# Chapter 12
# Micelles, Bicelles, Amphipols, Nanodiscs, Liposomes, or Intact Cells: The Hitchhiker's Guide to the Study of Membrane Proteins by NMR

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## 12.1 Introduction

The vast majority of biophysical studies of membrane proteins (MPs) at the atomic scale are performed in vitro with preparations as homogeneous as possible, where the protein is isolated in a nonnative environment. MP samples for nuclear magnetic resonance (NMR) spectroscopy are no exception to the rule, in particular because purification helps to clearly detect and unambiguously identify signals from the protein of interest. By *native environment,* we consider the original membrane(s) where the protein exerts its biological role. It is difficult to place artificial systems on a scale defining how well they mimic native membranes, especially when a functional test is difficult or impossible to set up. Indeed, liposomes or nanometric lipid bilayers still represent artificial environments, and, on the contrary, exotic surfactants like amphipols, which could be thought to be inappropriate given their chemical structures, have proven to keep numerous MPs stable and active in solution. Over the past decades, various membrane mimetics have been developed, chosen on the basis of the compatibility with the technique of investigation used, sometimes at the expense of the functionality of the protein. Paradoxically, after so many efforts to improve membrane substitutes, *in-cell* NMR has known significant advances during the past few years (Selenko and Wagner 2007; Ito and Selenko 2010), which represents a very attractive potential for future NMR studies of MPs in situ (Renault et al. 2012a, 2012b).

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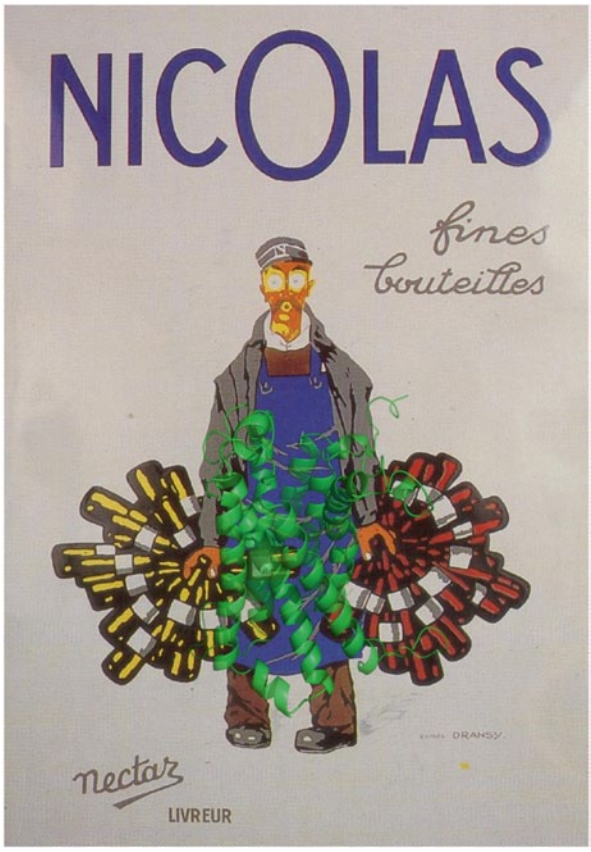
In this chapter, we describe the different environments available and their applications to MP studies by NMR spectroscopy. We treated solution- and solid-state NMR separately because sample preparations and methodologies are different, even though some environments are common to these two subtypes of NMR. In theory, protein size for solution-state NMR is limited, not in solid-state (vide infra § 12.3.1). Additional equipment is also required for solid-state NMR, such as high-power amplifiers, air compressor and dryer, etc. In order to be concise, each MP environment, with its own advantages and drawbacks, is briefly described; readers interested in more complete descriptions can find an exhaustive bibliography in Warschawski et al. (2011).

## 12.2 Solution-State NMR




Adapted from Dransy, 1923, courtesy of Nicolas

## *12.2.1 Detergents*

### 12.2.1.1 Generalities

For almost 40 years, detergents were used to characterize MPs in aqueous solutions (Helenius and Simons 1975; Tanford and Reynolds 1976), and most of NMR structural studies of MPs performed to date have been carried out in detergent solutions (Kang and Li 2011; Warschawski 2013). These molecules are amphiphilic, i.e., they possess both hydrophilic and hydrophobic parts, usually dubbed *head* and *tail,* respectively. Above a certain concentration and temperature, i.e., the critical micellar concentration (cmc) and *Kraft* temperature, detergent monomers form aggregates named *micelles,* and any addition of molecules of detergent create new micelles. In an aqueous solution, above the cmc, the hydrophilic heads are in contact with water molecules and the tails are in contact between each other. There is equilibrium between molecules of detergent associated in micelles with those existing as monomers (Fig. 12.1a). The form and size of micelles depend on the detergent chemical structure and experimental conditions, such as temperature, pH, and ionic strength. For instance, the detergent dodecyl-$\beta$-maltoside ($\beta$-DDM or C12-M), one of the most used detergents in structural biology, forms large oblate micelles in typical experimental conditions (Oliver et al. 2013), while dodecylphosphocholine (DPC or C12-PC, Fos-Choline-12 or MAPCHO-12) adopts preferentially a spherical shape (Tieleman et al. 2000). Detergents associated to MPs form complexes with a relatively small size compared to other solubilizing agents, and this is the main reason why they are the most frequently used molecules for solution-state NMR investigations of MPs.

Other detergent-based systems such as lipopeptide detergents (McGregor et al. 2003; Privé 2011) or peptide surfactants (Zhao et al. 2006) have been shown to substantially improve the stability of MPs in aqueous solutions compared to traditional detergents (McGregor et al. 2003; Yeh et al. 2005). The acyl-chain packing of these surfactants is more uniform compared to micelles, with a lateral pressure more comparable to the interior of a bilayer. They also display a low cmc, usually below the micromolar range, rendering them less dissociating than detergents (Privé 2011). These surfactants are interesting for solution-state NMR studies of MPs as they form complex sizes, once associated to MPs, similar to those measured with detergents. Indeed, the $\beta$-barrel protein PagP associated to lipopeptides gave rise to high-resolution NMR spectra (McGregor et al. 2003). Perhaps, one of the main drawbacks today remains the cost to produce these alternative molecules.

### 12.2.1.2 Illustrations

The glycophorin A was the first MP structure to be solved by NMR (MacKenzie et al. 1997, Fig. 12.1b). The experiments were conducted in DPC micelles. This study represented a major achievement, in both NMR and biochemistry: (1) by demonstrating the capacity of solution NMR to determine MP structures and

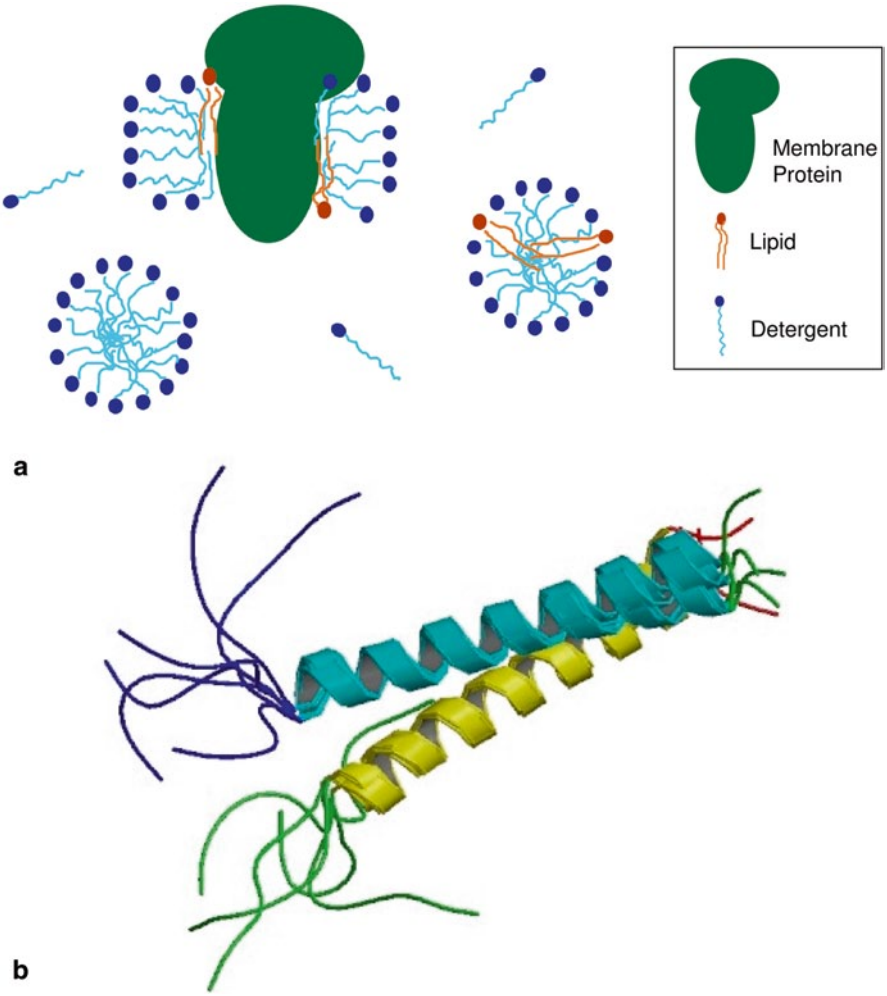



**Fig. 12.1** NMR studies of MPs in detergent solutions. **a** Schematic view of the coexisting entities in a detergent solution, the MP (in *green*), lipid cofactors (in *orange*), and detergent molecules (in *blue*). **b** The first three-dimensional structure of an MP determined by NMR: the dimeric TM domain of glycophorin A (GpA), a 40-residue peptide, in a detergent DPC solution. (Image from the RCSB PDB (www.pdb.org) of PDB ID 1AFO; MacKenzie et al. 1997)

(2) by maintaining the oligomeric state of the protein in a detergent solution, which is essentially based on van der Waals interactions in this case. Since then, more than 100 MP structures have been solved de novo by NMR in detergent solutions (Warschawski 2013), from small size bitopic MPs (e.g., Teriete et al. 2007; Lau et al. 2009; Yang et al. 2009; Wittlich et al. 2010) to larger systems (>30 kDa, which is equivalent to >70 kDa with the belt of surfactant, e.g., Schnell and Chou 2008; Hiller et al. 2008; Gautier et al. 2010). In addition, solution-state NMR studies of

MPs in detergent solutions can be used to get important structural and dynamics information, without performing a full structure determination. For instance, with the help of G protein-coupled receptor (GPCR) crystal structures, NMR spectroscopy brought fundamental observations regarding the activation of these receptors (Bokoch et al. 2010; Kofuku et al. 2012; Liu et al. 2012; Nygaard et al. 2013).

#### 12.2.1.3 Advantages

One of the best advantages using detergent for solution-state NMR is the resulting size of protein–detergents complexes that are usually smaller than complexes obtained with other classes of surfactant, despite their high propensity to inactivate MPs (Bowie 2001; Popot 2010). Novel promising detergents are regularly proposed, such as maltose-neopentyl glycol diacyl molecules (MNGs). Indeed, the thermal stability of several MPs could be substantially improved thanks to these new amphiphiles, such as the human $\beta_2$ adrenergic receptor-T4 lysozyme fusion protein or the muscarinic M3 acetylcholine receptor (Chae et al. 2010).

#### 12.2.1.4 Drawbacks

Detergents tend to destabilize MPs, essentially by disrupting intraprotein, protein–protein, or protein–lipid interactions (Fig. 12.1a). These dissociating properties explain why they were originally used to extract MPs from their native or host membranes. For a given detergent, it is usually recommended to work close to the cmc in order to reduce the presence of protein-free micelles that could absorb lipid cofactors that are essential for the stability and/or activity of the protein. Regarding the concentrations of protein and detergent required to perform an NMR study (e.g., McDonnell and Opella 1993; Arora et al. 2001), i.e., well above the cmc, the probability of working with an inactive protein is high. This is why, following the structure of the glycophorin A, pioneering studies in detergent solutions by NMR were carried out on rugged $\beta$-barrel MPs from *Escherichia coli* (*E. coli*; Arora et al. 2001; Fernández et al. 2001; Hwang et al. 2002).

Sometimes, especially with detergents that form spherical micelles, the organization of hydrophobic chains could not always accommodate MPs very well, which can be revealed by variations in NMR protein chemical shifts compared to other media. For instance, OmpX exhibits various backbone $^{15}N/^{1}H^{N}$ chemical shifts depending on the surfactant used (Fernández et al. 2001; Lee et al. 2008; Hagn et al. 2013). This mainly reflects modifications in the structure of the protein instead of transmembrane (TM) electronic environment variations, as whatever the surfactant used, the amino acids pointing towards the membrane mostly face $CH_n$ moieties of the surfactants.

Detergents can display a marked influence on the equilibrium kinetics between distinct MPs substates, depending of the cmc that is directly related to the detergent off-rate. This may be a drawback in studies that aim at looking at intra-MP

conformational exchanges. In a comparative study between the $\beta$2-adrenergic receptor solubilized in either $\beta$-DDM or MNG3 detergent solutions, different conformational exchanges of the GPCR have been observed: With $\beta$-DDM, that has a cmc four orders of magnitude higher than MNG3, faster exchanges between distinct functional states could be observed compared to the receptor associated to MNG3 (Chung et al. 2012).

Besides difficulties to maintain active or native-folded MPs with detergents, another drawback concerns the choice of the detergent to be used to perform NMR studies. Usually, any NMR investigation relies on an empirical screening of detergents and concentrations, which is quite demanding in time and costly. This is also one of the reasons that led to the development of new alternatives to conventional detergents, like bicelles (Sanders and Landis 1995), amphipols (Tribet et al. 1996), nanodiscs (Bayburt et al. 2002), lipopeptide detergents (McGregor et al. 2003), peptide surfactants (Zhao et al. 2006), or new milder detergents (Chae et al. 2010). Some of those alternative media are quite universal and can be used by following general rules. Despite the fact that most alternative media give rise to larger ensembles compared to detergents, they represent a powerful substitute, thanks to improvements in NMR methodology and instrumentation, and also in the development of new isotope-labeling schemes dedicated to the study of large proteins or protein complexes (e.g., Plevin and Boisbouvier 2012). These new environments allow the detection of well-resolved MP NMR signals (*vide infra*).

### *12.2.2 Mixed Detergent Solutions, Bicelles, or Detergents/Lipids Potpourris*

#### 12.2.2.1 Generalities

In order to improve the stability of MPs in detergent solutions, the simplest solution consists of adding lipids to detergent micelles. These binary or more complex assemblies are usually named *mixed micelles*. Indeed, lipid cofactors are known to be crucial for the activity or stability of many MPs (Lee 2004). For instance, the *sn*-1,2-diacylglycerol kinase of *E. coli* requires lipid cofactors to be active (Walsh and Bell 1986). Assays performed in mixed octylglucoside/dimirystoylphosphatidylcholine (OG/DMPC) micellar systems, showed a protein 50-fold more active compared to pure OG micelles (Badola and Sanders 1997). More recently, high-resolution atomic structures of GPCRs have revealed the presence of a conserved sterol-binding site along some TM helices (Cherezov et al. 2007; Hanson et al. 2008; Wacker et al. 2010; Warne et al. 2011; Rosenbaum et al. 2011). Resulting tests of stability performed in $\beta$-DDM/cholesterol hemisuccinate mixed micelles demonstrated an increase in stability by ~12 °C compared to the same measurements performed in a pure $\beta$-DDM solution (Thompson et al. 2011). Mixed micelles have also been used for solution-state NMR studies. For instance, the low-resolution structure of

the mitochondrial uncoupling protein 2 could be solved in mixed micelles of DPC and DMPC (Berardi et al. 2011), and low amount of mixed micelles were found to preserve the cytoplasmic domain of YgaP protein, in contrary to observations made with a protein solubilized in a pure detergent solution (Tzitzilonis et al. 2013).

From earlier studies of mixture of lipids and detergents in aqueous solutions (e.g., Gabriel and Roberts 1984), binary assemblies of detergents and lipids named *bicelles,* which contain usually a higher proportion of lipids than in mixed micelles, have been well characterized (Sanders and Prestegard 1990; Sanders and Schwonek 1992; Vold et al. 1997). Under appropriate conditions of temperature and concentration, bicelles are classically described as a planar bilayer of phospholipid stabilized by a swimming belt of detergents or short-chain lipids. Depending on the molar ratio $q$ of lipids versus detergents, two kinds of bicelles can be distinguished: large anisotropic ($q > 0.5$, *vide infra* Fig. 12.6) and small isotropic ($q \leq 0.5$) bicelles (Fig. 12.2a). Larger bicelles can be used for solid-state NMR studies of MPs (e.g., Triba et al. 2006a, *cf.* § 12.3.2. herein), while isotropic bicelles are used for solution-state NMR investigations (e.g., Czerski and Sanders 2000). MPs associated to isotropic bicelles give rise to longer overall correlation times, but still to detectable NMR signals (e.g., Lee et al. 2008, Fig. 12.2b).

#### 12.2.2.2 Illustrations

Complete structural studies of MPs in isotropic bicelles can be performed in solution by NMR (e.g., Bocharov et al. 2007, 2008; Lau et al. 2008). In an elegant comparison study, it has been shown that isotropic bicelles ($q = 0.33$) stabilize the functional form of a small multidrug-resistance transporter (Smr) compared to mono detergent solutions (Poget et al. 2007). Importantly, the authors succeeded to set up an in vitro ligand binding assay for this transporter, demonstrating that beautiful high-resolution two-dimensional (2D) $^{1}H,^{15}N$ correlation experiments obtained in various pure detergent solutions do not necessarily mean the protein is active (Poget and Girvin 2007). Despite broader NMR signals, the authors succeeded to assign signals of $^{1}H$, $^{13}C$, and $^{15}N$ nuclei of the protein backbone in bicelles (Poget et al. 2007, 2010, Fig. 12.2b).

#### 12.2.2.3 Advantages

Among some advantages, compared to pure detergent solutions, the immediate environment experienced by an MP is closer to a lipid bilayer. However, just as some mixed micelles may display some degree of organization, conversely, in the case of small isotropic bicelles, the architecture may be similar to mixed micelles rather than the idealized view of a well-segregated assembly between long-chain lipids and detergents (Triba et al. 2005, 2006b; Beaugrand 2014).

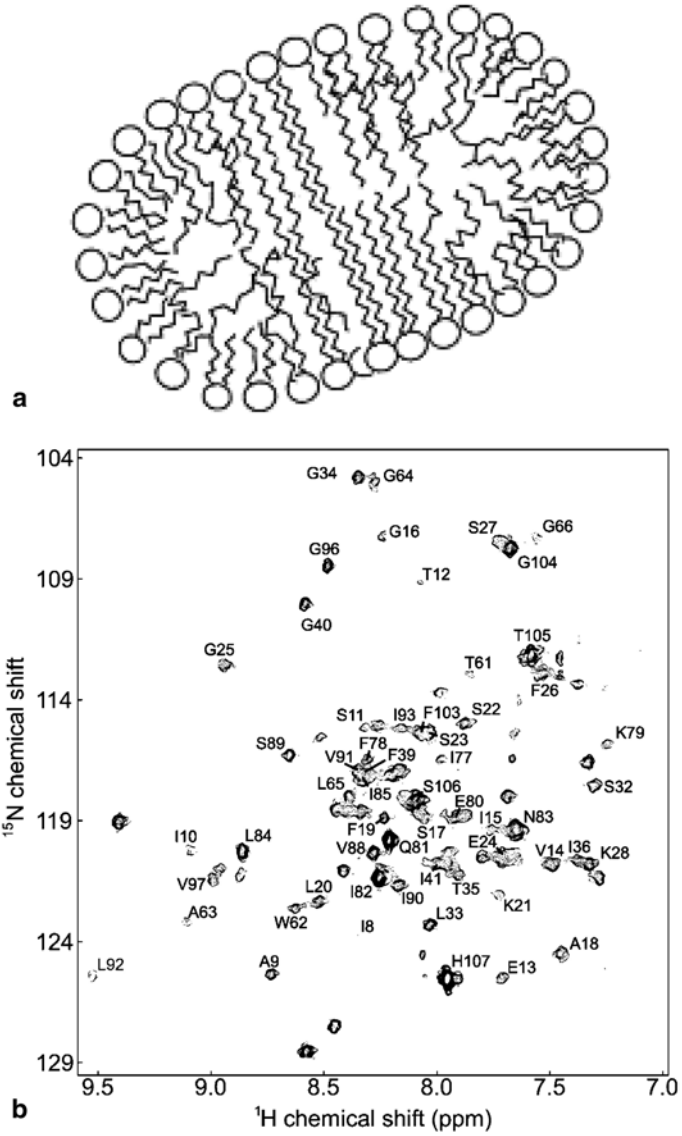



**Fig. 12.2** NMR studies of MPs in mixed micelles or bicellar solutions. **a** The cross-section of an isotropic bicelle model, in which the disk-shaped bicelle consists of a small planar bilayer domain, predominately composed of long-chain phospholipids, coated by a rim of short-chain phospholipids or detergents (reprinted from Whiles et al. 2002 with permission from Elsevier). **b** Example of high-resolution NMR data of an MP associated to isotropic bicelles ($q = 0.33$): $^{1}$H,$^{15}$N TROSY spectrum recorded at 900 MHz of 0.8 mM uniformly $^{2}$H,$^{13}$C,$^{15}$N-labeled protein Smr (pH 6.5 and 47 °C). (Reprinted from Poget et al. 2007 with permission from the American Chemical Society)

#### 12.2.2.4 Drawbacks

The detergent diffusion into the lipid disc (Triba et al. 2005, 2006b) may be a cause of protein instability. In addition, with isotropic bicelles, the current lipid compositions in use are limited, the best-characterized systems being composed of mixtures of DMPC and either dihexanoyl-sn-glycero-3-phosphocholine (DHPC) or cholamidopropyl-dimethylammonio-hydroxy-propanesulfonate (CHAPSO) as detergents. Regarding sample preparations, the molar ratio $q$ has to be kept constant in order to avoid any phase transition. This is not so trivial when buffer exchanges or temperature changes are required before reaching the NMR spectrometer or collecting NMR data.

### *12.2.3 Amphipols*

#### 12.2.3.1 Generalities

The term *amphipol* (APol) refers to short amphiphilic polymers highly chemically stable, that can substitute for detergents to keep integral MPs water soluble (Tribet et al. 1996, Fig. 12.3a). These polymers were developed to find a relevant substitute to detergents by multiplying attachment points along the TM domain of MPs. They provide: (1) a low off-rate dissociation constant for these polymers from the TM domain, rendering their association to the MP quasi-irreversible and (2) a small equilibrium dissociation constant, which means low equilibrium concentration of free surfactant. For more than 15 years, these compounds have been proved to be a valuable alternative to detergents (Popot 2010; Popot et al. 2011; Elter et al. 2014 in preparation). In addition to their stabilizing properties, APols are compatible with many biophysical techniques, including NMR spectroscopy.

#### 12.2.3.2 Illustrations

NMR studies of MP/APol complexes were carried out on several $\beta$-barrel MPs from the outer membrane of *E. coli* (Zoonens et al. 2005; Catoire et al. 2009, 2010a) or *Klebsiella pneumoniae* (Renault 2008; Planchard et al. 2014), and more recently with two $\alpha$-helical MPs, the GPCR BLT2 (Catoire et al. 2010b, 2011) and the bacteriorhodopsin (Raschle et al. 2010; Etzkorn et al. 2013). Even if no structure of MP associated to APols has been solved by NMR yet, all these studies clearly demonstrated that MP/APol complexes can give rise to highly resolved NMR signals in a short time: 2D heteronuclear $^{1}H,^{15}N$ correlation experiments to attest $\beta$-barrels associated to APols are correctly folded (Zoonens et al. 2005; Catoire et al. 2010a; Raschle et al. 2010; Etzkorn et al. 2013, Fig. 12.3b) or to look at slow dynamic chemical exchanges (Catoire et al. 2010a), 2D $^{1}H,^{13}C$ heteronuclear nuclear

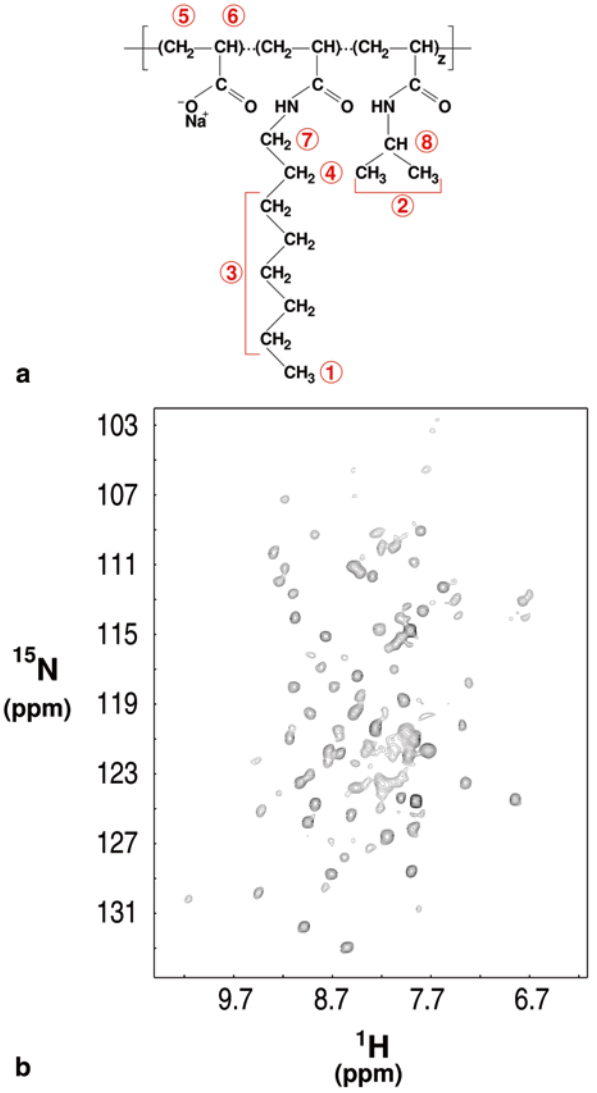



**Fig. 12.3** Solution NMR studies of MPs trapped with APols. **a** Primary chemical structure of the polyacrylate APol A8–35 (Tribet et al. 1996; Popot et al. 2011). APol A8–35 can be easily partially deuterated (named DAPol, *circled numbers 1, 2, 3, 4, 7, 8* indicating protons that are replaced by deuterons) or perdeuterated (named perDAPol). **b** Example of high-resolution NMR data of an MP associated to APols: $^{1}H,^{15}N$ TROSY spectrum recorded at 800 MHz of uniformly $^{2}H,^{15}N$-labeled TM domain of OmpA ([OmpA] = 1 mM, pH 7.9 and 30 °C). (Reprinted from Zoonens et al. 2005 with permission from the National Academy of Sciences, USA)

Overhauser spectroscopy experiments (Catoire et al. 2009), or three-dimensional (3D) $^{15}N$ edited-($^{1}H,^{1}H$) HSQC-NOESY-TROSY experiments to look at the organization of APols around MPs (Renault 2008; Planchard et al. 2014). Structures of protonated organic ligands in their protein-bound states could also be determined with MPs associated to partially deuterated APols (Catoire et al. 2010b, 2011). All these studies are reviewed in Planchard et al. (2014).

#### 12.2.3.3 Advantages

One of their major advantages over detergents is their stabilizing properties (e.g., Dahmane et al. 2009). This is particularly relevant in the context of NMR, which requires sometimes to work at high temperatures (typically 40–50 °C) during hours or days. Usually, MPs in APols come from a former stage where the protein is maintained soluble in a detergent solution. The oligomeric state of MPs in APols will depend whether or not the oligomerization has been conserved in detergents (see Planchard et al. 2014). Among some other advantages, these polymers have a very low critical aggregation concentration (equivalent to the cmc of detergents), which renders them irreversibly attached to MPs in the absence of competitive surfactants. This turns sample preparations and the handling of MPs associated to APols very easy. For instance, there is no need to add APols to the sample during buffer exchanges by dialysis. This has the practical advantage to limit the amount of APol consumed and to simplify sample preparations. Moreover, on an economical concern, APols are cost-effective compounds, which is quite interesting for NMR studies that usually require large amounts of material. These polymers can also be partially (Gohon et al. 2008) or totally deuterated (Giusti et al. 2014), which can greatly help to clearly identify protein signals in various homonuclear or heteronuclear NMR experiments.

#### 12.2.3.4 Drawbacks

MP/APol complex sizes are usually larger than the same protein trapped with detergents (Popot 2010; Popot et al. 2011). But this increase in the overall correlation time does not preclude the observation of well-resolved NMR signals, thanks to the high magnetic fields available associated to relevant methodology and isotope-labeling schemes dedicated to the studies of large proteins or protein complexes. These broader NMR signals are largely compensated by an MP stable and active, which renders highly safe any MP/APol NMR studies. Perhaps, one of the major drawbacks concerning APols is psychological, as they do not resemble at all to a lipid bilayer. But, the only relevant answer to "how far can we move away from the native lipid membrane experienced by one MP or how artificial can be the swimming belt?" relies in the fact that the protein is active, i.e., correctly folded and stable. To be noticed, APols are an artificial medium that favor the retention of lipids, in contrary to detergents, thus providing an environment that is finally closer to a membrane than what could be told by their primary chemical structures. For NMR, the polyacrylate-based APol, historically named A8–35, is highly soluble at pH > 7. In acidic solutions, carboxylate groups start to protonate, leading to the progressive aggregation of the polymer. Working in basic solutions can be detrimental to observing exchangeable protons (Wüthrich 1986). Consequently, a bunch of different APols soluble in the 0–14 pH range are now available and have been validated for NMR (see Dahmane et al. 2011; Bazzacco et al. 2012).

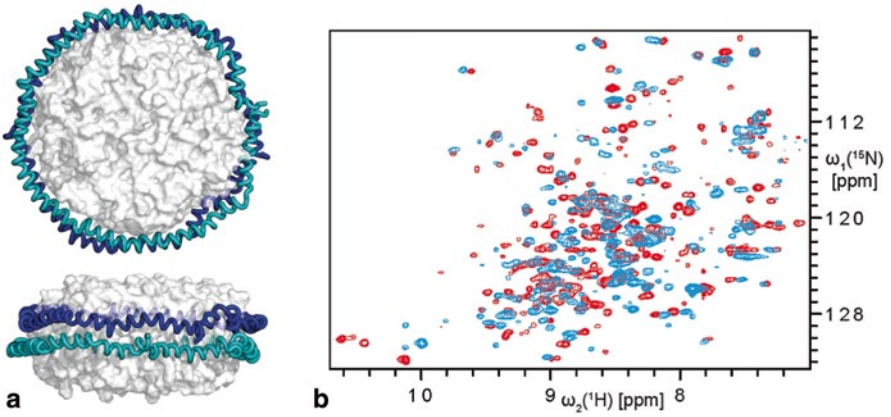



**Fig. 12.4** Solution NMR studies of MPs embedded in nanodiscs. **a** Schematic view of nanodiscs, modeled with POPC as lipid. Lipid bilayer fragment (*white space filling*) is encircled by two amphipathic helices of membrane scaffold proteins (*blue ribbons*) (reprinted from Nath et al. 2007 with permission from the American Chemical Society). **b** 2D [$^{1}$H,$^{15}$N]-TROSY spectrum of uniformly $^{2}$H,$^{15}$N-VDAC-1 in DMPC nanodiscs (*blue*) and in LDAO micelles (*red*). (Reprinted from Raschle et al. 2009 with permission from the American Chemical Society)

## *12.2.4 Nanometric Lipid Bilayers*

### 12.2.4.1 Generalities

Nanometric lipid bilayers, often referred to as *nanodiscs,* have been designed to conduct in vitro biophysical studies of MPs (Bayburt et al. 2002; Denisov et al. 2004; Ritchie et al. 2009). A nanodisc is a non-covalent assembly of a phospholipid bilayer surrounded by a dimer of a genetically engineered lipoprotein named *membrane scaffold protein* (MSP; Fig. 12.4a). Various MSP have been engineered from the original sequence of human serum apolipoprotein apoA-I, which physiological role, through discoidal high-density lipoprotein particles, consists in reversing the transport of cholesterol (Ohashi et al. 2005). The size of the MSP defines the size of the nanodiscs and various lipids or mix of lipids that can be used to form the discoidal bilayer architecture (Ritchie et al. 2009).

### 12.2.4.2 Illustrations

A tremendous number of studies of MPs embedded in nanodiscs have been described in the literature (Nath et al. 2007; Ritchie et al. 2009; Bayburt and Sligar 2010). MPs associated to these discs are amenable to either solid-state (*vide infra* § 12.3.4) or solution-state NMR studies. Indeed, high-resolution NMR spectra can be obtained in solution (e.g., Tzitzilonis et al. 2013; Glück et al. 2009; Shenkarev

et al. 2010; Yokogawa et al. 2012; Shenkarev et al. 2013). Among these examples, the human mitochondrial voltage-dependent anion channel (VDAC-1), a 19-stranded $\beta$-barrel MP, in DMPC nanodiscs gave rise to a high-resolution 2D $^{1}H,^{15}N$-TROSY spectrum (Raschle et al. 2009). These NMR data were sufficiently different from those obtained with the protein in dodecyl-dimethylamine-oxide (LDAO or C12-DAO) micelles (Fig. 12.4b), requiring a novel sequence-specific resonance assignment of VDAC-1 in nanodiscs. More recently, the first structure of an MP embedded in a nanodisc, OmpX, has been determined by NMR in solution with the help of truncated MSP variants (Hagn et al. 2013). A comparison of the different OmpX structures obtained in different mimetics indicated differences in both the extracellular loops and the length and relative orientation of TM $\beta$-strands. Dynamics measurements also showed substantial differences, all the data underlying the impact of the artificial medium chosen on the structural and dynamical properties of MPs outside their native membranes.

#### 12.2.4.3 Advantages

Nanodiscs offer a very convenient adjustable—in size and lipid composition—bilayer environment for in vitro studies of MPs. Like with APols, MPs associated to nanodiscs are highly stable, enabling the collection of NMR data at 50 °C during hours or days. Another advantage concerns studies of homo-oligomeric and hetero-oligomeric proteins. In most of the pre-discussed nonnative environments, it may sometimes be difficult to maintain the oligomeric state of proteins, especially in the framework of solution NMR studies that are conducted with high concentrations of surfactants. This is especially the case for systems involving the use of detergents. With nanodiscs, playing with the ratio of nanodiscs versus MP and the size of the nanodiscs at the reconstitution step, it is possible to adjust the oligomerization state of the protein (Ritchie et al. 2009).

#### 12.2.4.4 Drawbacks

Even if desorption of lipids has been evaluated to be 20 times faster than in liposomes (Nakano et al. 2009), MPs embedded in nanodiscs at concentrations compatible with NMR studies in solution have been proven to be highly stable (Nath et al. 2007; Ritchie et al. 2009; Bayburt and Sligar 2010). One of the major drawback concerns sample preparations, even though once the MSP has been produced, the reconstitution procedure is quite straightforward and universal. MSP production and purification, along with production of the tobacco etch virus (TEV) protease, is time consuming, but the MSP can be kept stable frozen at −80 °C for months. It may also be time consuming to find the appropriate lipid composition to get a fully active protein. Depending on the lipid composition used, sometimes it may be advantageous to work with deuterated lipids (e.g., Tzitzilonis et al. 2013). Unfortunately, many lipids are not available deuterated, even partially. Like with bicelles

and APols, the size of the MP/nanodisc complexes are much bigger than the corresponding size of the MPs in a detergent solution. This has led, for instance, to the construction of a new MSP variant leading to smaller nanodiscs (Hagn et al. 2013). Like with APols, broader NMR signals observed with nanodiscs can be compensated by working at higher temperatures.

## 12.3 Solid-State NMR

Courtesy of Edith Godard

### *12.3.1 A Little Bit of Theory*

Solid-state NMR is the application of NMR to molecules that do not *tumble fast and isotropically*. This is due to the fact that, in such a case, the effects of *orientation-dependent couplings* often manifest themselves as severe line broadening on the NMR spectra. Depending on the molecule, the temperature, the nuclei that will be observed, the magnetic field, etc., several couplings will be dominating. Those couplings involve, as often in NMR, energy transfer that can be expressed in units of *frequency rate*. The difference between *fast* and *slow* tumbling is thereby defined by comparison to the strongest effective coupling present.

Without going into too many details, it is important to state that most orientation-dependent couplings bear a $(3\cos^2\theta - 1)$ angular dependence, where $\theta$ is the angle between the magnetic field and the line that connects the two coupled nuclei. Without such knowledge, one would be tempted to try to tumble the sample fast and

*isotropically,* which is not very practical. Knowing the simple $(3\cos^2\theta - 1)$ angular dependence allows to suggest ways to get rid of the broadening.

Since we cannot force θ for all pairs of atoms in the sample, and at all times, to be such that $(3\cos^2\theta - 1) = 0$, or $\theta \sim 55°$ (also known as *the magic angle*), two options are offered:

- Aligning all molecules to a single orientation in the sample, so that instead of averaging out the couplings, they will have a single value throughout the sample, and the superimposition of resulting NMR spectra will be a narrow spectrum.
- Setting conditions to get the *average* value $(3\cos^2\theta - 1) = 0$, by placing the sample in a rotor macroscopically aligned at the magic angle, and spinning it fast, faster than the strongest effective coupling present.

In solution-state NMR, resonance lines broaden with protein size because they depend on the tumbling rate that averages $(3\cos^2\theta - 1)$ to zero when the molecular correlation time is around the nanosecond or faster. By contrast, in solid-state NMR, other approaches are at stake and linewidths are independent of the protein tumbling rate: therefore, theoretically, there is *no* protein size limit in solid-state NMR. Nevertheless, perfect alignment and infinite fast spinning rate are impossible to reach: Residual linewidths in solid-state NMR are broader than in solution-state NMR and $^{1}H - ^{1}H$ dipolar couplings that can reach 120 kHz are almost impossible to get rid of. In other words, biological solid-state NMR today is mostly applied to $^{13}C$ and $^{15}N$ NMR, in rotors of 2–7 mm diameter, 2–300-µl volume, and 5–50-kHz maximum spinning rate.

Combined with those mechanical approaches, another way to remove couplings between two nuclei is also to suppress one of the nuclei by replacing it with a *cold* isotope. Biochemistry has provided the NMR spectroscopist with a variety of isotopic labeling schemes, especially for the suppression of most large couplings in neighboring nuclei, leaving untouched either the smaller couplings among nuclei that are far away, or among selected isolated pairs of nuclei (Abdine et al. 2011). Typical examples involve growing proteins on selectively labeled glycerol (Castellani et al. 2002, Fig. 12.5) or glucose (Loquet et al. 2011).

## *12.3.2 Aligned Solid-State NMR: Glass Plates and Large Bicelles*

### 12.3.2.1 Generalities

Aligning all biomolecules to a single orientation at the blow of a whistle may seem like a foolish dream, but in the case of lipids, we are approaching this possibility (Dürr et al. 2007a; Warschawski et al. 2011). First, they spontaneously align on a glass plate, with the bilayer normal perpendicular to the plate plane. This approach was used in the 1980s and helped determine the first MP structures, mostly by the

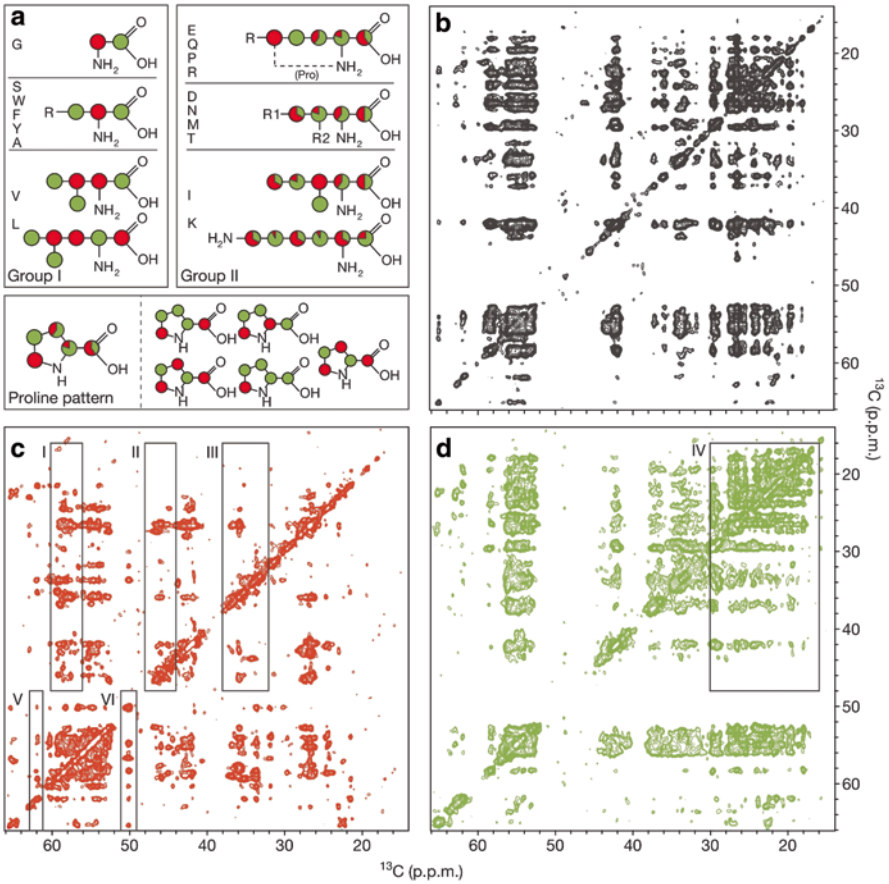



**Fig. 12.5** Labeling patterns and NMR spectra for different protein preparations. Schematic representation of the effective $^{13}C$ enrichment for indicated amino acids by growth on [1,3–$^{13}C$]glycerol (*green*) or [2–$^{13}C$]glycerol (*red*) (**a**). 2D $^{13}C$-$^{13}C$ solid-state NMR spectra under magic-angle spinning at 8 kHz on α-spectrin SH3 domain grown on uniformly labeled glucose (**b**), [2–$^{13}C$] glycerol (**c**), or [1,3–$^{13}C$]glycerol (**d**). (Reprinted from Castellani et al. 2002 with permission from Macmillan)

groups of Tim Cross and Stan Opella. Second, combined with short-chain lipids, long-chain lipids may form bicelles that also spontaneously align in the magnetic field. Bicelles were discovered in the 1990s (Sanders and Schwonek 1992) and are shaped as 500-Å diameter *camemberts* or *wheels,* composed of a planar lipid bilayer, generally made of around 6,000 DMPC molecules, surrounded by *tires* of around 2,000 short-chain lipids, usually DHPC. Depending on the ratio between both lipids, as well as temperature, bicelles can also be too small to align in a standard NMR magnetic field. In such a case (for example, 50 DMPC, 200 DHPC, and a 100-Å diameter), they are called *isotropic bicelles* and were described in an earlier section, for use in solution-state NMR (vide supra § 12.2.2. herein).

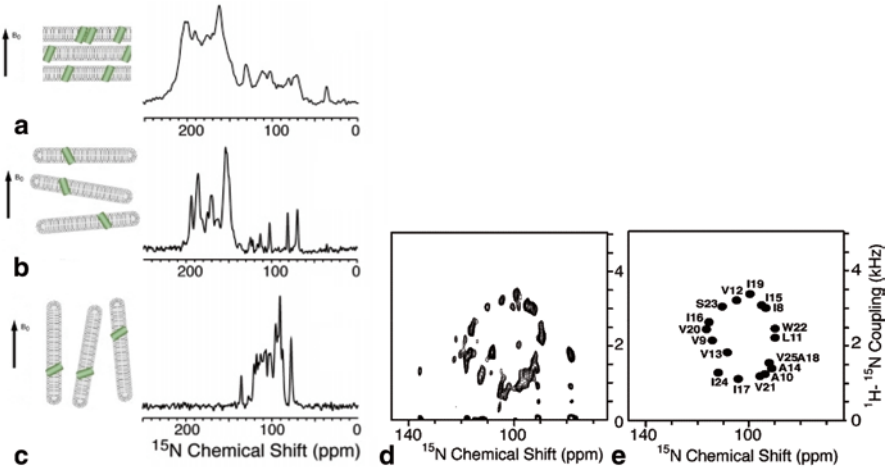



**Fig. 12.6** Solid-state NMR spectra of the TM domain of Vpu in lipid bilayers differently aligned: on glass plates (**a**), in flipped bicelles (**b**), or in normal bicelles (**c–e**). **a–c** are 1D $^{15}$N NMR spectra while **d** is a 2D $^{15}$N-$^{1}$H $-^{15}$N NMR spectrum allowing for resonance assignment (simulated and shown in (**e**)) and helix tilt determination (determined here to be approximately 30° with respect to the bilayer normal). (Adapted from De Angelis et al. 2004 with permission from the American Chemical Society. The cartoons on the left are adapted from Dürr et al. 2007a with permission from Elsevier)

#### 12.3.2.2 Illustrations

Recent progress in magic-angle spinning NMR has confined aligned solid-state NMR to mostly low-resolution structure determination, such as peptide orientation in the membrane, either on glass plates (Gong et al. 2004; Michalek et al. 2013) or bicelles (De Angelis et al. 2004; Triba et al. 2006a; Dürr et al. 2007b; Müller et al. 2007; Park et al. 2011a; Cook et al. 2011). Several high-resolution structures of small proteins were determined as well (Ketchem et al. 1993; Opella et al. 1999; Park et al. 2003; De Angelis et al. 2006; Hu et al. 2007; Traaseth et al. 2009; Ahuja et al. 2013), sometimes with the help of other complementary techniques such as solution-state NMR or X-ray crystallography (Warschawski 2013). Figure 12.6 shows typical NMR spectra of proteins reconstituted in lipids on glass plates, *normal* bicelles, or *flipped* bicelles (supplemented with small amount of lanthanides), of which the helix tilt is deduced.

#### 12.3.2.3 Advantages and Drawbacks

Aligned solid-state NMR is more useful for the determination of peptide orientation in the membrane than for ab initio MP complete structure determination. Lipids on glass plates can be aligned almost regardless of lipid composition and temperature, at any given orientation in the magnet. Large bicelles can only align with their

bilayer normal perpendicular or parallel to the magnetic field (*flipped* bicelles), and only with special lipid composition and in a specific temperature range. Nevertheless, bicelle samples are better hydrated, easier to prepare, and more convenient in case one needs to change the buffer. In addition, as can be seen on the spectral resolution in Fig. 12.6, the average lipid alignment is better in bicelles than on glass plates.

### *12.3.3 Magic-Angle Spinning: Liposomes*

#### 12.3.3.1 Generalities

Liposomes are mostly composed of lipids that can be chosen from an incredible variety of charges, headgroups, chain lengths, or insaturations. Spontaneously, lipids and water form heterogeneous multilamellar vesicles of around 1-µm diameter and up to a dozen bilayers. Several MP reconstitution methods are available, usually, but not necessarily, with the help of detergent molecules.

#### 12.3.3.2 Illustrations

Solid-state NMR of MPs in liposomes is almost exclusively performed under magic-angle spinning. Residual linewidths have hampered the structure determination of many MPs, but assignment is on the way for various proteins (Andronesi et al. 2005; Hiller et al. 2005; Lange et al. 2006; Frericks et al. 2006; Etzkorn et al. 2007; Shi et al. 2009; Abdine et al. 2010; Emami et al. 2013). Dynamic information can also be obtained (Ullrich et al. 2011; Yang et al. 2011; Williams et al. 2013). Recent structures include the small protegrin (Mani et al. 2006), influenza M2 channel (Cady et al. 2010), membrane domain of Mer F (Lu et al. 2013), as well as the large seven-helix G protein-coupled chemokine receptor CXCR1 (Park et al. 2012).

The structure of CXCR1 determined by solid-state NMR shares significant similarities with that of CXCR4 determined by X-ray crystallography (Park et al. 2012, Fig. 12.7). Differences mostly reflect the modifications made to the sequence of CXCR4 required for crystallization: In contrast with the NMR sample made of wild-type protein embedded in a liquid crystalline phospholipid bilayer, the crystal is made of a mutant protein, mostly by replacing the third intracellular loop (ICL3) by T4 lysosyme, and by removing the last 33 C-terminal residues. Removing ICL3 rendered CXCR4 incapable of activating G proteins, while CXCR1 in the NMR sample was fully active with respect to both G protein activation and chemokine binding. In addition, the C-terminus of CXCR1 forms a well-defined helix (H8) that aligns along the membrane surface. This helix, as well as the membrane, is absent from the crystal of the mutated CXCR4.

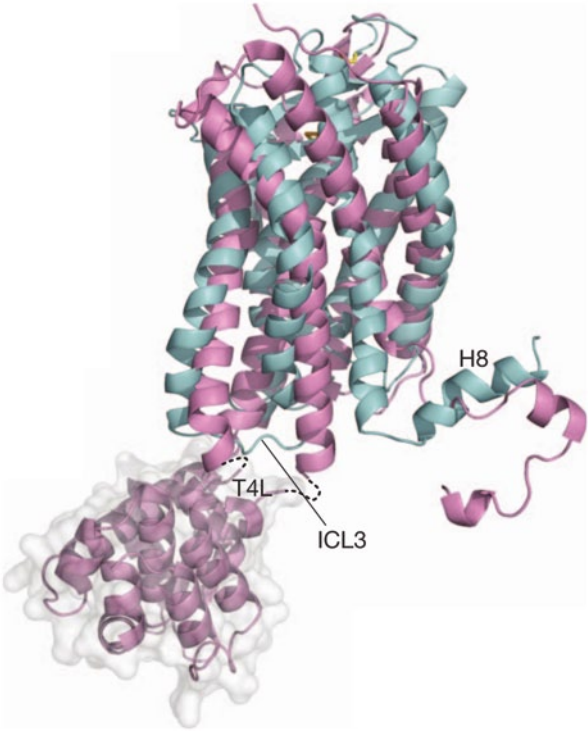



**Fig. 12.7** Backbone structural comparison of CXCR1 determined by solid-state NMR (PDB accession 2LNL, in *cyan*) and CXCR4 determined by X-ray crystallography (PDB accession 3ODU, in *pink*). The third intracellular loop (ICL3) of CXCR4 is replaced by T4 lysozyme (T4 L) present in the crystal. The C-terminus of CXCR1 forms a well-defined amphipathic helix (H8), whereas that of CXCR4 is only loosely helical. (Reprinted from Park et al. 2012 by permission from Macmillan)

#### 12.3.3.3 Advantages and Drawbacks

Magic-angle spinning NMR has shown its ability to determine high-resolution 3D structures of proteins. Liposomes are supposed to be the most natural local environment (in terms of size, shape, curvature, thickness, fluidity, lateral pressure, dielectric constant, hydration…) for MPs, where their structure and dynamics are supposed to be native-like, and where they can be studied in a functional state (Warschawski et al. 2011; Park et al. 2012). Lipids, which are the major constituent in the sample, can be chosen to suit the protein, if necessary. On the other hand, liposomes are heterogeneous multilamellar vesicles, where MPs may also experience heterogeneous conformations, slight differences between monomers in an oligomer, or slow and fast motion that, altogether, may broaden the NMR lines and affect the spectral resolution.

#### 12.3.3.4 Future Perspective

Dynamic nuclear polarization (DNP) is a technique that can be combined with solid-state NMR under magic-angle spinning for signal enhancement of up to 120 on frozen samples. It has successfully been applied to MPs in liposomes, such as the

SecYEG translocon, allowing to detect 40 nmol of peptide bound to the translocon (Reggie et al. 2011) or to the M2 proton transporter of Influenza A, allowing the precise positioning of rimantadine bound to the protein (Andreas et al. 2013). Such a progress will undoubtedly allow new applications for solid-state NMR, including structure determination of MPs in complex environment in a near future.

### *12.3.4 Nanodiscs and Other New Environments for Solid-State NMR*

#### 12.3.4.1 Nanodiscs

The smallest nanolipoproteins described in an earlier section of this chapter (*vide supra* § 12.2.4. herein) have a diameter of 100 Å, comparable to small isotropic bicelles. As opposed to bicelles, those nanodiscs, or larger discs dubbed *macrodiscs* of up to 300-Å diameter (Park et al. 2011b), could be precipitated for solid-state NMR, at any given temperature. Since they are monodisperse, proteins in precipitated nanodiscs are expected to be more homogeneous than in liposomes of various sizes and lamellarities. Such samples should therefore provide narrower NMR lines under magic-angle spinning. In some cases, nanodisc samples could also accommodate a higher protein-to-lipid ratio, thus providing more intense NMR lines. Nevertheless, few examples of solid-state NMR studies of MPs have been shown to use nanodiscs so far (Kijac et al. 2007; Mörs et al. 2013).

#### 12.3.4.2 Other Options

In the same line of thought, MPs can sometimes be (micro)crystallized to provide homogeneous, monodisperse samples that are known to give high-resolution solid-state NMR spectra (Castellani et al. 2002). Several MPs have followed this trend usually limited to soluble or fibrillar proteins: Structures of the small HNP1 defensin (Zhang et al. 2010), large complexes of DsbB (Tang et al. 2011, 2013), and the anchor domain of YadA (Shahid et al. 2012) have been determined by solid-state NMR using this procedure (Warschawski 2013). Other less common membrane mimetics for solid-state NMR, including APols, have also been described in a recent review (Warschawski et al. 2011).

### *12.3.5 Intact or Fragmented Cells Studied by NMR*

#### 12.3.5.1 Generalities

Cellular structural biology has known tremendous advances along the 2000s decade. Among the few techniques that can give rise to structural information at the

atomic level, NMR spectroscopy represents today a unique opportunity to work with intact or slightly modified biological samples. Solution-state NMR can give rise to highly resolved NMR signals of proteins in living cells (e.g., Inomata et al. 2009), opening the possibility to determine a complete protein structure de novo (Sakakibara et al. 2009). With MPs, the use of solution-state NMR techniques is essentially limited to observing ligand–protein interactions (e.g., Claasen et al. 2005; Assadi-Porter et al. 2008; Potenza et al. 2011), solid-state NMR being by far the most appropriate approach. Since solid-state NMR resolution is not affected by the large size or slow motion of the macromolecule observed, MPs can be studied in large liposomes or even the intact cells where they were produced or fragmented cellular membranes. Not only would the proteins be in their native environment (both the membrane and the soluble partners) but they will also be preserved from any potentially denaturing reconstitution protocol. Here, the main difficulty is to be able to isolate signals coming from the protein of interest in the forest of signals from all other molecules in the sample. $^{31}$P solid-state NMR of intact cells may also be a method of choice to follow the kinetics of membrane assembly, the production of lipids, and energetics parameters such as the adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio.

#### 12.3.5.2 Illustrations

Recent studies have tackled the proteosome and other biomolecules (MPs, polysaccharides, carotenoids, lipids, etc.) of several organisms such as *A. thaliana* (Dick-Pérez et al. 2011), *E. coli* (Renault et al. 2012a; Tardy-Laporte et al. 2013), *C. tepidum* (Kulminskaya et al. 2012), or *S. enterica* (Zandomeneghi et al. 2012). Others use bacterial cells to study individual proteins of interest (over-)expressed in *E. coli,* such as the human LR11 (Fu et al. 2011), the human FK506-binding protein (Reckel et al. 2012), the M2 from influenza virus (Miao et al. 2012), the diacylglycerol kinase (Shi et al. 2012), or the b subunit of the F1Fo ATP synthase (our current work).

Our studies of the b subunit are taking advantage of a mutant of *E. coli* called C43(λDE3) (Miroux and Walker 1996; Arechaga et al. 2000), known for overproducing *internal membranes* when this protein is over-expressed. Focusing on these internal membranes rather than on whole cells, and using various tricks (low or high temperatures, different pulse schemes, sample preparation, etc.), we manage to reduce background signals (such as those coming from lipids) and discriminate signals arising from proteins. We validate this approach by comparing the spectrum of biological samples with that of model membranes with the same lipid composition as these internal membranes (see Fig. 12.8).

#### 12.3.5.3 Future Perspective

DNP is particularly suited for the detection of small amount of active MPs in intact cells. It is not clear if structure determination is reachable, but attempts have already

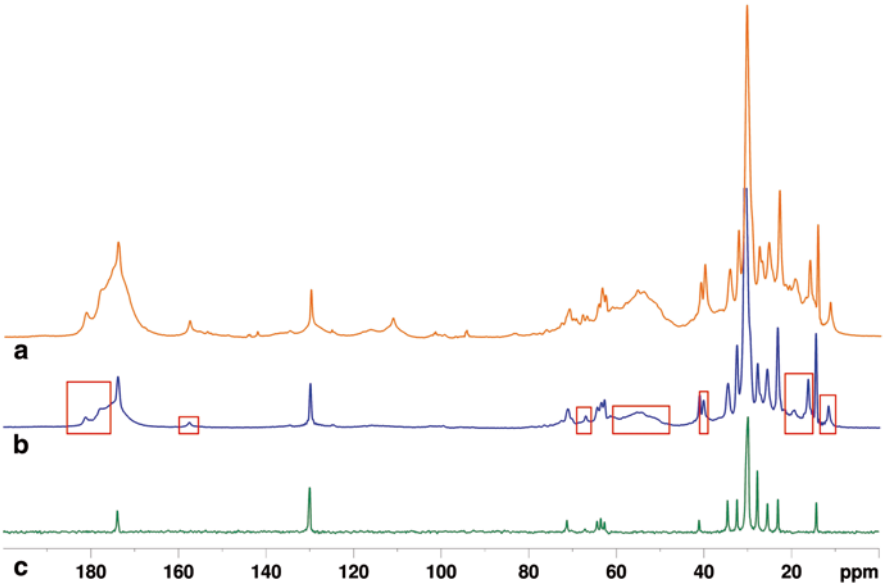



**Fig. 12.8** NOE-enhanced $^{13}C$ solid-state NMR spectra recorded at 700 MHz of samples under magic-angle spinning at 11 kHz. **a** Whole C43(λDE3) cells over-expressing the b subunit. **b** Purified internal membranes. **c** Model lipid membranes. Protein signals are highlighted by *red squares*

included bacteriorhodopsin (Bajaj et al. 2009), the acetylcholine receptor (Linden et al. 2011), mistic (Jacso et al. 2012), the whole proteosome of *E. coli* (Renault et al. 2012b), or the cell walls of *B. subtilis* (Takahashi et al. 2013).

## 12.4 Conclusion

This chapter is like a *hitch-hiker guide to the study of membrane proteins by NMR* in the jungle of surfactants and lipids for a clear overview on the best possible membrane mimetics. Detergents are a world of their own, which fascinates well beyond NMR: What would the biochemist do without detergents to solubilize, purify, transfer, renature, reconstitute, manipulate, and basically study MPs? If that was not enough, fellow scientists have gone out of their way to invent new molecules such as amphipathic polymers and nanoparticles of lipids and proteins, or new techniques such as magic-angle spinning. In the country of lipids, we have described artificial membranes of various sizes and lamellarity, and the quasi black magic that lets lipid self-align, either on glass plates or in the intriguing *bicelles*. Eventually, *native-like* membrane mimetics were replaced by real *native* cell membranes, the graal of MP structural biology. Two routes were suggested: either solid- or solution-state NMR, but always through the arduous path of biochemistry. With such a guide, the

biochemist can take his/her favorite MP on an NMR journey, and hopefully come back home with a picture of its 3D structure.

All those paths have shown to lead to structures, but they have also shown rough passes that require technical improvement. While it is not clear what new route will be opened, we can already suggest directions in which to look for:

- *Isotopic labeling.* In solution-state NMR, selective methyl labeling helps pushing the limit to studying protein–surfactant complexes beyond 100 kDa. In solid-state NMR, sparse labeling is necessary to improve the spectral resolution.
- *Ligand binding.* Both in solution- and solid-state NMR, focusing on the ligand in a protein–ligand complex is both easier and often a niche that is inaccessible to X-ray crystallography. In addition, it can freeze a single protein conformation that would improve the resolution in solid-state NMR.
- *Combination of various techniques.* Solution- and solid-state NMR, X-ray crystallography, small-angle X-ray scattering, electron, atomic-force or light microscopy, structural mass spectrometry, and molecular modeling should be combined in order to close the resolution scale gap between structural and cellular biology, including dynamic processes, of MPs in ever increasingly complex environments, and get a more complete picture of these proteins.

One exciting new development highlighted in our guide is *in-cell* NMR. Sensitivity and resolution issues impose long experiments under conditions that are unfavorable for cell survival. Thereby, in most experiments that we have described here, the cell membranes and proteins were untouched but the cells were most likely dead. With little technical improvement already tested by our colleagues in metabolomics (Gowda et al. 2012; Jordà et al. 2013), one can hope to develop efficient in vivo structural and dynamical NMR in the near future.

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