# Biophysical approaches to membrane protein structure determination

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Recently, there have been several technical advances in the use of solution and solid-state NMR spectroscopy to determine the structures of membrane proteins. The structures of several isolated transmembrane (TM) helices and pairs of TM helices have been solved by solution NMR methods. Similarly, the complete folds of two TM β-barrel proteins with molecular weights of 16 and 19 kDa have been determined by solution NMR in detergent micelles. Solution NMR has also provided a first glimpse at the dynamics of an integral membrane protein. Structures of individual TM helices have also been determined by solid-state NMR. A combination of NMR with site-directed spin-label electron paramagnetic resonance or Fourier transform IR spectroscopy allows one to assemble quite detailed protein structures in the membrane.

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**Abbreviations**

| | |
|---|---|
| **cmc** | critical micelle concentration |
| **DHPC** | dihexanoylphosphatidylcholine |
| **DPC** | dodecylphosphocholine |
| **EPR** | electron paramagnetic resonance |
| **FTIR** | Fourier transform IR |
| **MAS** | magic angle spinning |
| **NOE** | nuclear Overhauser effect |
| **PDB** | Protein Data Bank |
| **PISEMA** | polarization inversion with spin exchange at the magic angle |
| **TM** | transmembrane |
| **TROSY** | transverse relaxation optimized spectroscopy |

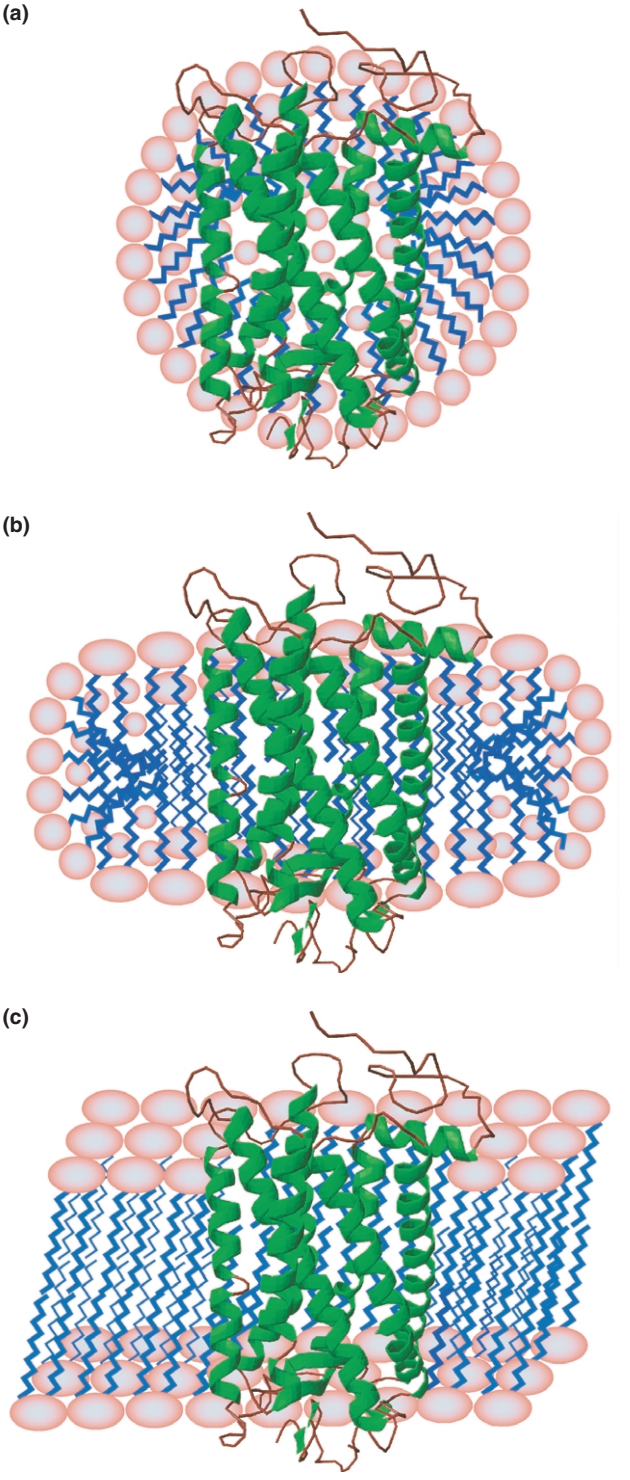
## Introduction

Determining the structures of membrane proteins is still a frontier of structural biology. Presently, <30 independent integral membrane protein structures have been solved. This contrasts sharply with ~15 000 soluble proteins solved by X-ray crystallography and NMR spectroscopy. If we assume that, firstly, the human genome codes for ~36 000 structural genes [1], secondly, there is an approximately fourfold redundancy in the database as a result of homologs and structures from different organisms that are not present in mammals and, finally, ~30% of all proteins in eukaryotic cells are membrane proteins [2], we realize that <0.2% of all membrane protein structures are known, whereas the complement of soluble proteins may be covered with ~10% of solved structures. (Vitkup *et al.* [3] very recently estimated that 6–10% of all proteins are structurally covered in a number of completely sequenced genomes.) As is the case for soluble proteins, most structures of membrane proteins have been solved by X-ray crystallography. Despite its relative success, X-ray crystallography of membrane proteins must still be considered a high art. It is very difficult to crystallize membrane proteins from detergent solutions and the search for appropriate crystallization conditions must sample a much larger space than a typical soluble protein crystallization screen. Therefore, although structural genomics initiatives to solve 'all' soluble protein structures appear to be a goal that can be reasonably achieved, a structural genomics approach to membrane protein structures still seems far out of practical reach.

A few membrane protein structures have been solved to atomic resolution by electron microscopic analysis of two-dimensional crystals. This method is particularly adequate for studying membrane proteins because they can be crystallized in their natural two-dimensional environment (i.e. the lipid bilayer). The practical problem with this method, however, is that many crystals are not ordered well enough to give resolution beyond ~4 Å.

NMR spectroscopy has been a very successful method for determining structures of soluble proteins up to molecular weights of ~30 kDa and, in a few cases, beyond. The use of NMR as a tool to determine structures of membrane proteins, however, has, until recently, been mostly in a developmental stage. Membrane samples are too large to tumble with a short enough correlation time to yield narrow and well-resolved resonance lines, as required for high-resolution NMR. Membrane proteins can, however, be analyzed in some detergent micelle systems by solution NMR techniques. So far, this approach has been mostly used with peptides of up to ~50 residues in length. However, recent developments that are discussed in this review raise our expectations that this approach can be extended to larger membrane proteins. An alternative is to study membrane proteins in lipid bilayers by solid-state NMR techniques. This approach has been successfully employed to determine the complete structures of a couple of peptides and there are strong efforts to extend these methods to larger proteins. NMR and other biophysical approaches to membrane protein structure determination need to be further developed in order to promote the field of structural biology of membrane proteins to a level that measures up to that of soluble proteins. In this review, we summarize recent developments in this area, with some emphasis on the current status and prospects of membrane protein structure determination by solution NMR techniques.

**Figure 1**



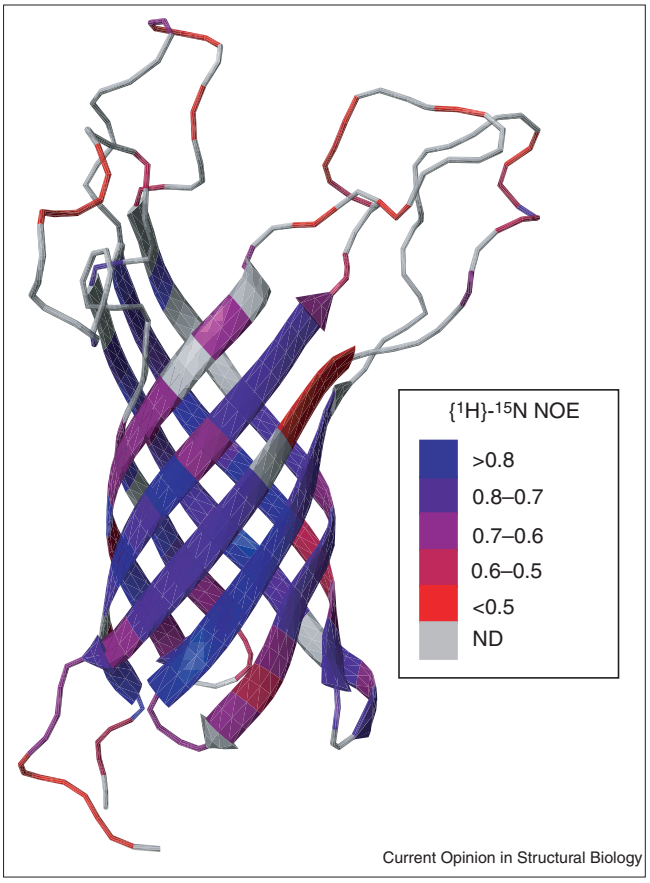

Membrane and membrane-like systems commonly used in biophysical studies of membrane proteins. **(a)** Detergent micelles are small, mostly spherical structures used in solution NMR and circular dichroism studies of membrane proteins. **(b)** Bicelles are disk-like structures composed of bilayer-forming lipids and detergents. They orient with their normal orthogonal to the magnetic field. Bicelles are used to orient soluble proteins in solution NMR studies and membrane-bound peptides in solid-state NMR studies. **(c)** Lipid bilayers, the natural environment of membrane proteins, are used in solid-state NMR, FTIR and spin-label EPR studies of membrane proteins. Structures of the seven-helix receptor rhodopsin (PDB accession number 1F88) are incorporated in each model system.

## Micelles, bicelles and bilayers

The structures of membrane proteins have been studied in different environments. Membrane proteins embedded in detergent micelles (Figure 1a) are most appropriate for studying membrane proteins by solution NMR techniques. Two important parameters characterize micellar solutions: the critical micelle concentration (cmc) and the aggregation number [4]. Detergents are monomeric below the cmc, but cooperatively assemble into micelles above the cmc. The aggregation number describes the number of monomers in a micelle and therefore is important for estimating apparent molecular weights of micelles. A survey of cmcs of many nonionic and zwitterionic detergents and short-chain phospholipids that preserve the structures of membrane proteins has been compiled recently [5]. Although aggregation numbers have been determined for the most important detergents, they are not as widely available as the cmcs. Cmcs and aggregation numbers depend, sometimes quite dramatically, on environmental parameters, such as temperature, ionic strength, pH and so on. Detergent micelles that have been widely used in solution NMR studies of membrane proteins include dodecylphosphocholine (DPC) and dihexanoylphosphatidylcholine (DHPC). The structures of smaller peptides and proteins that can be solved by $^{1}$H-NMR are best reconstituted in perdeuterated detergent systems in order to isolate the peptide protons from detergent deuterons. The use of deuterated detergents is not necessary for studying larger systems by heteronuclear NMR. DPC has a cmc of 1.5 mM and an estimated aggregation number of 70–80 at 25°C in 50 mM NaCl. This translates into an aggregate molecular weight of 25–28 kDa. The corresponding numbers for DHPC are 15.2 mM, ~35 and ~16 kDa [6,7].

Bicelles are disk-shaped aggregates of phospholipid and detergent (Figure 1b) that orient spontaneously perpendicular to an applied magnetic field owing to their diamagnetic moment. Several recipes to create bicelles of different sizes, shapes and orientation properties have been described [8]. Although originally devised to orient membrane proteins in the magnetic field for solid-state NMR studies, they have more recently gained more use in introducing small degrees of residual orientation of soluble proteins in order to determine dipolar couplings, which have proven extremely beneficial for structure determinations of soluble proteins by high-resolution NMR [9,10]. Apart from studying the structures of small membrane-bound peptides [11,12], bicelles have so far not found wide application in the structure determination of membrane proteins.

**Figure 2**




Dynamic structure of the OmpA TM domain. The structure was solved by heteronuclear solution NMR in DPC micelles. Backbone dynamics were probed by heteronuclear NOE measurements of all colored residues and mapped onto the structure using a color code ranging from blue (most rigid) to red (most dynamic). ND, not determined. Data taken from [24••].

Lipid bilayers (Figure 1c) are the natural environment of membrane proteins. Individual peaks can be resolved by solid-state NMR of proteins in membranes that either are mechanically oriented in the magnetic field or are unoriented, but spun at the magic angle in the NMR spectrometer.

## Protein expression and sample preparation

Efficient expression systems to introduce appropriate isotopes are essential for structure determinations by heteronuclear NMR. This is not a trivial problem for membrane proteins because their overexpression often causes lysis and cell death. Most heteronuclear NMR studies of membrane proteins to date have been carried out with proteins that were expressed in *Escherichia coli*, recovered from inclusion bodies and subsequently refolded. In some cases, signal sequences were deleted and/or purification tags (histidine tags, glutathione *S*-transferase, staphylococcal nuclease, maltose-binding protein, and so on, with appropriate proteolytic cleavage sites) were engineered into the expression vectors.

Purification can occur in detergent or, in the case of β-barrel membrane proteins, in denaturants such as urea or guanidinium chloride. Refolding conditions have to be carefully monitored for each membrane protein. In many cases, the lack of an appropriate refolding protocol has become a major obstacle in structure determinations of membrane proteins by NMR. It is hoped that future progress in the efficient expression of membrane proteins in their native form (e.g. in the yeast *Pichia pastoris*, which has many internal membranes) will avoid the often cumbersome refolding step.

## Advances in solution NMR spectroscopy

A major advance in solution NMR spectroscopy that has had a significant impact on the determination of membrane protein structures in detergent micelles has been the development of TROSY (transverse relaxation optimized spectroscopy) [13]. At the high magnetic fields available (currently up to proton frequencies of 900 MHz), transverse relaxation resulting from chemical shift anisotropy and dipolar interactions causes significant line broadening, which offsets some of the high-field advantages for resolution and sensitivity. In TROSY, the scalar heteronuclear spin–spin couplings are not decoupled, only one of the four peaks in the multiplet is retained and the chemical shift anisotropy relaxation (at high fields) is used to compensate dipolar relaxation. This procedure results in improved signal/noise ratios for proteins and complexes that are larger than ~20 kDa. These conditions are almost always met for membrane proteins in detergent micelles, which explains why TROSY has had a major impact on NMR of membrane proteins. Although the TROSY technique was originally introduced as an improvement of the $^{1}H$-$^{15}N$ HSQC (heteronuclear single-quantum coherence) experiment, TROSY-based analogs of the most important 3D and 4D heteronuclear experiments have since been developed [14–21].

Another indispensable tool for resolving and assigning NMR spectra of large complexes, including membrane proteins in detergent micelles, has been the selective and uniform deuteration of amino acid sidechains [22]. The gain in sensitivity comes from the lower gyromagnetic ratio of $^{2}H$ relative to $^{1}H$ and its correspondingly lower effectiveness in changing the rate of transverse relaxation of neighboring heteronuclei. This results in sharper resonance lines. As much sidechain information is lost by the complete deuteration of these moieties, schemes have been developed to reintroduce methyl protons in a number of aliphatic sidechains [23]. These latter schemes have not yet been extensively tried with membrane proteins. They are likely to produce valuable long-range nuclear Overhauser effects (NOEs) with helical membrane proteins, but may be less effective with β-barrel membrane proteins because most methyl groups face the lipid bilayer in this class of membrane protein.

TROSY and uniform sidechain deuteration have been used to determine the backbone structure of the transmembrane

(TM) domain of the outer membrane protein OmpA in DPC micelles [24••]. The OmpA TM domain comprises 177 residues (19 kDa) and is the largest membrane protein structure that has been solved by NMR. The detergent–protein complex has a molecular weight of the order of 45–50 kDa. OmpA forms an eight-stranded β barrel and functions as an ion channel in lipid bilayers. Quite large unstructured loops extend from the extracellular membrane surface and tight turns connect the individual β strands at the periplasmic membrane surface. The NMR structure generally resembles the crystal structure that had been determined previously [25]. In addition to the structural coordinates, however, heteronuclear NOE experiments also provide the first information on the picosecond to nanosecond dynamics of a membrane protein. A dynamic gradient increases from the center towards both ends of the barrel of OmpA (Figure 2). The increased dynamics of the protein in the region of both membrane surfaces opposes the dynamic gradient of the lipid bilayer itself (which is most dynamic in the center) and explains why the NMR signals of some residues in the highly mobile loops are eventually lost through conformational exchange in the millisecond time range.

The backbone structure of OmpX (148 residues, 16 kDa) in DHPC micelles has been determined using very similar techniques [26••]. This protein also folds into an eight-stranded TM β barrel and closely resembles the corresponding crystal structure [27]. The function of OmpX is not known, but the protein has been speculated to be part of a cellular defense system. NMR experiments probing the dynamics of OmpX have not yet been reported. Most experiments with OmpA and OmpX were carried out at 750 MHz proton frequency.

Before the advent of TROSY and the common use of very high magnetic fields, the structures of a small number of helical TM peptides had been solved by NMR. The TM domain of glycophorin A forms a helical dimer in detergent micelles and membranes. The structure, including sidechains, of the 40-residue peptide has been solved in DPC micelles using heteronuclear solution NMR [28]. The dimer structure, including the dimer interface, was modeled based on six intermonomer NOEs and the crossing angle between the two helices was determined to be –40°. The structures of subunits *c* and *b* of the *E. coli* $F_oF_1$ ATP synthase have been determined using heteronuclear and $^1$H-NMR, respectively, in organic solvent–water mixtures [29,30,31••]. Although we generally discourage the use of organic solvents as an adequate environment for membrane proteins, these extremely hydrophobic small proteins may form the exception to the rule and may still adopt their native conformations in the solvent mixtures that were chosen. This view is supported by, firstly, the fact that the resulting NMR structures of subunit *c* could be fitted quite well into the 3.9 Å electron density map calculated from X-ray diffraction data of the $F_oF_1$ ATP synthase [32] and, secondly, extensive chemical cross-linking data. The NMR structures of subunit *c* obtained in the protonated and unprotonated states [31••] are also consistent with biochemical evidence, lending further support to the observation of a physiological conformational change in this highly reduced system. The structure of the 52-residue peptide phospholamban has also been determined by solution NMR in organic solvents [33]. The structure is described as two helices connected by a β-turn-type hinge. As the more hydrophilic N-terminal domain is known to extend from the lipid bilayer into the aqueous phase, it is unclear whether this NMR structure represents the native conformation of phospholamban.

Other systems may be near a structural solution by NMR. For example, well-resolved TROSY spectra of the 40 kDa homotrimeric protein diacylglycerol kinase (the 13 kDa monomer comprises 121 residues, 3 TM helices and 1 interfacial helix) in DPC micelles have been reported [34•]. This work emphasizes the importance of appropriate refolding protocols as a prerequisite to obtain interpretable NMR spectra. It is also shown that generating some superstable mutants (with mutated residues in the helix–helix contact region) may suppress slow conformational exchange and thereby increase the intensity of NMR peaks in this and perhaps other helical membrane proteins. Diacylglycerol kinase has also been subjected to a new type of amide hydrogen–deuterium exchange protocol to probe the stability of secondary structure elements in the membrane [35]. Although amide hydrogen–deuterium exchange is a potentially very useful structural method, it is somewhat limited for complex membrane proteins because it is hard to find aprotic solvents that completely solubilize membrane proteins for post-exchange analysis. The multidrug transporter EmrE (12 kDa, 110 residues) forms a bundle of four hydrophobic TM helices. Because it contains only a small number of hydrophilic residues, a structure determination was attempted in an organic solvent mixture [36]. All resonances have been assigned and a complete determination of the secondary structure based on medium-range NOEs, chemical shifts and J couplings has been achieved. It proved very difficult, however, to obtain long-range NOEs in this system. The resonances of the methyl groups of hydrophobic sidechains that are expected to form the major tertiary contacts in this protein were highly overlapped and could not be resolved. It is possible that some organic solvent molecules penetrated the structure and thereby diminished tertiary contacts and/or induced slow conformational exchange, problems that were observed in helices 2 and 3. Although conducted at 800 MHz (but without TROSY), this work illustrates very well the challenges that helical membrane proteins still pose to the experienced NMR spectroscopist.

Although we expect them to become a useful additional tool in membrane protein structure determination, residual dipolar couplings have not yet been widely used in solution NMR studies of membrane proteins. An interesting, although somewhat special, method to weakly align

spherical membrane protein–detergent complexes in the magnetic field is by binding lanthanides to adventitious [37] or engineered [38] sites in this class of protein. The phage coat proteins fd and Pf1, and the mercury transporter MerF have adventitious sites for $Yb^{3+}$ and $Dy^{3+}$, respectively. At lanthanide:protein ratios near 10, residual dipolar couplings between –15 and +20 Hz have been observed [37]. The 81-residue channel protein Vpu from HIV does not have an adventitious lanthanide-binding site, but an 'EF hand' calcium-binding site could be engineered to the N terminus of the protein, which resulted in residual dipolar couplings between –6 and +6 Hz after binding of $Yb^{3+}$ or $Dy^{3+}$ [38].

## Advances in solid-state NMR spectroscopy

We focus here on solid-state NMR methods that are ultimately aimed at the complete structure determination of membrane proteins. These methods have been reviewed recently in this journal and elsewhere [39•–41•]. As is the case for structure determination by solution NMR, one may distinguish between distance and orientational constraint approaches to structure determination by solid-state NMR. Common to both approaches is the need for complete resonance assignments before constraint measurements. Limits of resolution, which are partially determined by sample preparation, have been the major obstacles to obtaining full assignments of membrane proteins in the 'solid state.' ('Solid state' in this context refers to proteins in liquid-crystalline bilayers that do not tumble fast on the NMR timescale.) For oriented membrane samples, PISEMA (polarization inversion with spin exchange at the magic angle) experiments have been a major breakthrough [42]. $^{15}N$, $^{13}C$ and $^{1}H$ chemical shifts and $^{1}H$-$^{15}N$ dipolar couplings have been separated in 3D PISEMA spectra [43,44]. It has been recognized that, in PISEMA spectra, the resonances of regular secondary structures fall on ellipses, so-called PISA wheels [45–47]. The positions and elliptical dimensions of the PISA wheels are good indicators of the secondary structure and orientation of the protein in the membrane. The development of 2D correlated spectra of samples that are spun at the magic angle (MAS [magic angle spinning] NMR) is also making rapid progress. Highly resolved and almost completely assigned spectra of the α-spectrin SH3 domain (62 residues) in the solid state have been obtained recently [48,49•]. One may expect that these and similar solid-state NMR experiments will also find application in assigning resonances of uniformly labeled membrane proteins, which is a critical step on the way to a complete structure determination. It has been pointed out in these (e.g. see [48]) and many previous solid-state NMR studies, however, that the best resolution is achieved with highly ordered samples. Whether the natural ordering of membrane proteins in liquid-crystalline lipid bilayers suffices to yield completely resolved MAS spectra remains to be demonstrated. Similarly, the degree of ordering of membrane proteins in oriented lipid bilayers is currently the main limiting factor of the orientation approach to membrane protein structure determination. The sharpest resonance lines would be obtained with perfectly uniaxially aligned samples, which are difficult to obtain with membrane proteins. A combined approach, MAOSS (magic angle oriented sample spinning), has also been proposed [50].

The first structure of a membrane protein to be solved by solid-state NMR was that of the peptide channel gramicidin A [51,52]. Numerous isotopic labels were introduced individually to arrive at this structure. The advent of PISEMA led to the structure of the M2 helix of the nicotinic acetylcholine receptor from a uniformly labeled sample [53]. The structure comprises a single helix that is tilted 12° from the bilayer normal. A membrane pore was constructed from five such helices by molecular modeling. Other structures are under investigation by similar techniques. The M2 protein from influenza virus forms an ion channel lined by four helices that are tilted ~35° from the membrane normal [54•,55]. Vpu forms an analogous channel in the membrane of HIV. A comparison of the experimental PISEMA spectrum with calculated PISA wheels shows that the TM domain of Vpu forms a helix that is tilted ~15° from the bilayer normal [56]. In another recent example, the structure of an 18-residue peptide whose sequence corresponded to part of the sixth TM domain of the α-factor receptor was determined in lipid bilayers [57]. About nine residues were in a helical conformation and the helix axis was ~8° from the membrane normal. This structure is too short to span the lipid bilayer. A difficulty that is often encountered in solution NMR of membrane proteins is the small (sometimes zero) number of observable interhelical NOEs. Distance measurements by rotational resonance in the solid state can provide a very valuable complement to solution NMR studies in these cases. Interhelical distances between various residues of the glycophorin A TM domain dimer have been measured by rotational resonance NMR [58•]. The crossing angle of the two helices was –35°, that is, similar to that found by solution NMR, but the interhelical interfaces in the modeled structure were rotated by ~25° relative to the structure that was modeled based on the solution data only [28]. These differences could result from the larger number of constraints in the solid-state data or from true differences between the structures in detergent micelles and lipid bilayers.

## Combining solution NMR with lower-resolution techniques

It is now clear that the structures of small membrane proteins or individual domains of larger membrane proteins can be solved by solution NMR. The challenge then becomes to correctly assemble these structures in the lipid bilayer. As described, selected solid-state NMR experiments are one option to solve this problem. In many cases, however, similar information can be obtained from lower-resolution techniques, such as Fourier transform IR (FTIR) or electron paramagnetic resonance (EPR) spectroscopy, or from chemical cross-linking. Polarized attenuated total

reflection (ATR)-FTIR spectroscopy on oriented and fully hydrated supported lipid bilayers yields information about secondary structure and helix or β-sheet orientation in the bilayer [59,60]. Global information is obtained from unlabeled samples and local information can be obtained from samples that are $^{13}$C-labeled at selected sites [61–64]. Site-directed spin labeling of membrane proteins (at engineered cysteines) has become a very powerful technique to probe the architecture and disposition of membrane proteins in lipid bilayers [65•]. For example, conformational changes of the KcsA potassium channel from *Streptomyces lividans* were measured with quite high precision using this technique [66••]. Another novel approach is to combine high-resolution NMR data in micelles with lower-resolution EPR data in lipid bilayers. This approach led to the determination of the complete structure and a pH-triggered conformational change of the fusion peptide of influenza hemagglutinin in lipid bilayers [67••]. In this work, the atomic coordinates of the two NMR structures were fitted to two sets of 18 EPR distance constraints obtained in lipid bilayers. The two structures reveal a fusion-triggering conformational change that involves a deeper and more angled membrane insertion of the V-shaped molecule in the fusogenic state relative to the nonfusogenic state. Finally, a future avenue to assemble structures of larger membrane proteins may be to fit high-resolution structures of fragments (domains) that are obtained by solution (or solid-state) NMR into medium-resolution (4–7 Å) structures of the whole protein obtained by electron crystallography. These are the ranges at which each of these methods works best and therefore may complement each other. This approach will of course only work if the selected domains fold as independent units in lipid bilayers and detergent micelles, as proposed in the two-stage model of membrane protein folding [68].

## Conclusions

Significant progress has been made in recent years in the determination of the structures of small membrane proteins or domains of membrane proteins by solution or solid-state NMR spectroscopy. The largest structures (folds) that have been solved *de novo* by solid-state NMR in lipid bilayers are single TM helices. TM helix dimers and β-barrel membrane proteins up to 19 kDa have been solved by solution NMR in detergent micelles. TROSY and the high magnetic fields currently available have greatly facilitated the progress of solution NMR of membrane proteins. Similarly, new 2D and 3D experiments have advanced solid-state NMR of membrane proteins. In our opinion, currently the major limitations of solid-state NMR for membrane protein structure determination are difficulties in producing highly ordered samples, which are required to improve line-widths and resolution. In solution NMR, the main current limitations are the size of the proteins and the relatively small chemical shift dispersion of residues in hydrophobic helices. Improvements on all these fronts may be expected in the next few years and one may look at a bright future for the structure determination of membrane proteins by NMR spectroscopy. The combination of NMR with lower-resolution techniques to solve larger structures and determine their precise disposition in the lipid bilayer looks particularly promising.

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- •• of outstanding interest

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