Published in final edited form as:

Methods. 2007 April ; 41(4): 398–408. doi:10.1016/j.ymeth.2006.08.011.

NMR of membrane proteins in micelles and bilayers: The FXYD family proteins

Carla M. Franzin, Xiao-Min Gong, Khang Thai, Jinghua Yu, and Francesca M. Marassi*

The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, USA

Abstract

Determining the atomic resolution structures of membrane proteins is of particular interest in contemporary structural biology. Helical membrane proteins constitute one-third of the expressed proteins encoded in a genome, many drugs have membrane-bound proteins as their receptors, and mutations in membrane proteins result in human diseases. Although integral membrane proteins provide daunting technical challenges for all methods of protein structure determination, nuclear magnetic resonance (NMR) spectroscopy can be an extremely versatile and powerful method for determining their structures and characterizing their dynamics, in lipid environments that closely mimic the cell membranes. Once milligram amounts of isotopically labeled protein are expressed and purified, micelle samples can be prepared for solution NMR analysis, and lipid bilayer samples can be prepared for solid-state NMR analysis. The two approaches are complementary and can provide detailed structural and dynamic information. This paper describes the steps for membrane protein structure determination using solution and solid-state NMR. The methods for protein expression and purification, sample preparation and NMR experiments are described and illustrated with examples from the FXYD proteins, a family of regulatory subunits of the Na, K-ATPase.

Keywords

NMR; Lipid; Micelle; Bilayer membrane; Protein; Expression; Structure; FXYD

1. Introduction

Helical integral membrane proteins constitute approximately one third of all expressed genes, and are major regulators of the most basic cellular functions as well as major therapeutic targets [1,2]. However, despite their prevalence and importance, the examples of membrane proteins whose structures have been determined with atomic resolution are exceptional, and only hundreds of structures have been deposited in the Protein Data Bank (PDB; www.rcsb.org/pdb) compared to the tens of thousands deposited for globular proteins to date. This disproportion reflects the difficulties associated with the over-expression, purification, and crystallization of lipophilic membrane proteins, whose structures and functions are stabilized by their association with the lipid bilayer membrane. Furthermore, since the physical interactions of the lipid bilayer with membrane proteins are often more important in determining protein stability and fold than the binding interactions with specific lipids, it is desirable to determine protein structures within the lipid bilayer environment. Nuclear magnetic resonance (NMR) spectroscopy is very well suited for this goal, since it can be applied to molecules in all physical states, including liquid crystalline bilayers and

*Corresponding author. Fax: +1 858 713 6268., fmarassi@burnham.org (F.M. Marassi).

micelles formed by the lipids that associate with membrane proteins. Solution NMR methods can be used on samples of proteins in lipid micelles, while solid-state NMR methods can be applied to samples of membrane proteins in lipid bilayers, enabling structures to be determined in a native-like environment. The two approaches are complementary, and can be used in combination to obtain detailed descriptions of the structures and dynamics of membrane proteins.

Recent developments in bacterial expression systems for the preparation of recombinant isotopically labeled membrane proteins, methods for sample preparation, pulse sequences for high-resolution spectroscopy, and structural indices that guide the structure assembly process, have greatly extended the capabilities of these techniques, and the structures of a variety of helical membrane proteins have been determined by NMR in micelles and in bilayers [3–11].

High-quality solution NMR spectra can be obtained for some fairly large membrane proteins in micelles. However, for helical membrane proteins the range of measurements that can be made is limited compared to what is possible with samples of soluble globular proteins, because of the severe line broadening which results from the slow molecular tumbling of the protein associated with the micelle. Typically, it is very difficult to measure and assign a sufficient number of long-range NOE (nuclear Overhauser effect) distance constraints to determine protein folds, however, this limitation can be overcome by preparing weakly aligned micelle samples for the measurement of residual dipolar couplings (RDCs) and residual chemical shift anisotropies (RCSAs) that provide key orientation constraints for structure determination. Beta-barrels, the second major class of membrane proteins, have been more successfully studied in micelles, since their compact structures lend themselves to the applications of transverse relaxation optimized spectroscopy (TROSY) [12] pulse sequences for improved resolution and to NOE-based methods of structure determination [13–15].

High-resolution solid-state NMR spectra can be obtained for membrane proteins that are expressed, isotopically labeled, and reconstituted in uniaxially oriented planar lipid bilayers. The spectra have frequencies that reflect the orientation of their respective protein sites relative to the direction of the magnetic field, and, since the lipid bilayer plane is perpendicular to the magnetic field direction, each resonance frequency reflects the orientation of its corresponding protein site in the membrane [16]. In addition, the spectra display characteristic patterns of peaks that directly reflect protein structure and topology. This direct relationship between spectrum and structure provides the basis for methods that enable the simultaneous sequential assignment of resonances and the measurement of orientation restraints for protein structure determination [6].

In this paper, the methods are described and illustrated with examples from the FXYD proteins, a family of tissue-specific and physiological-state-specific subunits of the Na,K-ATPase, that help to regulate the distribution of Na and K ion concentrations across animal cell membranes [17–19]. The FXYD protein sequences are highly conserved through evolution, and are characterized by a 35-amino acid FXYD homology domain, which includes the short signature motif PFXDYD (Pro, Phe, X, Tyr, Asp) and the transmembrane domain (Fig. 1C). Conserved basic residues flank the transmembrane domain, the extracellular N-termini are acidic, and the cytoplasmic C-termini are basic.

FXYD1 (PLM; phospholemman) is the principal substrate of hormone-stimulated phosphorylation by cAMP-dependent protein kinase A and C in heart sarcolemma [20]. FXYD2 (γ) and FXYD4 (CHIF; channel-inducing factor; corticosteroid hormone induced factor), are each expressed in distinct, specialized segments of the kidney, with

unique expression patterns that help explain the physiological differences in Na,K-ATPase activity among the nephron segments [21–25]. FXYD3 (Mat8; mammary tumor protein 8 kDa) is expressed in cancers and plays a role in tumor progression [26,27]. Despite their relatively small sizes ranging from about 60–160 amino acids, all FXYD proteins are encoded by genes with six to nine small exons, and NMR has shown that the protein structures reflect the structures of their corresponding genes, suggesting that they were assembled from modules through exon shuffling [28].

2. Materials

The pBCL plasmids for protein expression (Fig. 1) were developed in our laboratory and are available upon request. The *Escherichia coli* C41(DE3) cells for protein over-expression were developed by Miroux and Walker [29], and were obtained from Avidis (www.overexpress.com). All phospholipids were from Avanti Polar Lipids (www.avantilipids.com), octyl-glucopyranoside (OG) was from Fluka (www.sigmaaldrich.com), and deuterated sodium-dodecyl-sulfate (SDS) and dodecyl-phosphocholine (DPC) were from Cambridge Isotopes Laboratories (www.isotope.com). Isotopically labeled salts, sugar, amino acids, and D₂O, used to produce ¹⁵N-, ¹³C-, and ²H-labeled proteins by bacterial expression were from Cambridge Isotopes Laboratories (www.isotope.com). Ion exchange and size exclusion chromatography were performed with FF-S and S-200HR columns and an AKTA-prime chromatography system, all from Amersham (www.amershambiosciences.com). Reverse-phase HPLC (high-performance liquid chromatography) was performed with a Delta-Pak C4 column using a Breeze HPLC system, all from Waters (www.waters.com). Glass slides (11 × 11 mm or 11 × 20 mm) for oriented solid-state NMR experiments were purchased from Paul Marienfeld (www.superior.de). They are 0.06–0.08 mm thick, and may be used directly after washing in detergent and rinsing in distilled water.

Solution NMR experiments were performed on a Bruker AVANCE 600 MHz spectrometer using a triple-resonance ¹H/¹³C/¹⁵N probe equipped with three-axis pulsed field gradients (www.bruker-biospin.com). Solid-state NMR experiments were performed on a Bruker AVANCE 500 MHz (www.bruker-biospin.com) spectrometer with a wide-bore 500/89 Magnex magnet (www.magnex.com). The double-resonance (¹H/¹⁵N or ¹H/³¹P) probes with square radiofrequency coils wrapped directly around the samples were built at the UC San Diego NIH Resource for Molecular Imaging of Proteins (nmrresource.ucsd.edu). The NMR data were processed using NMRPipe [30], and the spectra were assigned and analyzed using Sparky [31].

3. Methods

3.1. Protein expression and purification

3.1.1. The pBCL plasmid—The FXYD proteins PLM, gamma, Mat-8, and CHIF, were expressed using the pBCL plasmid vector, which was developed for the large-scale expression of membrane proteins [32]. This plasmid directs the expression of a target polypeptide fused to the C-terminus of a mutant form of the anti-apoptotic protein Bcl-XL, where the hydrophobic C-terminus has been deleted, to be replaced with a hydrophobic polypeptide gene of interest by insertion at an engineered *Afl*/*III*/*Xho*I cloning site, and Met residues have been mutated to Leu to facilitate CNBr cleavage after a single Met inserted at the beginning of the target sequence (Fig. 1A and B). In cases where the target protein contains Met residues that cannot be mutated, separation from the fusion partner can be obtained by introducing amino acid sequences specific for cleavage by other chemical means, such as hydroxylamine (Asn-Gly), or for cleavage by one of the commonly used proteases: thrombin, factor Xa, entero-kinase, and tobacco etch virus protease. Chemical

cleavage is an attractive option because it eliminates the difficulties—poor specificity and enzyme inactivation—often encountered with protease treatment of hydrophobic proteins in detergents. The plasmid utilizes a T7 expression system [33], and the fusion tag has an N-terminal (His)₆ sequence for protein purification by Ni-affinity chromatography.

Fig. 1D compares the expression levels of PLM obtained using two variants of the pBCL plasmid, with those obtained using the Trp Δ LE (pTLE) [34–36], or the ketosteroid isomerase (pKSI) [37] fusion protein expression systems. pBCL is clearly superior for the expression of PLM, and it enabled us to obtain milligram quantities of pure, isotopically labeled protein easily and quickly. Our experience further suggests that pBCL is also useful for the expression of other membrane proteins with multiple transmembrane helices. After cleavage from the fusion partner, highly pure FXYD proteins were obtained using a combination of Ni-affinity, ion exchange, size exclusion, and reverse-phase chromatography, with yields in the range of 10 mg of purified protein per liter of culture in M9 minimal medium (Fig. 1E). The cloning procedure has been described in detail in reference [32].

3.1.2. Protein expression—For protein expression, 5–10 μ L of transformed C41(DE3) cells from a frozen glycerol stock, were used to inoculate 10 mL of LB media and grown for 5 h at 37 °C with vigorous shaking, then, 1 mL of this starter culture was added to 100 mL of minimal M9 media and grown overnight. All media contained 100 μ g/mL of ampicillin. In the morning, 1 L of fresh M9 media was inoculated with the overnight culture, and the cells were grown to a cell density of OD₆₀₀ = 0.7. Protein expression was induced by the addition of 1 mM IPTG for 4 h at 37 °C. The cells were subsequently harvested by centrifugation and stored at –20 °C overnight. For uniformly ¹⁵N- and ¹³C-labeled proteins, (¹⁵NH₄)₂SO₄ and ¹³C-labeled glucose were supplied to the M9 salts. ²H-labeled proteins were obtained by growing the bacteria in M9 media dissolved in D₂O, and selectively ¹⁵N-labeled samples were obtained by supplying individual ¹⁵N-labeled amino acids to the media. SDS–PAGE was performed with the Tris–tricine system [38], and gels were stained with Coomassie blue G250.

3.1.3. Protein purification—Frozen cells from 1 L of culture were lysed by French press in 30 mL of buffer A (50 mM Tris–HCl, pH 8.0, 15% glycerol). The soluble fraction was removed by centrifugation (48,000g, 4 °C, 30 min), and the pellet was washed twice by resuspension in 30 mL of buffer A, followed by centrifugation (48,000g, 4 °C, 30 min) to remove the soluble fraction. The resulting pellet was dissolved in 30 mL of 6 M guanidinium hydrochloride (GdnHCl), and again centrifuged (48,000g, 4 °C, 2 h) to remove any insoluble materials. If needed, the fusion protein can be purified by Ni-affinity chromatography in GdnHCl before CNBr cleavage.

For CNBr cleavage, the 6 M GdnHCl protein solution was adjusted to 0.1 N HCl (pH 0.2), a 100-fold molar excess of solid CNBr was added, and the mixture was allowed to react overnight, in the dark, at room temperature. In the morning, the reaction mixture was dialyzed against water until the pH reached about 5.0 (6 h with several changes of 4 L of water, in a dialysis membrane with molecular weight cutoff of 1 kDa), lyophilized to powder, and dissolved in buffer B (20 mM Tris–HCl, pH 7.0, 8 M urea).

The FXYD proteins were purified by ion exchange chromatography with a NaCl gradient (FF-S column), followed by exchange into buffer C (20 mM Tris–HCl, pH 7.0, 4 mM SDS), and preparative reverse-phase HPLC with a gradient of acetonitrile in water and 0.1% trifluoroacetic acid. Alternatively, after CNBr cleavage the proteins can be purified by size exclusion chromatography in buffer C, followed by reverse-phase HPLC. Purified proteins were stored as lyophilized powder at –20 °C.

3.2. Solution NMR structural studies in micelles

3.2.1. Solution NMR experiments—All NMR experiments were performed at 40 °C using a 1 s recycle delay. The chemical shifts were referenced to the $^1\text{H}_2\text{O}$ resonance, set to its expected position of 4.5999 ppm at 40 °C [39]. The standard fHSQC (fast heteronuclear single quantum correlation) experiment was used for isotropic samples with 1024 points in t_2 and 256 in t_1 [40]. Backbone resonance assignments were made using a standard HNCA experiment with constant time evolution for ^{15}N , and solvent suppression was accomplished with a water flip-back pulse after the original ^1H – ^{15}N magnetization transfer [41–43]. For the FXYD proteins, the spectra from selectively ^{15}N -labeled protein samples were necessary to resolve assignment ambiguities due to the extensive overlap among the Ca resonances that is typical of helical membrane proteins in micelles.

^1H – ^{15}N heteronuclear NOE measurements were made using difference experiments with and without 3 s of saturation of the ^1H resonances between scans [44]. Hydrogen deuterium (HD) exchange experiments were performed by dissolving the lyophilized protein in SDS buffer with 100% D_2O , and then acquiring HSQC spectra at 0.5 h time intervals.

For the measurement of RDCs, the protein–SDS micelles were weakly aligned in 7% polyacrylamide gels, using either vertical compression [45,46] or expansion [47]. The ^1H – ^{15}N RDCs were measured using a sensitivity-enhanced ^1H – ^{15}N IPAP (in-phase-anti-phase) experiment modified for suppression of the NH_2 signals from the acryl-amide in the gel [46,48,49]. The contribution to the residual dipolar coupling splitting from the isotropic scalar coupling was determined by performing the same experiment on an isotropic micelle sample, and subtracting the value of the isotropic J -coupling obtained from that measured for the weakly aligned gel sample. The methods for the measurement and analysis of RDCs are described in detail in two recent review articles [50,51].

3.2.2. Detergent selection—The first step in solution NMR structural studies of membrane proteins is to find a suitable detergent that yields high-resolution spectra and supports protein structure and function. With the increasing level of activity in the field of solution NMR of membrane proteins, many new suggestions for lipids and combinations of lipids are emerging [52,53]. Since the key solution NMR measurements are performed with samples that are weakly aligned in polyacryl-amide gels, it is also necessary to simultaneously optimize the conditions for the various gel samples.

For the FXYD proteins, we examined the $^1\text{H}/^{15}\text{N}$ HSQC spectra of the proteins in the best characterized micelle-forming detergents: DPC (dodecyl-phosphocholine), DHPC (di-heptanoyl-phosphocholine), LPPG (lyso-palmitoyl-phosphoglycerol), OG (octyl-glucopyranoside), SDS (sodium-dodecyl-sulfate), at various conditions (protein, detergent, and salt concentration; pH; temperature) (Fig. 2A). The highest quality spectra were obtained in SDS micelles at 40 °C, by dissolving each lyophilized FXYD protein in NMR buffer (20 mM sodium citrate, pH 5, 10 mM DTT, and 1 mM sodium azide, 90% H_2O , 10% D_2O) plus 500 mM SDS (Fig. 2B). Focus, for example, on the resonances corresponding to the Gly residues for each protein, in the region between 105 and 110 ppm in ^{15}N chemical shift (Fig. 2B): the peaks have uniform intensities and are well dispersed, indicative of well-behaved samples.

Although SDS is widely assumed to be a universal protein-denaturant because of its common use in electrophoresis of globular proteins, many membrane proteins retain their three-dimensional and oligomeric structures in SDS micelles [54]. Notable recent examples include a homotetrameric bacterial potassium channel, KcsA [55], and a bacterial mercury transport protein MerF [11]. Furthermore, since SDS purification of the Na,K-ATPase maintains the non-covalent associations of α , β , and FXYD subunits, and yields highly

functional enzyme [56–58], we reasoned that this detergent would be also be a good choice for FXYP structural studies.

The HSQC spectra show that PLM, gamma, Mat-8, and CHIF adopt unique folded structures in SDS. These spectra are highly sensitive monitors of the entire expression, purification, and sample preparation process. The 1.5 ppm dispersion of the amide ^1H chemical shifts is typical of native helical membrane proteins in micelles. Any doubling or anomalous broadening of resonances are warning signs that the polypeptide is aggregated or improperly folded. Resolved HSQC spectra like those in Fig. 2 set the stage for structural studies by solution NMR, and provide assurance that it is worthwhile to proceed with the preparation of bilayer samples for solid-state NMR experiments.

3.2.3. H/D exchange—The comparison of HSQC spectra of samples in H_2O and D_2O solutions gives a useful view of the topology of membrane proteins in micelles, by identifying the subset of the most stable helical residues. For the FXYP proteins, the HSQC spectra obtained in D_2O revealed a core region in each protein, with amide protons that exchange very slowly with the surrounding aqueous solvent (Fig. 3B). These regions match the hydrophobic transmembrane segments of the proteins identified by the hydropathy plots (Fig. 3A), and reflect the strong intra-molecular hydrogen bonds present in transmembrane helices in the low dielectric environment of the membrane, or, in this case, the micelle interior.

3.2.4. Residual dipolar couplings and chemical shifts—To determine the protein secondary structures of the FXYP proteins we relied primarily on ^1H – ^{15}N RDCs measured from weakly aligned samples and analyzed in terms of dipolar waves [59] (Fig. 3C). These were supplemented with chemical shifts, analyzed in terms of CSI (chemical shift indices) [60] (Fig. 3D), and with TALOS [61]. Dipolar waves, obtained by plotting the magnitudes of the RDCs as a function of residue number, and fitting the plot to a sine wave with a period of 3.6 residues, are highly effective at identifying the helical residues and the relative orientations of the helical segments. The quality of fit is monitored by a scoring function in a four-residue sliding window and the phase of the fit. Alignment-induced changes in chemical shift frequencies can provide complementary conformational constraints.

The RDCs were analyzed using MATLAB scripts as described [59,62,63]. Helical regions were identified by applying a sliding window algorithm to fit the experimental RDCs. The RDCs within a five-residue window were fit to a sinusoid of periodicity 3.6, and the RMSD between the sinusoid and the data were plotted as a function of residue number. Continuous stretches of amino acids with low RMSD (less than the experimental error of 1.5 Hz) were identified as helices and fitted to a single sinusoid. Higher RMSDs were generally interpreted as deviations from ideality, including kinks, curvature, and loops. This analysis relating the orientation of the helix to the amplitude, average value, and phase of the sinusoid is an initial step toward structure determination, and can determine the relative orientations of helices to within four degenerate solutions [50,51,62,64]. Furthermore, the membrane or micelle geometries impose a constraint that can be very important for selecting the correct solution among the relative orientation possibilities.

3.2.5. Backbone dynamics—The protein backbone dynamics were characterized with measurements of the heteronuclear ^1H – ^{15}N NOE and of the resonance intensities. Within the core transmembrane helical regions of the proteins, all residues have similar positive values of ^1H – ^{15}N NOE, reflecting similar rotational correlation times, and indicating that the helices are rigidly connected (Fig. 3E). Lower negative values of the ^1H – ^{15}N NOE, reflecting additional backbone motions, are present in residues near the N- and C-termini, and at the helix boundaries. Furthermore, all of the resonances from amino acids within the

transmembrane central helical regions of the three proteins have similar peak intensities that plateau at minimum values (Fig. 3F), indicating that the helices are rigidly connected. In contrast, residues at the boundaries of the transmembrane domain, and at the terminal regions of the proteins have greater intensities reflecting narrower line widths that result from the increased dynamics in these regions.

3.2.6. Interaction of the protein with the micelle—To probe the interactions between membrane proteins and micelles, it is useful to examine the effect of MnCl_2 on their $^1\text{H}/^{15}\text{N}$ HSQC spectra. Mn^{2+} ions are excluded from the hydrophobic interior of the micelle, and their paramagnetic electrons induce distance-dependent broadening of the peaks from protein sites that are solvent-exposed or near the aqueous environment, while residues in the micelle, such as those in transmembrane helices, are unaffected.

To examine the micelle insertion of PLM and Mat-8, we monitored the MnCl_2 -induced line broadening by measuring the $^1\text{H}/^{15}\text{N}$ peak intensities in the s spectrum of each protein dissolved in SDS, before and after the addition of 0.5, 0.8, and 1.6 mM MnCl_2 . The addition of MnCl_2 to PLM in micelles resulted in substantial line broadening and the disappearance of the HSQC peaks from amino acids in helix 1, in the flexible connecting segment between helices 3 and 4, and in the N- and C-terminal regions (Fig. 4). In contrast, peaks from residues in the transmembrane helix (helix 2), and in helices 3 and 4, retained significant intensity. This result indicates that helices 3 and 4 of PLM are also tightly associated with the micelle, and is consistent with the solid-state ^{15}N NMR spectra of PLM in lipid bilayers, which indicate the presence of helical segments associated with the membrane surface. A similar profile is observed for Mat-8, although in this case protection from MnCl_2 extends over the four helices. In addition to Mn, paramagnetic oxygen has been developed by Prosser and coworkers to probe the interaction of membrane proteins with micelles or membranes [65,66].

3.3. Solid-state NMR structural studies in lipid bilayers

3.3.1. Solid-state NMR experiments—All NMR experiments were performed at 23 °C using a recycle delay of 6 s. The ^{15}N and ^{31}P chemical shifts were referenced to 0 ppm for liquid ammonia and phosphoric acid. The ^{15}N spectra were obtained with single contact 1 ms CPMOIST (cross polarization with mismatch-optimized IS polarization transfer) [67,68], and the ^{31}P spectra with a single pulse. Both were acquired with continuous ^1H irradiation (rf field strength 63 kHz) to decouple the ^1H - ^{15}N and ^1H - ^{31}P dipolar interactions.

The PISEMA (polarization exchange with exchange at the magic angle) experiment gives high-resolution, two-dimensional, ^1H - ^{15}N dipolar coupling, and ^{15}N chemical shift correlation spectra of oriented membrane proteins where the individual resonances contain orientation restraints for structure determination [69]. The spectra were obtained with a cross polarization contact time of 1 ms, a ^1H 90° pulse width of 5 μs , and continuous ^1H decoupling of 63 kHz rf field strength. The two-dimensional data were acquired with 512 accumulated transients and 256 complex data points, for each of 64 real t_1 values incremented by 32.7 μs .

3.3.2. Sample preparation—Bilayers and large bicelles are the most desirable lipid assemblies for structural studies of membrane proteins because they closely mimic the properties of biological membranes. They require the use of solid-state NMR methods since the associated polypeptides are immobile on the 10^4 Hz timescale of the dipolar and chemical shift interactions. Bilayers can be completely aligned between glass plates, and the resulting spectra from the incorporated proteins have line widths that are similar to those of single crystals [70]. Bicelles, prepared by mixing long chain and short chain lipids, can be

aligned magnetically perpendicular to the direction to the magnetic field, or flipped to the parallel orientation by the addition of lanthanide ions [71], and have been used recently to obtain high-resolution spectra of membrane proteins [72].

Samples of membrane proteins in lipid bilayers oriented on glass slides can be prepared by deposition from organic solvents followed by evaporation and lipid hydration, or by fusion of reconstituted unilamellar lipid vesicles with the glass surface. The method of choice depends on the individual protein, and the choice of solvents in the first method, and of detergents in the second, is critical for obtaining highly oriented lipid bilayer preparations. In both methods, the choice of lipid can be used to control the lateral spacing between neighboring phospholipid molecules as well as the vertical spacing between bilayers. The use of phospholipids with unsaturated chains leads to more expanded and fluid bilayers, and the addition of negatively charged lipids increases inter-bilayer repulsions leading to larger interstitial water layers between bilayer leaflets. In all cases the thinnest available glass slides are utilized to obtain the best filling factor in the coil of the probe. With carefully prepared samples it is possible to obtain ^{15}N resonance line widths of less than 3 ppm [70].

The FXYP protein samples were prepared by first dissolving 2 mg of ^{15}N -labeled protein in 0.5 mL of TFE with 50 μL of β -mercaptoethanol, and then adding 100 mg of lipid, di-oleoyl-phosphatidyl-choline/di-oleoyl-phosphatidyl-glycerol (DOPC/DOPG; 8/2 molar), in 1 mL of CHCl_3 . After spreading this solution on the surface of 35 glass slides (dimensions $11 \times 11 \times 0.06$ mm), the solvents were removed under vacuum overnight, and the slides were stacked. Oriented lipid bilayers were formed by equilibrating the stacked slides for 24 h, at 40 $^\circ\text{C}$, in a chamber containing a saturated solution of ammonium phosphate, which provides an atmosphere of 93% relative humidity. The samples were wrapped in parafilm and then sealed in thin polyethylene film prior to insertion in the NMR probe. The samples for HD exchange experiments were prepared by exposing the stacked oriented bilayer samples to an atmosphere saturated with $^2\text{H}_2\text{O}$. This was achieved by placing the sample in a closed chamber containing $^2\text{H}_2\text{O}$ and incubating at 40 $^\circ\text{C}$ for 24 h.

3.3.3. ^{31}P NMR spectra of the membrane lipids—The phospholipid phase and the degree of phospholipid bilayer alignment can be assessed with ^{31}P NMR spectroscopy of the lipid phosphate headgroup. The ^{31}P NMR spectrum obtained for unoriented bilayer vesicles containing CHIF (Fig. 5D) is characteristic of lipids in a liquid crystalline bilayer arrangement, while the spectrum for oriented lipids with CHIF has a single resonance near 30 ppm that is characteristic of oriented lipid bilayer membranes (Fig. 5I). The presence of a single peak demonstrates that the samples are highly oriented, as required for NMR structure determination.

3.3.4. ^{15}N chemical shift spectra—The spectra of ^{15}N -labeled FXYP proteins in oriented lipid bilayers (Fig. 5E–H) display significant resolution throughout the frequency range of the ^{15}N amide chemical shift, and reflect the presence of transmembrane and in-plane helical segments. In each spectrum, the resonance intensity near 200 ppm is from backbone amide sites in the transmembrane helix, with amide NH bonds nearly perpendicular to the plane of the membrane, while the intensity near 80 ppm is from sites in the N- and C-termini of the protein, with NH bonds nearly parallel to the membrane surface. The peak near 35 ppm is from the amino groups of the lysine sidechains and the N-terminus. These spectra are strikingly different from those of unoriented samples, which provide no resolution among resonances (Fig. 5A–C). Most of the backbone sites are structured and immobile on the timescale of the ^{15}N chemical shift interaction (10 kHz), contributing to the characteristic amide powder pattern between about 220 and 60 ppm. Some of the backbone sites, in the loop and terminal regions, are mobile, and give rise to the resonance band

centered near 120 ppm, the isotropic frequency, although some resonances near 120 ppm may reflect specific orientations of their corresponding sites.

The narrow chemical shift dispersion in the frequency range near 200 ppm associated with transmembrane helices indicates that all three proteins cross the membrane with only a very small tilt angle. Taken together, the ^{15}N and ^{31}P spectra provide evidence that the FXYD proteins insert in membranes without disruption of the membrane integrity.

3.3.5. H/D exchange—Amide hydrogen exchange rates are useful for identifying residues that are involved in hydrogen bonding, and that are exposed to water. Although lipid bilayers are permeable to water and other small polar molecules, the amide hydrogens in transmembrane helices can have very slow exchange rates due to strong hydrogen bonds in the low dielectric of the lipid bilayer environment, and their ^{15}N chemical shift NMR signals can persist for days after exposure to D_2O . Faster exchange rates are observed for transmembrane helices that are not tightly hydrogen bonded and are exposed to bulk water because they participate in channel pore formation [73], and for other water-exposed helical regions of proteins with weaker hydrogen bonded networks.

When the CHIF sample was exposed to D_2O , the hydrogens in the transmembrane helix did not exchange while those in the rest of the protein did, and, because the signals are generated by cross polarization from ^1H , the amide resonances disappeared from the spectrum (Fig. 5H). This indicates that, in lipid bilayers, the CHIF transmembrane helix forms a tight hydrogen bonding network that is resistant to hydrogen exchange.

3.3.6. $^1\text{H}/^{15}\text{N}$ PISEMA spectra—The PISEMA spectra of uniaxially oriented proteins provide ^{15}N chemical shift and ^1H - ^{15}N dipolar coupling frequencies for each ^{15}N -labeled amide site in the protein. For membrane proteins in oriented lipid bilayers, these frequencies reflect the orientation of their corresponding molecular site with respect to the membrane. The spectra also serve as sensitive indices of protein secondary structure and topology because they exhibit characteristic wheel-like patterns of resonances, called Pisa wheels, that reflect helical wheel projections of residues in both α -helices and β -sheets [74–76]. When a Pisa wheel is observed, no assignments are needed to determine the tilt of a helix, and a single resonance assignment can be sufficient to determine the helix rotation in the membrane. This information is extremely useful for determining the structures and orientations of membrane proteins in membranes.

The shape and position of the PISA wheel in the spectrum depends on the protein secondary structure and its orientation relative to the lipid bilayer surface, as well as the amide N–H bond length and the magnitudes and orientations of the principal elements of the amide ^{15}N chemical shift tensor. This direct relationship between spectrum and structure makes it possible to calculate solid-state NMR spectra for specific structural models of proteins, and provides the basis for a method that has been developed for backbone structure determination from a limited set of uniformly and selectively ^{15}N -labeled samples [6,77].

The two-dimensional PISEMA spectra of uniformly and selectively Leu ^{15}N -labeled CHIF in lipid bilayers are shown in Fig. 6. The Pisa wheel that is observed in the region from 6 to 10 kHz and 180 to 220 ppm in the spectrum, provides definitive evidence that the protein associates with the lipid bilayer as a transmembrane helix. To estimate the tilt of the CHIF transmembrane helix we compared the experimental spectrum with those calculated for an ideal α -helix, with 3.6 residues per turn and identical backbone dihedral angles for all residues ($\phi, \psi = 57^\circ, -47^\circ$), tilted at $10^\circ, 15^\circ, 20^\circ$, and 25° relative to the lipid bilayer normal. This comparative analysis demonstrates that the CHIF helix is tilted by about 15° in the membrane (or 75° from the membrane surface). The data suggest that the peaks in the

PISEMA spectrum will have to be fitted with Pisa wheels of different tilts, in agreement with the solution NMR studies in micelles, showing that helices 1 and 2 have different orientations. According to the data in micelles, the peaks in the spectrum of ^{15}N -Leu labeled CHIF should account for Leu 17 and 19 in helix 1 preceding the transmembrane helix (helix 2), and Leu 22, 27, 28, 35, 37 in the transmembrane helix.

3.4. Structural features of the FXYD proteins

The structure of PLM in micelles (Fig. 7) was determined by combining the constraints provided by RDCs, chemical shift, H/D exchange, and Mn protection factors measured by solution NMR in micelles, and the 15° tilt of the transmembrane helix measured by solid-state NMR in oriented lipid bilayers. Protein structures were calculated from the experimental data using a basic simulated annealing protocol in the program X-PLOR-NIH [78], as described [11], and the relative orientations of helical segments were determined using the programs and REDCAT [64]. Helices 1, 2, and 3 are rigidly connected, and their relative orientations are determined. Helix 4 is connected to the others by a long flexible loop, and its orientation relative to the rest of the protein can have one of four symmetry related solutions. The solution in Fig. 7 satisfies the amphiphilic association of helix 4 with the membrane, as indicated by the Mn binding studies in Fig. 4.

An important finding of these NMR structural studies was that the helical secondary structures of the FXYD family members, PLM, gamma, Mat-8, and CHIF, reflect the structures of their corresponding genes [28]. Despite their relatively small sizes (60–160 residues), the FXYD family proteins are all encoded by genes with six to nine small exons, and the coincidence of intron–exon junctions with helical structures and flexible connecting segments, support the hypothesis that the FXYD proteins were assembled from discrete structural modules through exon shuffling. The multiple exon organization of the FXYD genes could serve to confer high structural and functional diversity among the family members.

4. Concluding remarks

NMR approaches that exploit the properties of aligned samples are crucial for structure determination of proteins that are not compactly folded, such as helical membrane proteins. Membrane proteins in lipid bilayers are immobile on the millisecond timescale, and can be completely aligned for solid-state NMR experiments, with a degree of alignment similar to that observed in single crystals of peptides. In contrast, membrane proteins in micelles have rotational correlation times around 10 ns, and can be weakly aligned in samples where the extent-of-alignment is about 0.1% of that in the completely aligned bilayer samples. For both solution NMR and solid-state NMR, sample alignment provides a way to map protein structure onto the NMR spectra through the orientation-dependent frequencies of the dipole–dipole and chemical shift interactions.

Acknowledgments

This research was supported by grants from the National Institutes of Health (CA082864, GM065374). The NMR studies utilized the Burnham Institute NMR Facility and the UCSD Resource for Molecular Imaging of Proteins, each supported by grants from the National Institutes of Health (P30 CA030199, P41EB002031).

References

1. Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman RD, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott

- KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA 3rd, Venter JC. *Science* 1995;270:397–403. [PubMed: 7569993]
2. White SH, Wimley WC. *Annu Rev Biophys Biomol Struct* 1999;28:319–365. [PubMed: 10410805]
3. Ketchum RR, Hu W, Cross TA. *Science* 1993;261:1457–1460. [PubMed: 7690158]
4. Opella SJ, Marassi FM, Gesell JJ, Valente AP, Kim Y, Oblatt-Montal M, Montal M. *Nat Struct Biol* 1999;6:374–379. [PubMed: 10201407]
5. Wang J, Kim S, Kovacs F, Cross TA. *Protein Sci* 2001;10:2241–2250. [PubMed: 11604531]
6. Marassi FM, Opella SJ. *Protein Sci* 2003;12:403–411. [PubMed: 12592011]
7. Park SH, Mrse AA, Nevzorov AA, Mesleh MF, Oblatt-Montal M, Montal M, Opella SJ. *J Mol Biol* 2003;333:409–424. [PubMed: 14529626]
8. Oxenoid K, Chou JJ. *Proc Natl Acad Sci USA* 2005;102:10870–10875. [PubMed: 16043693]
9. Zamoon J, Mascioni A, Thomas DD, Veglia G. *Biophys J* 2003;85:2589–2598. [PubMed: 14507721]
10. Sorgen PL, Cahill SM, Krueger-Koplin RD, Krueger-Koplin ST, Schenck CC, Girvin ME. *Biochemistry* 2002;41:31–41. [PubMed: 11772000]
11. Howell SC, Mesleh MF, Opella SJ. *Biochemistry* 2005;44:5196–5206. [PubMed: 15794657]
12. Pervushin K, Riek R, Wider G, Wuthrich K. *Proc Natl Acad Sci USA* 1997;94:12366–12371. [PubMed: 9356455]
13. Arora A, Abildgaard F, Bushweller JH, Tamm LK. *Nat Struct Biol* 2001;8:334–338. [PubMed: 11276254]
14. Fernandez C, Adeishvili K, Wuthrich K. *Proc Natl Acad Sci USA* 2001;98:2358–2363. [PubMed: 11226244]
15. Hwang PM, Choy WY, Lo EI, Chen L, Forman-Kay JD, Raetz CR, Prive GG, Bishop RE, Kay LE. *Proc Natl Acad Sci USA* 2002;99:13560–13565. [PubMed: 12357033]
16. Marassi FM. *Concepts Magn Reson* 2002;14:212–224.
17. Sweadner KJ, Rael E. *Genomics* 2000;68:41–56. [PubMed: 10950925]
18. Crambert G, Geering K. *Sci STKE* 2003;2003:RE1. [PubMed: 12538882]
19. Garty H, Karlisch SJ. *Annu Rev Physiol* 2006;68:431–459. [PubMed: 16460279]
20. Palmer CJ, Scott BT, Jones LR. *J Biol Chem* 1991;266:11126–11130. [PubMed: 1710217]
21. Mercer RW, Biemesderfer D, Bliss DP Jr, Collins JH, Forbush B 3rd. *J Cell Biol* 1993;121:579–586. [PubMed: 8387529]
22. Attali B, Latter H, Rachamim N, Garty H. *Proc Natl Acad Sci USA* 1995;92:6092–6096. [PubMed: 7597086]
23. Arystarkhova E, Wetzel RK, Asinowski NK, Sweadner KJ. *J Biol Chem* 1999;274:33183–33185. [PubMed: 10559186]
24. Shi H, Levy-Holzman R, Cluzeaud F, Farman N, Garty H. *Am J Physiol Renal Physiol* 2001;280:F505–F512. [PubMed: 11181413]
25. Wetzel RK, Sweadner KJ. *Am J Physiol Renal Physiol* 2001;281:F531–F545. [PubMed: 11502602]
26. Morrison BW, Leder P. *Oncogene* 1994;9:3417–3426. [PubMed: 7970700]
27. Kaye H, Kleeff J, Kolb A, Ketterer K, Keleg S, Felix K, Giese T, Penzel R, Zentgraf H, Buchler MW, Korc M, Friess H. *Int J Cancer*. 2005
28. Franzin CM, Yu J, Thai K, Choi J, Marassi FM. *J Mol Biol* 2005;354:743–750. [PubMed: 16288923]
29. Miroux B, Walker JE. *J Mol Biol* 1996;260:289–298. [PubMed: 8757792]
30. Delaglio F, Grzesiek S, Vuister GW, Zhu G, Pfeifer J, Bax A. *J Biomol NMR* 1995;6:277–293. [PubMed: 8520220]
31. Goddard, TD.; Sneller, DG. SPARKY 3. University of California; San Francisco: 2004. Available from: <www.cgl.ucsf.edu/home/sparky/>
32. Thai K, Choi J, Franzin CM, Marassi FM. *Protein Sci* 2005;14:948–955. [PubMed: 15741345]
33. Studier FW, Rosenberg AH, Dunn JJ, Dubendorff JW. *Methods Enzymol* 1990;185:60–89. [PubMed: 2199796]

34. Miozzari GF, Yanofsky C. J Bacteriol 1978;133:1457–1466. [PubMed: 346574]
35. Kleid DG, Yansura D, Small B, Dowbenko D, Moore DM, Grubman MJ, McKercher PD, Morgan DO, Robertson BH, Bachrach HL. Science 1981;214:1125–1129. [PubMed: 6272395]
36. Staley JP, Kim PS. Protein Sci 1994;3:1822–1832. [PubMed: 7531529]
37. Kuliopulos A, Nelson NP, Yamada M, Walsh CT, Furie B, Furie BC, Roth DA. J Biol Chem 1994;269:21364–21370. [PubMed: 8063763]
38. Schagger H, von Jagow G. Anal Biochem 1987;166:368–379. [PubMed: 2449095]
39. Cavanagh, J. Protein NMR Spectroscopy: Principles and Practice. Academic Press; San Diego: 1996.
40. Mori S, Abeygunawardana C, Johnson MO, Vanzijl PCM. J Magn Reson B 1995;108:94–98. [PubMed: 7627436]
41. Ikura M, Kay LE, Bax A. Biochemistry 1990;29:4659–4667. [PubMed: 2372549]
42. Sattler M, Schleucher J, Griesinger C. Prog Nucl Magn Reson Spectrosc 1999;34:93–158.
43. Grzesiek S, Bax A. J Am Chem Soc 1992;114:6291–6293.
44. Farrow NA, Zhang O, Forman-Kay JD, Kay LE. J Biomol NMR 1994;4:727–734. [PubMed: 7919956]
45. Sass HJ, Musco G, Stahl SJ, Wingfield PT, Grzesiek S. J Biomol NMR 2000;18:303–309. [PubMed: 11200524]
46. Ishii Y, Markus MA, Tycko R. J Biomol NMR 2001;21:141–151. [PubMed: 11727977]
47. Chou JJ, Gaemers S, Howder B, Louis JM, Bax A. J Biomol NMR 2001;21:377–382. [PubMed: 11824758]
48. Ottiger M, Delaglio F, Bax A. J Magn Reson 1998;131:373–378. [PubMed: 9571116]
49. Ding K, Gronenborn AM. J Magn Reson 2003;163:208–214. [PubMed: 12914836]
50. Prestegard JH, Mayer KL, Valafar H, Benison GC. Methods Enzymol 2005;394:175–209. [PubMed: 15808221]
51. Bax A, Kontaxis G, Tjandra N. Methods Enzymol 2001;339:127–174. [PubMed: 11462810]
52. Vinogradova O, Sonnichsen F, Sanders CR 2nd. J Biomol NMR 1998;11:381–386. [PubMed: 9691283]
53. Krueger-Koplin RD, Sorgen PL, Krueger-Koplin ST, Rivera-Torres IO, Cahill SM, Hicks DB, Grinius L, Krulwich TA, Girvin ME. J Biomol NMR 2004;28:43–57. [PubMed: 14739638]
54. Tanford C, Reynolds JA. Biochim Biophys Acta 1976;457:133–170. [PubMed: 135582]
55. Chill JH, Louis JM, Miller C, Bax A. Protein Sci 2006;15:684–698. [PubMed: 16522799]
56. Jorgensen PL. Methods Enzymol 1988;156:29–43. [PubMed: 2835612]
57. Maunsbach AB, Skriver E, Jorgensen PL. Methods Enzymol 1988;156:430–441. [PubMed: 2835625]
58. Ivanov AV, Gable ME, Askari A. J Biol Chem 2004;279:29832–29840. [PubMed: 15123703]
59. Mesleh MF, Lee S, Veglia G, Thiriot DS, Marassi FM, Opella SJ. J Am Chem Soc 2003;125:8928–8935. [PubMed: 12862490]
60. Wishart DS, Sykes BD. Methods Enzymol 1994;239:363–392. [PubMed: 7830591]
61. Cornilescu G, Delaglio F, Bax A. J Biomol NMR 1999;13:289–302. [PubMed: 10212987]
62. Mesleh MF, Veglia G, DeSilva TM, Marassi FM, Opella SJ. J Am Chem Soc 2002;124:4206–4207. [PubMed: 11960438]
63. Mesleh MF, Opella SJ. J Magn Reson 2003;163:288–299. [PubMed: 12914844]
64. Valafar H, Prestegard JH. J Magn Reson 2004;167:228–241. [PubMed: 15040978]
65. Luchette PA, Prosser RS, Sanders CR. J Am Chem Soc 2002;124:1778–1781. [PubMed: 11853456]
66. Prosser RS, Luchette PA, Westerman PW. Proc Natl Acad Sci USA 2000;97:9967–9971. [PubMed: 10954744]
67. Pines A, Gibby MG, Waugh JS. J Chem Phys 1973;59:569–590.
68. Levitt MH, Suter D, Ernst RR. J Chem Phys 1986;84:4243–4255.
69. Wu CH, Ramamoorthy A, Opella SJ. J Magn Reson A 1994;109:270–272.

70. Marassi FM, Ramamoorthy A, Opella SJ. Proc Natl Acad Sci USA 1997;94:8551–8556. [PubMed: 9238014]
71. Prosser RS, Hunt SA, DiNatale JA, Vold RR. J Am Chem Soc 1996;118:269–270.
72. De Angelis AA, Nevzorov AA, Park SH, Howell SC, Mrse AA, Opella SJ. J Am Chem Soc 2004;126:15340–15341. [PubMed: 15563135]
73. Tian C, Gao PF, Pinto LH, Lamb RA, Cross TA. Protein Sci 2003;12:2597–2605. [PubMed: 14573870]
74. Marassi FM, Opella SJ. J Magn Reson 2000;144:150–155. [PubMed: 10783285]
75. Wang J, Denny J, Tian C, Kim S, Mo Y, Kovacs F, Song Z, Nishimura K, Gan Z, Fu R, Quine JR, Cross TA. J Magn Reson 2000;144:162–167. [PubMed: 10783287]
76. Marassi FM. Biophys J 2001;80:994–1003. [PubMed: 11159466]
77. Marassi FM, Opella SJ. J Biomol NMR 2002;23:239–242. [PubMed: 12238596]
78. Schwieters CD, Kuszewski JJ, Tjandra N, Marius Clore G. J Magn Reson 2003;160:65–73. [PubMed: 12565051]
79. Kyte J, Doolittle RF. J Mol Biol 1982;157:105–132. [PubMed: 7108955]

Fig. 1.

(A) Organization of the pBCL173 and pBCL99 fusion protein expression plasmids. The target sequence with N-terminal Met is inserted between the *AflIII* and *XhoI* cloning sites. BCL173 has a cleavable Met after the His tag, while BCL99 does not. (B) Amino acid sequence of the Bcl-XL fusion protein. Both BCL173 and BCL99 lack the Bcl-XL hydrophobic C-terminus (highlighted in the gray box), and BCL173 (single underline) also lacks the flexible loop of Bcl-XL, while BCL99 (double underline) lacks the first 116 residues. (C) Amino acid sequences of the FXYD proteins, PLM, gamma-b, Mat-8, and CHIF. The transmembrane domains are in the gray box. (D) Expression of PLM with four different fusion protein plasmid vectors: pBCL173, pBCL99, pKSI, and pTLE. The gel shows total lysates from cells, transformed with each plasmid, and harvested before (–) or after (+) induction with IPTG. Fusion protein over-expression is marked by the appearance of a distinct band (arrows) at the molecular weight of the corresponding fusion protein: BCL173-PLM (29.8 kDa); BCL99-PLM (21.2 kDa); KSI-PLM (23.4 kDa); and TLE-PLM (22.1 kDa). (E) Purified FXYD family proteins run as monomers on SDS–PAGE: PLM (8.4 kDa); gamma-a (7.3 kDa); gamma-b (7.3 kDa); Mat-8 (7.1 kDa); CHIF (7.3 kDa).

Fig. 2. Two-dimensional $^1\text{H}/^{15}\text{N}$ HSQC spectra of uniformly ^{15}N -labeled PLM, γ -b, Mat-8, and CHIF, in detergent micelles. The samples were prepared by dissolving each protein in buffer (20 mM sodium citrate, pH 5, 10 mM DTT, and 1 mM sodium azide, in 90% H_2O , 10% D_2O) plus detergent. (A) The spectra of γ -b in 500 mM DPC, 180 mM DHPC, 100 mM LPPG, and 200 mM OG. (B) The spectra of PLM, γ -b, Mat-8, and CHIF in 500 mM SDS.

Fig. 3. Summary of NMR parameters, plotted as a function of residue number, for PLM, gamma-b, Mat-8, and CHIF. The protein secondary structures are shown at the top of the figure. The positions of intron–exon junctions are marked by vertical dotted lines. The residue numbers for the three proteins begin after the signal sequences. (A) Kyte–Doolittle hydropathy plot [79]. (B) Amide H/D exchange profiles, with exchange rates classified as rapid (less than 1 h), short (less than 3 h), medium (less than 12 h), or long (more than 24 h). (C) Values of ^1H – ^{15}N RDCs with the characteristic periodicity in the α -helical regions of the proteins fitted to sinusoids. (D) $^{13}\text{C}\alpha$ chemical shift indices. (E) $^1\text{H}/^{15}\text{N}$ heteronuclear NOEs. (F) Normalized $^1\text{H}/^{15}\text{N}$ HSQC peak intensities. Positions that are left blank correspond to prolines, or overlapped resonances.

Fig. 4. (A) Normalized $^1\text{H}/^{15}\text{N}$ HSQC peak intensities for PLM and Mat-8 in the absence of MnCl_2 ($I_{[-\text{Mn}]}$). (B) Normalized $^1\text{H}/^{15}\text{N}$ HSQC peak intensities obtained in the presence of MnCl_2 ($I_{[+\text{Mn}]}$) relative to those obtained in the absence of MnCl_2 ($I_{[-\text{Mn}]}$).

Fig. 5. Solid-state NMR ^{15}N and ^{31}P chemical shift spectra of uniformly ^{15}N -labeled FXYP proteins in unoriented (A–D) and oriented (E–I) lipid bilayers. (A and E) PLM, (B and F) Mat-8, and (C, G, H, and I) CHIF. Resonances near 200 ppm are from amino acid residues in the FXYP transmembrane helix. Amide hydrogens in the transmembrane helix of CHIF are resistant to hydrogen exchange, and their resonances are visible after HD exchange, while the resonances from exchangeable hydrogens disappear (H).

Fig. 6. $^1\text{H}/^{15}\text{N}$ solid-state NMR PISEMA spectra of CHIF in oriented lipid bilayers. The region corresponding to the transmembrane segment is shown. (A) The spectrum of uniformly ^{15}N -labeled CHIF is superimposed on the PISA wheels calculated for ideal α -helices with different tilts in the lipid bilayer. (B) The spectrum of ^{15}N -Leu labeled CHIF is also shown in (A) as red dots. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

Fig. 7. Structure of PLM determined by combining the NMR constraints obtained in micelles with those obtained in lipid bilayers. The consensus phosphorylation sites, Ser 63 and Ser 68, in the cytoplasmic helix of PLM are highlighted.