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Minireview

NMR solution structure determination of membrane proteins reconstituted in detergent micelles

César Fernández*, Kurt Wüthrich

Institut für Molekularbiologie und Biophysik, Eidgenössische Technische Hochschule Zürich, CH-8093 Zürich, Switzerland

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Abstract As an alternative to X-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy in solution can be used for three-dimensional structure determination of small membrane proteins, preferably proteins with β-barrel fold. This paper reviews recent achievements as well as limiting factors encountered in solution NMR studies of membrane proteins. Our particular interest has been focused on supplementing structure determination with data on the solvation of the proteins in the mixed micelles with detergents that are used to reconstitute membrane proteins for the NMR experiments. For the *Escherichia coli* outer membrane protein X (OmpX) in dihexanoylphosphatidylcholine (DHPC) micelles, such studies showed that the central part of the protein is covered with a fluid monolayer of lipid molecules, which seems to mimic quite faithfully the embedding of the protein in the lipid phase of the biological membrane. The implication is that the micellar systems used in this instance for the NMR studies of the membrane protein should also be suitable for further investigations of functional interactions with other proteins or low-molecular weight ligands.
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Key words: Transverse relaxation-optimized spectroscopy-nuclear magnetic resonance; Membrane protein structure; Isotope labeling; Mixed protein–detergent micelle

1. Introduction

In structural proteomics projects, membrane proteins present a particular challenge. On the one hand, membrane proteins take part in a large number of important physiological functions, and constitute key targets for drug development, so that knowledge of their three-dimensional (3D) structures could contribute decisively to better understanding of biological processes at the molecular level. On the other hand, structural studies of membrane proteins have traditionally been limited by technical and practical difficulties. High-yield expression, purification and refolding of membrane proteins for studies either by X-ray crystallography or by nuclear magnetic resonance (NMR) spectroscopy are still much more demanding than the corresponding work with soluble proteins, and it is generally difficult to crystallize membrane proteins from detergent solutions. For solution NMR experiments, membrane proteins have to be solubilized in polar solvents by incorporation in model membrane systems, and in the past, the size of the resulting protein/detergent/lipid supramolecular assemblies was typically too large for a structure determination in solution (Fig. 1b).

Modern solution NMR techniques now enable studies of much larger structures through the application of the principles of transverse relaxation-optimized spectroscopy (TROSY) [1–5] (Fig. 1c) and cross-correlated relaxation-enhanced polarization transfer (CRINEPT) [6,7], with recent applications to particles in the molecular mass range 50–900 kDa (see, for example, [8–10]). Small isotope-labeled membrane proteins in an environment of unlabeled detergents are ideal objects for the use of TROSY-NMR, since in spite of the overall large size of the mixed micelles, the complexity of the resulting NMR spectra is manageable [2,3].

For biophysical, structural and functional studies of membrane proteins, detergent micelles, bicelles, lipid bilayers or lipid vesicles are commonly used as a replacement of the natural membrane environment (for example, [11–14]). For solution NMR studies, the combined demands of modest overall size of the protein/detergent/lipid supramolecular structure and preservation of the functional structure of the protein have so far most promisingly been met by reconstitution in micellar structures (Fig. 1). This paper is therefore primarily focused on NMR applications with mixed protein–detergent micelles.

2. Producing membrane protein samples for solution NMR studies

At the outset of a project, it is especially important for membrane proteins to obtain a high-yield expression system for the desired protein, because membrane proteins typically have to be labeled with the stable isotopes ^{2}H, ^{13}C and ^{15}N for multidimensional heteronuclear NMR experiments [15]. Isotope labeling is intrinsically quite expensive, and deuteration often causes a drastic yield reduction of protein synthesis

*Corresponding author. Present address: Novartis Pharma AG, P.O. Box, 4002 Basel, Switzerland. Fax: (41)-61-324 2686.
E-mail address: cesar.fernandez@pharma.novartis.com (C. Fernández).

Abbreviations: 3D, three-dimensional; DHPC, dihexanoylphosphatidylcholine (1,2-dihexanoyl-*sn*-glycero-3-phosphocholine); NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; OmpA, outer membrane protein A from *Escherichia coli*; OmpX, outer membrane protein X from *Escherichia coli*; TROSY, transverse relaxation-optimized spectroscopy

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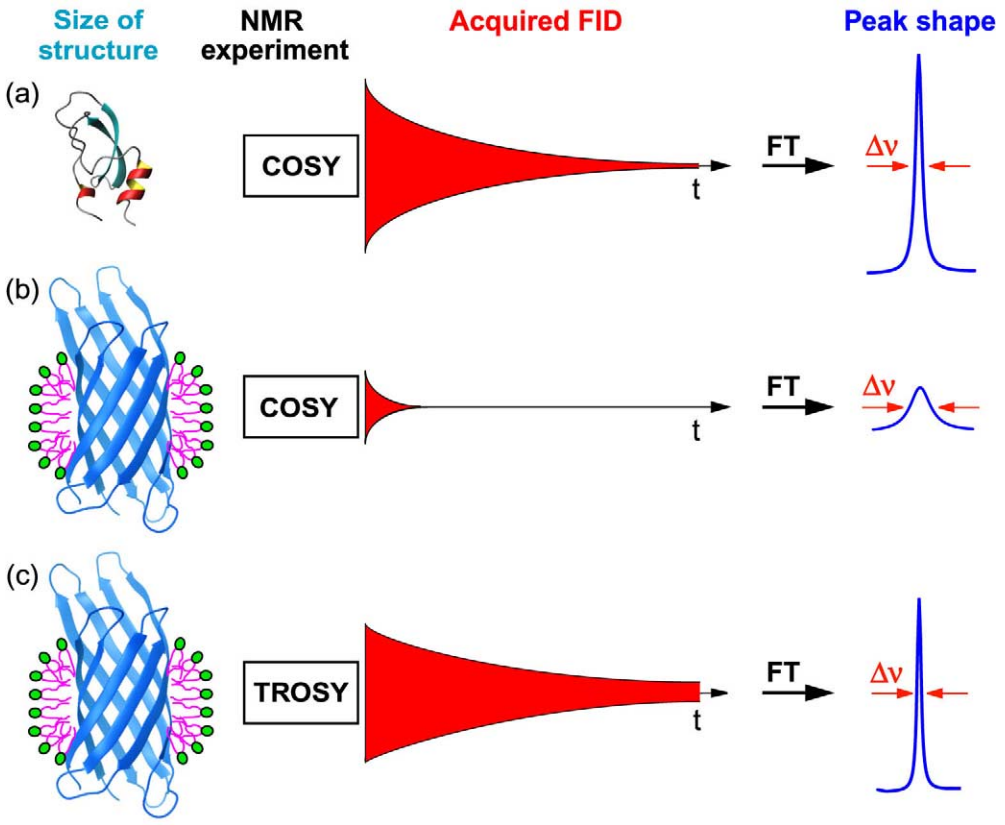

Fig. 1. Visualization of key features of solution NMR spectroscopy with polypeptide chains in smaller and larger structures. a: For small proteins, high-frequency Brownian motion results in slow loss of magnetization by transverse relaxation during the entire experiment. As a result, the free induction decay (FID) recorded during the acquisition period starts with high amplitude, because of the slow relaxation during the preceding elements of the NMR experiment, and decays slowly. Correspondingly, high sensitivity and narrow linewidths are obtained in the frequency domain spectrum after Fourier transformation (FT) of the FID. b: Slow tumbling of larger structures, such as membrane proteins in detergent micelles, results in rapid loss of magnetization due to fast transverse relaxation. The acquisition therefore starts with a smaller amplitude, the recorded FID decays rapidly, and correspondingly low sensitivity and broad lines are obtained in the NMR spectrum. c: Using TROSY and ^{2}H labeling of the protein, rapid transverse relaxation can be largely suppressed, which results in improved spectral resolution and sensitivity (see also Fig. 3).

due to the negative influence of the deuterated medium on the cell metabolism. For the structure determinations of the β-barrel *Escherichia coli* outer membrane proteins OmpX [16–18], OmpA [19] and PagP [20], the proteins were expressed in high yields in inclusion bodies in *E. coli*. The proteins were then dissolved in concentrated urea or guanidinium hydrochloride solutions, and subsequently refolded in the presence of detergent micelles (Fig. 2). For α-helical membrane proteins, there is so far no generally recommended refolding protocol available. Attempts at sample preparation are therefore also based on constitutive expression, where the protein may in favorable cases be isolated in the folded form from the membrane (Fig. 2), but low yields may make the preparation of isotope-labeled NMR samples prohibitively expensive. Wherever protein expression cannot be achieved in bacteria, which might be the case with many eukaryotic membrane

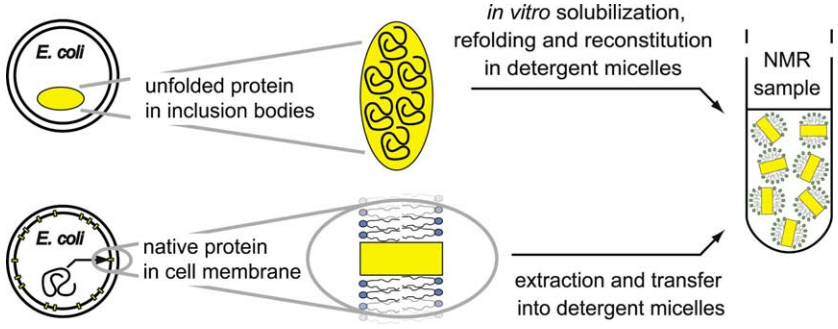

Fig. 2. Two production strategies of membrane proteins in *E. coli* for NMR studies. a: The protein is overexpressed into and extracted from inclusion bodies, followed by refolding in vitro and reconstitution in detergent micelles. b: The protein is constitutively expressed and folded, and subsequently extracted from the membranes and transferred into detergent micelles.

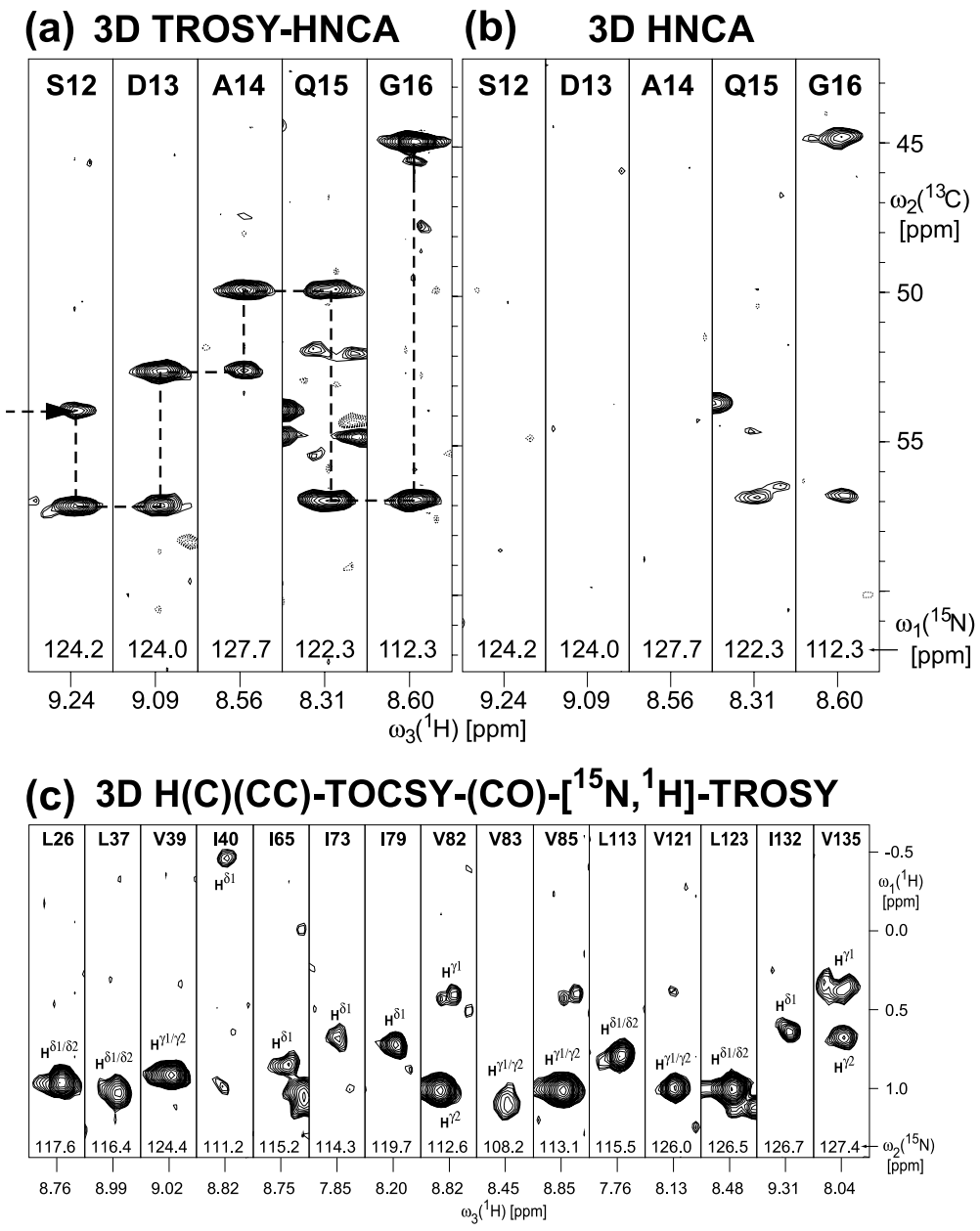

Fig. 3. NMR spectroscopy with the uniformly [^{2}H,^{13}C,^{15}N]-labeled membrane protein OmpX in unlabeled DHPC micelles (2 mM solution of OmpX, aqueous solvent containing 20 mM phosphate, 100 mM NaCl, 0.05% NaN_3, 200 mM DHPC and 5% $^{2}H_2O$, pH = 6.8, T = 30°C). a: 3D [^{15}H,^{1}H]-TROSY-HNCA spectrum. b: Conventional 3D HNCA spectrum. c: 3D H(C)(CC)-TOCSY-(CO)-[^{15}N,^{1}H]-TROSY spectrum measured with selectively methyl-protonated and otherwise uniformly [^{2}H,^{13}C,^{15}N]-labeled OmpX, [u-^{2}H,^{13}C,^{15}N/L,V,Iδ^{1}-$^{13}CH_3$]OmpX/DHPC. The spectra a and b were recorded on a Bruker DRX-750 spectrometer, spectrum c on a Bruker DRX-500 spectrometer equipped with a triple-resonance cryoprobe.

proteins, possible resort to eukaryotic expression systems may again result in prohibitively high costs. In this situation, preparative cell-free protein synthesis systems might become a valid alternative also for the production of membrane proteins [21–23].

The search for appropriate solution conditions for the NMR samples may need to consider a larger number of variable parameters for membrane proteins than for soluble proteins. In addition to the temperature, the pH and the ionic strength, one has to consider the choice of the detergent, the detergent concentration, and the protein-to-detergent ratio. Moreover, membrane protein solutions tend to deteriorate in the NMR sample tubes, especially at the elevated temperatures, typically above 30°C, that are commonly preferred for NMR spectroscopy. Long-time stability of the sample is thus an additional variable to take in account during the optimization process.

3. Refined isotope labeling strategies for membrane proteins

Uniform ^{2}H labeling is required to benefit optimally from the TROSY effect in NMR experiments with large molecules or macromolecular complexes, such as membrane proteins in detergent micelles [1–5]. However, extensive deuteration limits the data collection to spin systems with labile protons, i.e. in practice to the ^{15}N-^{1}H groups. From the limited set of nuclear

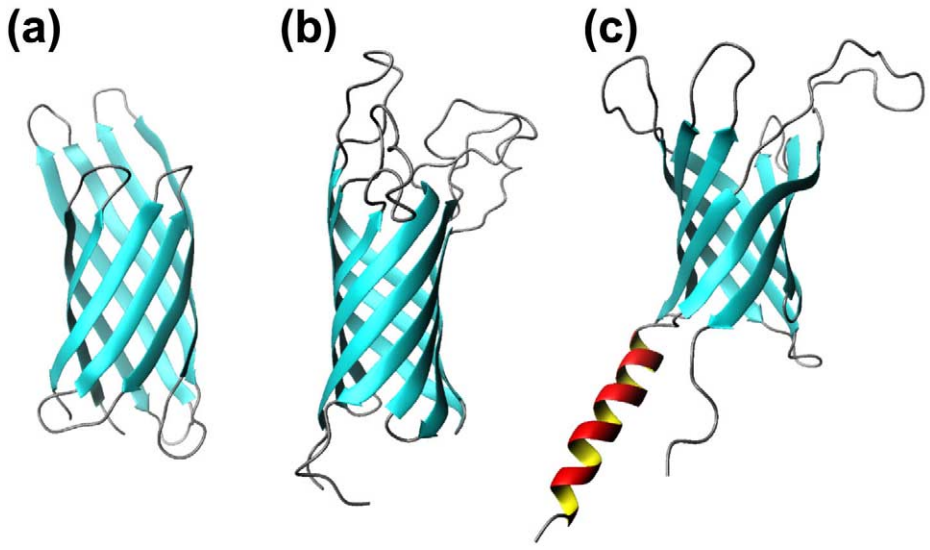

Fig. 4. Solution NMR structures of three β-barrel membrane proteins. a: OmpX in DHPC micelles. b: OmpA in DPC micelles. c: PagP in *n*-octyl-β-D-glucoside micelles. The figures have been prepared with the program MOLMOL [53].

Overhauser effect (NOE) distance constraints thus accessible, low-precision structures can be obtained for β-barrel proteins, whereas for α-helical membrane proteins usually only the secondary structure can be determined. Additional selective protonation of specific positions in the molecule, such as protonation of methyl groups in the predeuterated background, can result in greatly improved precision of the structure determination [15,24], using NMR experiments that correlate the $^{13}CH_3$ resonances with backbone ^{15}N-^{1}H resonances via the $^{13}CD_n$ moieties separating the protonated methyl groups and the backbone ^{15}N-^{1}H moieties. This strategy was recently applied with the protein OmpX in mixed micelles with dihexanoylphosphatidylcholine (OmpX/DHPC), with protonation of the Val, Leu and Ile(δ1) methyl groups [25]. To this end, the isotope-labeled amino acid precursors α-ketoisovalerate and α-ketobutyrate were added to the growth medium before induction of protein overexpression [26]. With this sample, 3D H/C(CC)-TOCSY-(CO)-[^{15}N,^{1}H]-TROSY experiments allowed the sequence-specific assignment of all protonated protein methyl groups in OmpX/DHPC [25] (Fig. 3c). Subsequent analysis of the 3D ^{15}N-resolved [^{1}H,^{1}H]-NOESY and ^{13}C-resolved [^{1}H,^{1}H]-NOESY spectra yielded a five-fold increase of the number of NOE distance constraints and a concomitantly greatly improved precision of the NMR structure of OmpX/DHPC [18]. Supplementary experimental constraints for membrane protein structure determination can presently be primarily expected to result from measurement of residual dipolar couplings [27], and from the use of paramagnetic spin labels [28,29].

4. NMR spectroscopy and structure determination of membrane proteins in micelles

The use of TROSY-based NMR experiments combined with the aforementioned isotope labeling strategies enabled the presently discussed NMR structure determinations of integral membrane proteins reconstituted in detergent micelles. For these large structures, the advantages of TROSY are particularly remarkable when performing multidimensional triple-resonance experiments, where the application of TROSY gives sensitivity gains of more than an order of magnitude [10,30–33]. As illustrated in Fig. 3 for OmpX in DHPC micelles, this advance with TROSY-based NMR actually enabled the resonance assignment and structure determination procedure. It appears that all presently available NMR structures of larger integral membrane proteins (Fig. 4) have been determined during the last three years using TROSY-based NMR techniques [11,16–20].

In our studies of OmpX and OmpA in DHPC micelles, we used the same techniques for the collection of NOE distance constraints and the structure calculation as with soluble proteins. Thereby, due consideration was given to the fact that longer ^{1}H-^{1}H distances are observable with (^{1}H,^{1}H)-NOEs in a deuterated background. Similarly, structure calculations of membrane protein in other laboratories appear to have been performed with the established methodology for soluble proteins.

5. Some recent membrane protein structure determinations by NMR

The NMR structure of OmpX (148 residues) in DHPC micelles of about 60 kDa molecular mass was solved based on data collection with a sample containing selectively protonated Val, Leu and Ile(δ1) methyl groups on a perdeuterated background [18] (Fig. 4a). The polypeptide backbone fold of OmpA (177 residues) has been determined in dodecylphosphocholine (DPC) micelles of 50 kDa molecular mass [19]. However, in DPC [19] as well as in DHPC micelles [17] the NMR signals of the residues located at the interface between the well-structured central part of the β-barrel and the peripheral loops (Fig. 4b) were not observed, presumably due to conformational exchange line broadening. The backbone fold of the outer membrane enzyme PagP (164 residues) has been determined both in DPC and *n*-octyl-β-D-glucoside micelles of size 50–60 kDa (Fig. 4c) [20]. The architecture of each of these three proteins consists of an eight-stranded antiparallel β-barrel, where sequentially successive β-strands are connected by loops on the extracellular and the periplasmic sides (Fig. 4).

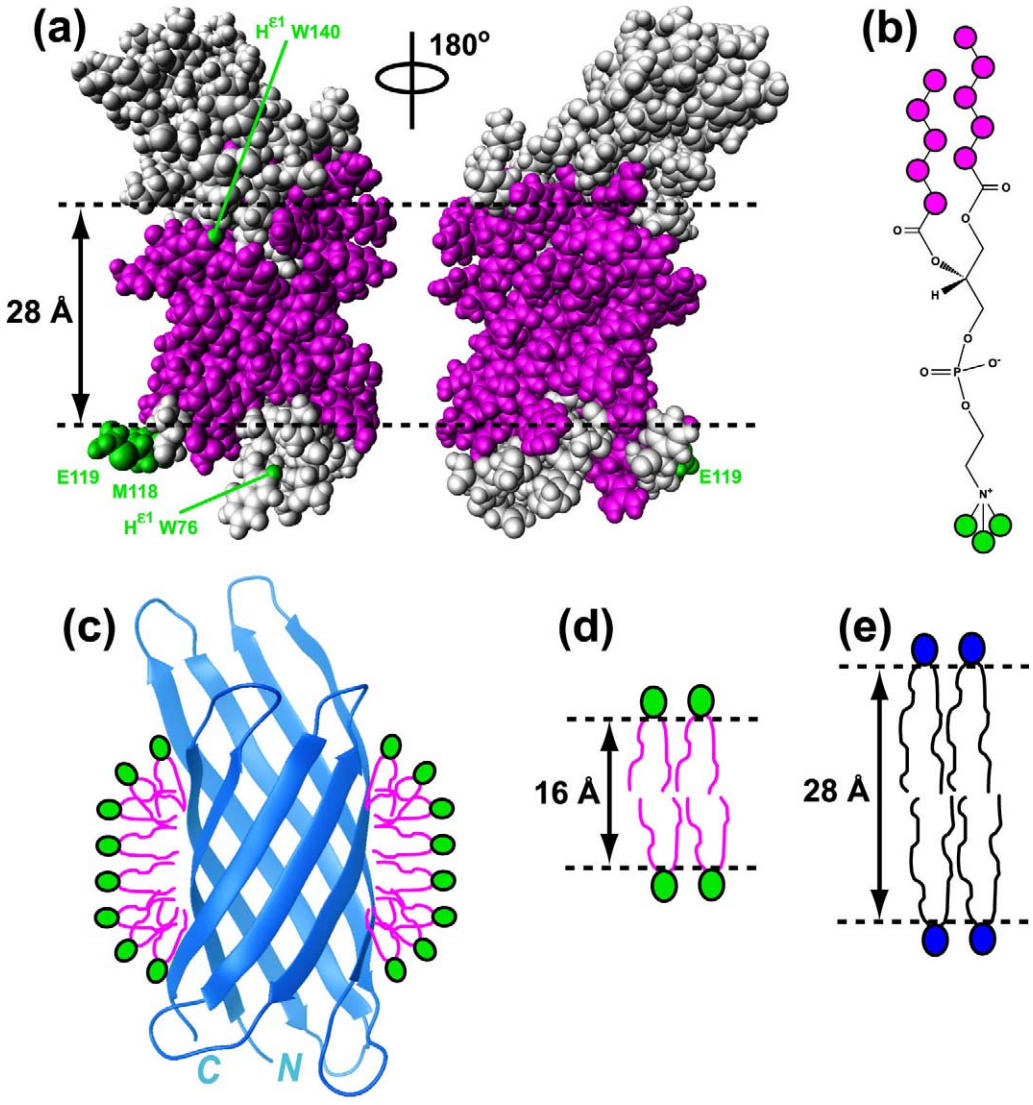

Fig. 5. NMR data on lipid–protein interactions in DHPC micelles containing the integral membrane protein OmpX. a: Space-filling all-atom representation of the NMR structure of OmpX in DHPC micelles. The drawing on the right was generated by a 180° rotation about a vertical axis of the molecular model on the left. The residues that showed NOEs between protons of OmpX and the hydrophobic tails of DHPC are colored magenta. Residues with NOEs from the amide proton to the polar head methyl groups of DHPC are green and identified with the one-letter amino acid symbol and the sequence position; for NOEs to side chain hydrogens, the atom position is also indicated. b: Chemical structure of DHPC. Magenta circles represent the CH_n groups of the hydrophobic tails, and green circles denote the polar head methyl groups. c: Schematic drawing of OmpX/DHPC protein–lipid micelles. In addition to this general arrangement of protein and lipid molecules, the NMR data show that the detergent phase is 'fluid', with lateral diffusion of the DHPC molecules, and possibly exchange of DHPC molecules in and out of the mixed micelles on a sub-millisecond timescale (see text). d: Hypothetical DHPC bilayer, with indication of the thickness of the hydrophobic phase. e: Schematic drawing of the *E. coli* outer membrane, with indication of the thickness of the hydrophobic phase.

Although for the membrane-associated α-helical 29-residue polypeptide hormone glucagon in DPC micelles the secondary structure was determined by NMR already in the early 1980s [34], NMR structure determination of α-helical membrane proteins has so far yielded less complete results than for the β-barrel proteins of Fig. 4. Examples of systems studied include the dimeric transmembrane domain of human glycoporin A (2×40 residues) [35], the light-harvesting 1β subunit of *Rhodobacter sphaeroides* (48 residues) [36], the coat proteins from the filamentous bacteriophages M13 (50 residues) [37] and fd (50 residues) [38], the bacteriorhodopsin fragment comprising residues 1–71 [39], the 81-residue human immunodeficiency virus (HIV) membrane-associated protein Vpu [40] and the 80-residue bacterial membrane transport protein MerF [41]. With regard to future developments, recently reported results for the 39-kDa homotrimeric protein diacylglycerol kinase (DAGK) in micellar complexes with overall sizes larger than 100 kDa [42,43] suggest that NMR-based structure determination of membrane proteins as large and complex as some members of the G-protein-coupled receptor family may soon be envisaged.

6. NMR studies of the solvation of membrane proteins in micelles

Studies of the solvation of the protein surface in mixed OmpX/DHPC micelles started from the observation that there was only one set of NMR lines for the ensemble of all DHPC

molecules in the micelles. This indicated that lateral diffusion and possibly exchange of individual detergent molecules in and out of a given micelle are fast on the NMR chemical shift timescale [44]. A similar situation was thus encountered as with solvent water, which typically also presents only a single NMR line. A similar approach could thus be taken as for NMR studies of the hydration of proteins in solution, which has been described in detail [45]. It turned out that numerous intermolecular protein–detergent (^{1}H-^{1}H)-NOEs could be observed [46]. The negative sign of these NOEs showed that the lifetime of the detergent molecules is longer than about 1 ns and that the effective correlation time for the (^{1}H-^{1}H)-NOEs is dominated by the overall rotational tumbling of the mixed micelles. Compared to the studies of solvation with water, the work with detergents is facilitated in this regime because of minimal interference from the presence of hydroxyl-bearing amino acid side chains [47]. Similar to the studies with hydration water, all spatial information has to be based on sequence-specific resonance assignments of the protein, and because of the dependence of the NOEs on the inverse sixth power of the intervening distance, only a first layer of solvating detergent molecules will be detected. Supplementary information, when compared with the situation with water, is obtained on the orientation of the detergent molecules relative to the protein surface, i.e. close approach either by the lipophilic tail end or by the hydrophilic headgroup (Fig. 5c).

In the remainder of this section we illustrate the above general considerations with an investigation of the OmpX/DHPC mixed micelles [46]. Analysis of the intermolecular NOEs revealed that close contacts between the DHPC hydrophobic tails and the amide protons cover the surface of OmpX over a range of approximately 28 Å centered about the middle of the β-barrel (magenta in Fig. 5a), which coincides closely with the hydrophobic surface area of OmpX. In contrast to this homogeneous and continuous distribution of hydrophobic DHPC contacts on the OmpX surface, intermolecular NOEs of the polar moieties of DHPC with OmpX are confined to highly specific locations of the protein surface (green in Fig. 5a) at the periphery of the barrel formed by the residues with hydrophobic contacts with DHPC molecules. These interactions appear to be amino acid-type specific, and such observations might eventually contribute new insights into the widely different behavior of membrane proteins in solutions with different detergents [12,14]. In the OmpX/DHPC micelles, the detergent molecules appear to be predominantly oriented perpendicular to the protein surface, forming a cylindrical belt around the hydrophobic surface. The experiments would thus be in line with previous model considerations on the embedding of membrane proteins in detergent micelles [48,49]. The formation of a torus-like DHPC bilayer around the protein, which would cause a mismatch of about 10 Å relative to the height of the hydrophobic area on the OmpX surface of 28 Å (Fig. 5a), can in this system be excluded from the NMR data.

NMR data on protein–detergent contacts may also lead to information about the stoichiometry and size of the mixed micelles. For OmpX/DHPC, the molecular mass estimated assuming dense packing of DHPC molecules on the lipophilic OmpX surface (Fig. 5a) is about 52 kDa, since about 80 DHPC molecules can simultaneously contact OmpX in this area. From experimental measurements, the approximate molecular mass of the OmpX/DHPC micelles is known to be in the range 50–70 kDa. The NOEs observation that the hydrophobic tails of DHPC form a continuous layer on the hydrophobic OmpX surface thus also leads to the conclusion that a single protein molecule is present in each OmpX/DHPC micelle.

Considering that membrane proteins will probably in most or all instances need to be reconstituted in artificial milieus for 3D structure determination, it is of interest to compare these model systems with the natural environment of the membrane proteins. The presently discussed NMR data show for OmpX/DHPC mixed micelles that the protein surface area covered by DHPC (Fig. 5a) corresponds closely to the area that is assumed to be lipid exposed in a biological lipid bilayer membrane (Fig. 5e). Although the orientation of the lipid molecules relative to the protein surface is obviously different in the micelles and in lipid bilayers, a similar hydrophobic coating appears to be achieved with the two different arrangements of the lipid molecules (Fig. 5c and e). These observations indicate that the DHPC micellar system should be suitable also for functional studies of OmpX. The experimental approach illustrated here with OmpX/DHPC should be applicable as well with other membrane proteins and different detergents or lipids.

7. Conclusions and outlook

The use of solution NMR techniques for studies of integral membrane proteins reconstituted in detergent micelles has only just started. The initial results are encouraging, indicating that NMR may also provide complementary information to the structural data that can be obtained by diffraction methods. The next steps in further establishing the utility of the solution NMR approach will be structure determination of new types of transmembrane proteins with β-structures, for which less direct leads from other methods will be available than for some of the presently discussed β-barrels, and of transmembrane proteins with α-helical secondary structure. Finally, the initial results about the solvation of a membrane protein in detergent micelles indicate additional potentialities of the method for functional studies of membrane-standing receptor proteins, including SAR by NMR [50–52].

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