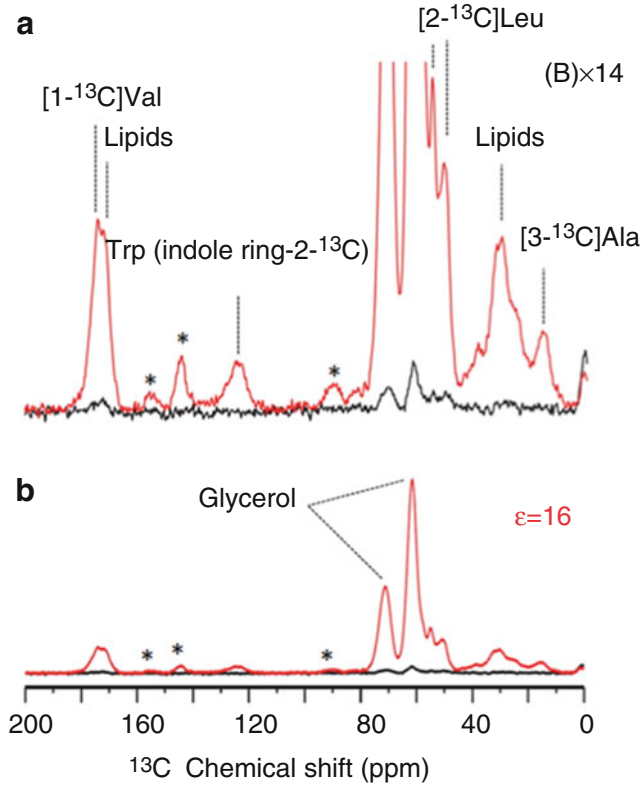

Fig. 3.6 DNP-enhanced solid-state NMR spectroscopy of E. coli cells. One-dimensional in-cell ^{13}C CPMAS NMR spectra of cytochrome b5 in E. coli cells using CPMAS with microwave irradiation on (*red*), and off (*black*); magnified (**a**) and full height (**b**) (Yamamoto et al. 2015)

tion with MAS NMR can boost sensitivity of current solid-state NMR tools by a factor ε 30–90 (Bajaj et al. 2007) and in some cases above 150, which opens the possibility of studying membrane protein structures within native membrane environments (Barbet-Massin et al. 2014) (Fig. 3.6). Photoinduced CDNP studies of intermediate states in bacterial photoreaction centre in *Heliobacter* (Thamarath et al. 2012) provide information on transition states and electron transport. The electronic structure of the electron donor of purple bacteria reaction centres has yielded atomic resolution detail from photo-CIDNP MAS NMR studies (Daviso et al. 2009). Reaction photocentre has also been studied in sulphur bacteria by DNP MAS NMR (Roy et al. 2007) and information on the electron transport and intermediate states has been obtain from photosystem II by DNP MAS NMR (Diller et al. 2007). Recent use of DNP has offered new insights into electron transport in plants (Janssen et al. 2012). A major qualitative advancement in spectroscopy, delivered through the DNP signal enhancement, is the possibility of deriving spectroscopic information from isolated membranes, cell envelopes and from intact cells, such as in studies of cyanobacteria (Janssen et al. 2010) and *E. coli* (Renault et al. 2012; Yamamoto et al. 2015). Substrate binding to multidrug resistance pump EmrE has been observed by DNP MAS NMR, allowing further analysis of the drug binding site (Ong et al. 2013). Using DNP MAS NMR, it has been possible to study protein export by the SecYEG system and to obtain two dimensional spectra from as low as 40 nmoles of bound cargo peptide (Reggie et al. 2011). And, in drug discovery, DNP signal enhancement has made it possible to observe the binding of an inhibitor to the proton transporter from influenza A (Andreas et al. 2013).

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References

Anderson TM, Clay MC, Cioffi AG, Diaz KA, Hisao GS et al (2014) Amphotericin forms an extramembranous and fungicidal sterol sponge. Nat Chem Biol 10(5):400–406

Andreas LB, Barnes AB, Corzilius B, Chou JJ, Miller EA et al (2013) Dynamic nuclear polarization study of inhibitor binding to the M2(18–60) proton transporter from influenza A. Biochemistry 52(16): 2774–2782

Bajaj VS, Hornstein MK, Kreischer KE, Sirigiri JR, Woskov PP et al (2007) 250 GHz CW gyrotron oscillator for dynamic nuclear polarization in biological solid state NMR. J Magn Reson 189(2): 251–279

Barbet-Massin E, Pell AJ, Retel JS, Andreas LB, Jaudzems K, Franks WT, Nieuwkoop AJ, Hiller M, Higman V et al (2014) Rapid proton-detected NMR assignment for proteins with fast magic angle spinning. J Am Chem Soc 136(35):12489–12497

Berardi MJ, Shih WM, Harrison SC, Chou JJ (2011) Mitochondrial uncoupling protein 2 structure determined by NMR molecular fragment searching. Nature 476(7358):109–113

Bonev BB (2013) High-resolution solid-state NMR of lipid membranes. Adv Planar Lipid Bilayers Liposomes 17:299–329

Butterwick JA, MacKinnon R (2010) Solution structure and phospholipid interactions of the isolated voltage-sensor domain from KvAP. J Mol Biol 403(4):591–606

Carravetta M, Eden M, Zhao X, Brinkmann A, Levitt MH (2000) Symmetry principles for the design of radiofrequency pulse sequences in the nuclear magnetic resonance of rotating solids. Chem Phys Lett 321(3–4):205–215

Chen H, Ji F, Olman V, Charles KM, Liu Y et al (2011) Optimal mutation sites for PRE data collection and membrane protein structure prediction. Structure 19(4):484–495

Daviso E, Prakash S, Alia A, Gast P, Neugebauer J, Jeschke G, Matysik J (2009) The electronic structure of the primary electron donor of reaction centers of purple bacteria at atomic resolution as observed by photo-CIDNP C-13 NMR. Proc Natl Acad Sci U S A 106(52):22281–22286

Diller A, Roy E, Gast P, van Gorkom HJ, de Groot HJM et al (2007) N-15 photochemically induced dynamic nuclear polarization magic-angle spinning NMR analysis of the electron donor of photosystem II. Proc Natl Acad Sci U S A 104(31):12767–12771

Dominguez C, Boelens R, Bonvin A (2003) HADDOCK: a protein-protein docking approach based on biochemical or biophysical information. J Am Chem Soc 125:1731–1737

Eichmann C, Tzitzilonis C, Bordignon E, Maslennikov I, Choe S, Riek R (2014) Solution NMR structure and functional analysis of the integral membrane protein YgaP from Escherichia coli. J Biol Chem 289(34):23482–23503

Fan Y, Shi L, Ladizhansky V, Brown L (2011) Uniform isotope labeling of a eukaryotic seven-transmembrane helical protein in yeast enables high-resolution solid-state NMR studies in the lipid environment. J Biomol NMR 49(2):151–161

Fogh R, Ionides J, Ulrich E, Boucher W, Vranken W, Linge JP et al (2002) The CCPN project: an interim report on a data model for the NMR community. Nat Struct Mol Biol 9(6):416–418

Gautier A, Nietlispach D (2012) Solution NMR studies of integral polytopic α-helical membrane proteins: the structure determination of the seven-helix transmembrane receptor sensory rhodopsin II, pSRII. In: Membrane protein structure and dynamics. Humana Press, New York, pp 25–45

Gautier A, Mott HR, Bostock MJ, Kirkpatrick JP, Nietlispach D (2010) Structure determination of the seven-helical transmembrane receptor sensory rhodopsin II by solution NMR spectroscopy. Nat Struct Mol Biol 17(6):768–774

Gulati S, Jamshad M, Knowles TJ, Morrison KA, Downing R et al (2014) Detergent-free purification of ABC (ATP-binding-cassette) transporters. Biochem J 461(2):269–278

Haeberlen U, Waugh JS (1968) Coherent averaging effects in magnetic resonance. Phys Rev 175(2): 453–467

Hagn F, Etzkorn M, Raschle T, Wagner G (2013) Optimized phospholipid bilayer nanodiscs facilitate high-resolution structure determination of membrane proteins. J Am Chem Soc 135(5): 1919–1925

Hiller S, Garces RG, Malia TJ, Orekhov VY, Colombini M, Wagner G (2008) Solution structure of the integral human membrane protein VDAC-1 in detergent micelles. Science 321(5893):1206–1210

Horst R, Stanczak P, Serrano P, Wüthrich K (2012) Translational diffusion measurements by micro-coil NMR in aqueous solutions of the Fos-10 detergent-solubilized membrane protein OmpX. J Phys Chem B 116(23):6775–6780

Jamshad M, Charlton J, Lin Y-P, Routledge SJ, Bawa Z et al (2015) G-protein coupled receptor solubilization and purification for biophysical analysis and functional studies, in the total absence of detergent. Biosci Rep 35(2):e00188

Janssen GJ, Daviso E, van Son M, de Groot HJM, Alia A, Matysik J (2010) Observation of the solid-state photo-CIDNP effect in entire cells of cyanobacteria Synechocystis. Photosynth Res 104(2–3):275–282

Janssen GJ, Roy E, Matysik J, Alia A (2012) N-15 photo-CIDNP MAS NMR to reveal functional heterogeneity in electron donor of different plant organisms. Appl Magn Reson 42(1):57–67

Kainosho M, Torizawa T, Iwashita Y, Terauchi T, Ono AM, Güntert P (2006) Optimal isotope labelling for NMR protein structure determinations. Nature 440(7080):52–57

Klammt C, Maslennikov I, Bayrhuber M, Eichmann C, Vajpai N et al (2012) Facile backbone structure determination of human membrane proteins by NMR spectroscopy. Nat Methods 9(8):834–839

Knowles TJ, Finka R, Smith C, Lin Y-P, Dafforn T, Overduin M (2009) Membrane proteins solubilized intact in lipid containing nanoparticles bounded by styrene maleic acid copolymer. J Am Chem Soc 131(22):7484–7485

Kofuku Y, Ueda T, Okude J, Shiraishi Y, Kondo K, Maeda M, Tsujishita H, Shimada I (2012) Efficacy of the β(2)-adrenergic receptor is determined by conformational equilibrium in the transmembrane region. Nat Commun 3:1045

Koppisetti RK, Fulcher YG, Jurkevich A, Prior SH, Xu J et al (2014) Ambidextrous binding of cell and membrane bilayers by soluble matrix metalloproteinase-12. Nat Commun 5:5552

Kufareva I, Lenoir M, Dancea F, Sridhar P, Raush E et al (2014) Discovery of novel membrane binding structures and functions. Biochem Cell Biol 92(6):555–563

Lemmon MA (2008) Membrane recognition by phospholipid-binding domains. Nat Rev Mol Cell Biol 9:99–111

Lenoir M, Grzybek M, Majkowski M, Rajesh S, Kaur J et al (2015a) Structural basis of dynamic membrane recognition by trans-Golgi network specific FAPP proteins. J Mol Biol 427(4):966–981

Lenoir M, Kufareva I, Abagyan R, Overduin M (2015b) Membrane and protein interactions of the pleckstrin homology domain superfamily. Membranes (Basel) 5(4):646–663

Liu W, Chun E, Thompson AA, Chubukov P, Xu F, Katritch V et al (2012) Structural basis for allosteric regulation of GPCRs by sodium ions. Science 337(6091):232–236

Lu GJ, Tian Y, Vora N, Marassi FM, Opella SJ (2013) The structure of the mercury transporter MerF in phospholipid bilayers: a large conformational rearrangement results from N-terminal truncation. J Am Chem Soc 135(25):9299–9302

Madono M, Sawasaki T, Morishita R, Endo Y (2011) Wheat germ cell-free protein production system for post-genomic research. Nat Biotechnol 28(3):211–217

Mao J, Do NN, Scholz F, Reggie L, Mehler M et al (2014) Structural basis of the green-blue color switching in proteorhodopsin as determined by NMR spectroscopy. J Am Chem Soc 136(50):17578–17590

Mowrey D, Cui T, Jia Y, Ma D, Makhov AM et al (2013) Open-channel structures of the human glycine receptor α1 full-length transmembrane domain. Structure 21(10):1897–1904

Ong YS, Lakatos A, Becker-Baldus J, Pos KM, Glaubitz C (2013) Detecting substrates bound to the secondary multidrug efflux pump EmrE by DNP-enhanced solid-state NMR. J Am Chem Soc 135(42):15754–15762

Opella SJ (2013) Structure determination of membrane proteins in their native phospholipid bilayer environment by rotationally aligned solid-state NMR spectroscopy. Acc Chem Res 46(9):2145–2153

Overduin M, Cheever ML (2001) Signaling with phosphoinositides: better than binary. Mol Interv 3:10

Park SH, Das BB, Casagrande F, Tian Y, Nothnagel HJ, Chu M et al (2012) Structure of the chemokine receptor CXCR1 in phospholipid bilayers. Nature 491(7426):779–783

Reckel S, Gottstein D, Stehle J, Löhr F, Verhoefen MK et al (2011) Solution NMR structure of proteorhodopsin. Angew Chem Int Ed 50(50):11942–11946

Reggie L, Lopez JJ, Collinson I, Glaubitz C, Lorch M (2011) Dynamic nuclear polarization-enhanced solid-state NMR of a C-13-labeled signal peptide bound to lipid-reconstituted sec translocon. J Am Chem Soc 133(47):19084–19086

Renault M, Bos MP, Tommassen J, Baldus M (2011) Solid-state NMR on a large multidomain integral membrane protein: the outer membrane protein assembly factor BamA. J Am Chem Soc 133(12):4175–4177

Renault M, Pawsey S, Bos MP, Koers EJ et al (2012) Solid-state NMR spectroscopy on cellular preparations enhanced by dynamic nuclear polarization. Angew Chem Int Ed 51(12):2998–3001

Rout AK, Strub M-P, Piszczek G, Tjandra N (2014) Structure of transmembrane domain of lysosome-associated membrane protein type 2a (LAMP-2A) reveals key features for substrate specificity in chaperone-mediated autophagy. J Biol Chem 289(51):35111–35123

Roy E, Gast P, van Gorkom H, de Groot HJ (2007) Photochemically induced dynamic nuclear polarization in the reaction center of the green sulphur bacterium chlorobium tepidum observed by (13)C MAS NMR. Biochim Biophys Acta -Bioenergetics 1767(6):610–615

Sanghera N, Correia BE, Correia JR, Ludwig C et al (2011) Deciphering the molecular details for the binding of the prion protein to main ganglioside GM1 of neuronal membranes. Chem Biol 18(11):1422–1431

Shahid SA, Bardiaux B, Franks WT, Krabben L, Habeck M et al (2012) Membrane-protein structure determination by solid-state NMR spectroscopy of microcrystals. Nat Methods 9:1212–1217

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387(6633):569–572

Sobhanifar S, Reckel S, Junge F, Schwarz D, Kai L et al (2010) Cell-free expression and stable isotope labelling strategies for membrane proteins. J Biomol NMR 46(1):33–43

Sounier R, Mas C, Steyaert J, Laeremans T, Manglik A et al (2015) Propagation of conformational changes during [mgr]-opioid receptor activation. Nature 524(7565):375–378

Tang M, Sperling LJ, Berthold DA, Schwieters CD, Nesbitt AE et al (2011) High-resolution membrane protein structure by joint calculations with solid-state NMR and X-ray experimental data. J Biomol NMR 51(3):227–233

Thamarath SS, Alia A, Daviso E, Mance D, Golbeck JH et al (2012) Whole cell nuclear magnetic resonance

characterization of two photochemically active states of the photosynthetic reaction center in heliobacteria. Biochemistry 51(29):5763–5773

Van Horn W, Ogilvie M, Flynn P (2008) Use of reverse micelles in membrane protein structural biology. J Biomol NMR 40(3):203–211

Van Horn WD, Kim HJ, Ellis CD, Hadziselimovic A et al (2009) Solution NMR structure of membrane-integral diacylglycerol kinase. Science 324(5935):1726–1729

Vinarov DA, Newman CLL, Markley JL (2006) Wheat germ cell-free platform for eukaryotic protein production. FEBS J 273(18):4160–4169

Vostrikov VV, Mote KR, Verardi R, Veglia G (2013) Structural dynamics and topology of phosphorylated phospholamban homopentamer reveal its role in the regulation of calcium transport. Structure 21(12):2119–2130

Warschawski DE, Arnold AA, Beaugrand M, Gravel A, Chartrand E, Marcotte I (2011) Choosing membrane mimetics for NMR structural studies of transmembrane proteins. Biochim Biophys Acta-Biomembranes 1808(8):1957–1974

Williamson PTF, Verhoeven A, Miller KW, Meier BH, Watts A (2007) The conformation of acetylcholine at its target site in the membrane-embedded nicotinic acetylcholine receptor. Proc Natl Acad Sci U S A 104(46):18031–18036

Wu CH, Ramamoorthy A, Opella SJ (1994) High-resolution heteronuclear dipolar solid state NMR spectroscopy. J Magn Reson A 109(2):270–272

Yamamoto K, Caporini MA, Im S-C, Waskell L, Ramamoorthy A (2015) Cellular solid-state NMR investigation of a membrane protein using dynamic nuclear polarization. Biochim Biophys Acta-Biomembranes 1848(1):342–349

Zech SG, Olejniczak E, Hajduk P, Mack J, McDermot AE (2004) Characterization of protein-ligand interactions by high-resolution solid-state NMR spectroscopy. J Am Chem Soc 126(43):13948–13953

Zhou Y, Cierpicki T, Jimenez RHF, Lukasik SM, Ellena JF et al (2008) NMR solution structure of the integral membrane enzyme DsbB: functional insights into DsbB-catalyzed disulfide bond formation. Mol Cell 31(6):896–908