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Authors

Cross, Timothy A
Murray, Dylan T
Watts, Anthony

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Helical Membrane Protein Conformations and their Environment

Timothy A. Cross[1,2,3], **Dylan T. Murray**[2,3], and **Anthony Watts**[4]

Timothy A. Cross: cross@magnet.fsu.edu; Anthony Watts: anthony.watts@bioch.ox.ac.uk

[1]Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306, USA

[2]Institute of Molecular Biophysics, Florida State University, Tallahassee, FL 32306, USA

[3]National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL 32310, USA

[4]Biomembrane structure Unit, Biochemistry Department, University of Oxford, South Parks Road, Oxford OX1 3QU, UK

Abstract

Evidence that membrane proteins respond conformationally and functionally to their environment is gaining pace. Structural models, by necessity, have been characterized in preparations where the protein has been removed from its native environment. Different structural methods have used various membrane mimetics that have recently included lipid bilayers as a more native-like environment. Structural tools applied to lipid bilayer-embedded integral proteins are informing us about important generic characteristics of how membrane proteins respond to the lipid environment as compared with their response to other non-lipid environments. Here, we review the current status of the field, with specific reference to observations of some well-studied α-helical membrane proteins, as a starting point to aid the development of possible generic principals for model refinement.

Keywords

Membrane proteins; solid state NMR; lipids; membrane protein crystallography

I. Introduction

Every biochemist knows that when the environment of a protein is changed by the addition of a denaturant at high concentration that the membrane protein structure changes, and yet the environments used for membrane protein structural characterization differ greatly from their native environment, often not including any lipid. Even if lipids are captured in a structural model, their functional influence is unclear without independent further study. However, very few questions have been raised about the native-like character of these membrane protein structures. For some of these structures, a correlation between function and structure provides some assurance that the structure is native-like, but for many membrane protein structures the correlation is not obvious. Typically, functional assays need to be performed in a different and more native-like environment. Recently, it has become possible to obtain high-resolution structural restraints for membrane proteins in lipid bilayer environments, even cellular membrane environments (Miao et al. 2012; Fu et al. 2011; Renault et al. 2012; Kamihira et al. 2005; Sivertsen et al. 2009), and for some cases, it has been suggested that lipids visualized in crystal diffraction models, are not representative of the entire protein interface (Marsh and Pali 2013).

Membrane proteins have evolved to perform functions that exploit the properties of membranes, as well as ways of responding to the membrane as a modulator of function.

Functions include transport, recognition, signaling and energy production, properties that require the asymmetry and vectorial nature of the membrane. Membrane proteins may change location during their lifetime, for example during internalization, or transit from one compartment to another. Each of these membranes may have different and changing lipid compositions, and hence may present to the protein a varying physicochemical environment.

In efforts to produce atomic resolution structural models of membrane proteins, mimetics of many forms have been developed. Functional consequences for the protein are likely to vary depending on the protein and the mimetic environment, and this is expected to be reflected in more or less conformational perturbations for the protein. In an analogous way, well studied soluble proteins show variable folding characteristics in various solvents, and so it is to be expected that membrane proteins will respond to their local “solvent” environment. Indeed, membrane proteins are likely to be more sensitive to environmental change than soluble proteins because of the increased hydrophobic amino acid composition of the transmembrane (TM) domain that minimizes specific interhelical interactions (Zhou and Cross 2013b).

Here, the current evidence about the influence of membrane biophysical properties on membrane protein conformations is discussed. The influence of specific lipid-protein interactions on membrane protein function is a relatively mature field (Marsh and Watts 1982; Lee 2003; Watts 1998; Hunte and Richers 2008), but here the focus is on the influence of membrane properties on α–helical TM protein structure and conformation at higher resolution than previously possible, as a result of recent developments in several methodologies. Unfortunately, with one exception, bacteriorhodopsin (Grigorieff et al. 1996), it has not been possible to characterize high resolution 3D structures of membrane proteins in their native membrane environment. Instead, the environment has to be modeled to achieve the samples necessary for NMR spectroscopy, x-ray diffraction or electron diffraction. Here, we will discuss not only the properties of membranes, but also those that are known for membrane mimetic environments. Based on the differences in the membrane and membrane mimetic properties, we will discuss the perturbing influence of these mimetic environments on the structural characterization of membrane proteins. Structural examples will inform a view about the differences, sometime subtle, between the various environments, and provide new insights into the energetics of tertiary and quarternary structural stability under these various environmental conditions. The result is enhanced recognition of the structural perturbations that can be induced by varying the environmental conditions. We conclude with a section on how to recognize a native-like TM domain structure of a α-helical membrane protein and suggestions for validating the native-like structural character of such proteins.

II. Sophistication of the Membrane Environment and Implication for Membrane Protein Structure, Dynamics and Function

A. Overview of the heterogeneous membrane environment that makes life possible

Membrane proteins, both peripheral and integral, have evolved together with the properties of lipid bilayers to affect their functional, dynamic, and structural roles. These semipermeable barriers are essential for life, controlling all that goes in and out of cells and organelles. It is the membrane, with its embedded and peripheral proteins, that conducts these essential functions. TM proteins themselves take unique advantage of the membrane properties to achieve their functional roles. The membrane is far more than a simple solvent, described in many textbooks by a few solvent slabs, and the spectrum of permissible conditions that support native conformations for integral membrane proteins have been shown to be rather narrow (Popot and Engelman 2000), whereas for soluble proteins, they

are relatively broad (Timmasheff 1993). This sensitivity of membrane proteins for their environment originates in the complex nature of the membrane, that can be regarded as an anisotropic solvent with numerous physical and chemical gradients across it, and separates bulk aqueous environments by a thin ~50 Å span. It is this phyiso-chemical complexity that generates a heterogeneous environment for membrane proteins. Neutron and X-ray scattering profiles reporting on nuclear and electron densities across the membrane have been modeled to characterize the distribution of lipid groups in a highly dynamic liquid-crystalline environment (Wiener and White 1992; White and Wiener 1996). In addition, a dynamic equilibrium is essential for membrane turn-over, redistribution of components, and assembly of functional molecular determinants, such as ion channel components and signaling cascades, some of which are transient in nature.

Hundreds to thousands of chemically distinguishable molecules, including membrane proteins, form native membranes generating an environment that influences the structure, dynamics and function of these same membrane proteins. Although not the subject of this review, membrane proteins can also modify the properties of the membrane environment. Native membranes are composed of two amphipathic lipid monolayers with their polar domains providing an interaction surface with the bulk aqueous environment, and their hydrophobic domains oriented away from the aqueous environment. The composition of the two monolayers is often distinct. For instance, in erythrocytes the outer leaflet is dominated by phosphatidylcholine and sphingomyelin, while the inner leaflet is dominated by phosphatidylethanolamine and phosphatidylserine (Verkleij et al. 1973). For all membrane proteins, a unique orientation in this asymmetric membrane environment is achieved by the cellular machinery that inserts the proteins into or onto the membrane, and this topogenesis must clearly be lipid-dependent. Across these bilayers there are substantial chemical and electrical potentials, all of which may vary for different membranes, such as the plasma membrane, Golgi apparatus, and inner mitochondrial membranes. Specific membrane proteins may be exposed to a range of membrane lipid environments during their lifetime, as clearly described by Sanders (Sanders and Mittendorf 2011), but while the lipid composition may vary, the physical properties for a given membrane environment may remain relatively constant as sensed by a functional membrane protein.

Much has been written about specific lipid-protein interactions that may be largely electrostatic in nature to aid, for example, assembly or insertion of TM proteins (Rothman and Lenard 1977; Dowhan and Bogdanov 2009), or the stability of oligomeric structures (Raja et al. 2007), or as essential requirements for functional activity (Marius et al. 2008; Powl et al. 2008). Examples exist for both polar moieties acting like ligands or substrates to facilitate membrane protein function, as well as the hydrophobic lipid components to modulate the protein interface, and its physiochemical properties, within the membrane. Here, the focus is on the influence of the collective physical properties of the membrane, such as hydrophobicity of the membrane, its hydrophobic thickness, surface curvature and lateral pressure profile on the structure of membrane proteins.

Polar head groups are highly mobile and possess the anionic, neutral or zwitterionic charged moieties of the lipids. Together these groups with inorganic as well as organic ions bound in the head group region, account for the charge state of each membrane surface. Phosphatidyl-ethanolamine has a special involvement in protein folding and insertion in some systems (Dowhan and Bogdanov 2009). The polar head groups provide an interface of non-water mediated H-bonds (Sixl and Watts 1982) and reduced mobility for water between the aqueous phase and the hydrophobic core. Outside of the head group region, and away from the membrane, the environment may have yet further chemical complexity from, for example, oligosaccharides or cytoskeletal protein networks. Despite this complexity, membrane protein domains outside of the membrane behave essentially like soluble

proteins, as with the ligand binding domains of class C GPCRs (G-protein coupled receptors) and of large ligand-gated ion channels (Figure 1), as just two examples where a signal is received extramembranously, and then signals are communicated through the complexities of the membrane to the cellular interior.

The fatty acyl and sterol composition of membranes make up the hydrophobic domain of the membrane interstices. For the fatty acids there is a complex mixture of chain lengths, straight chains, saturated and unsaturated, methylated as well as small cyclic hydrocarbon structures. As an extreme case, the outer membrane of *Mycobacterium tuberculosis* has chains of mycolic acids with up to 90 carbons (Barry 1998), whereas a typical eukaryotic plasma membrane has chains lengths of 16 or 18 carbons. The hydrophobic thickness of membranes can influence membrane protein function (Marsh 2008) and the tilt of TM helices (Killian and Nyholm 2006; Strandberg et al. 2004; Holdbrook et al. 2010; Park and Opella 2005), even in oligomeric structures (Duong-Ly 2005). Changing the length of TM helices can alter the targeted membrane for a K^+ channel from the mitochondrial to plasma membrane (Balss et al. 2008). Raft-like domains with high cholesterol and sphingomyelin have a much greater hydrophobic thickness that selectively solubilizes specific proteins (Zhou and Cross 2013b; Lingwood and Simons 2010).

B. Water and dielectric gradients

The environment for membrane proteins includes the aqueous environment with a dielectric constant (ε) of ~80 at 20°C and the hydrocarbon interior of the bilayer that is essentially devoid of water with a low dielectric of ε~2, typical of an alkane environment (Figure 2). Recent profiling of the dielectric constant across a lipid bilayer has this very low value for ±10Å from the bilayer center, and between ±10Å and ±17Å in POPC bilayers, a dielectric constant of 3 or 4 (Stern and Feller 2003; Nymeyer and Zhou 2008), while in the headgroup region ε reaches 200 or more due to the high density of dipoles from the charged groups (Figure 2). This range of approximately 10^2 in dielectric constant has a profound impact on the strength and distance dependence of electrostatic interactions. While substantial energy is required to stabilize partial charges exposed to the very low dielectric of the membrane interstices, long-range (distances ≥10Å) electrostatic interactions have been shown to be functionally important in this region of the membrane (Hu and Cross 1995; Steffen et al. 1994). For the headgroup region, electrostatic interactions are scaled by the large dielectric constant even more so than in an aqueous environment, and therefore significant electrostatic interactions will be restricted to much shorter distances.

Water permeation through synthetic bilayers (rates ~2.2×10^{-3} cm/s) has been the subject of considerable debate for many decades (Finkelstein 1976), however, there are many native membranes that have a very low permeability to water (Mathai et al. 2001). Cholesterol, sphingomyelin and methylated acyl chains are known to reduce such permeability (Finkelstein 1976; Marsh 2001; Simon et al. 1982; Mathai et al. 2001). The relatively higher permeability in unbranched diacyl lipids is thought to result from jumps between ‘void defects’ in the hydrophobic interstices of the bilayer generated by *trans-gauche* isomerization (Traüble 1971). Despite the significant permeability to water, this rate is small compared to the amount of water that cells transport via aquaporins (Agre 1999). Consequently, there is a dramatic water concentration gradient of many orders of magnitude from the middle of the lipid bilayer to the bulk aqueous environment. Moreover, the surface of membrane proteins near the center of the bilayer is essentially that of a hydrocarbon with very few hydrophilic residues and no charged residues exposed to the fatty acyl chains. It can be anticipated that in the presence of cholesterol, sphingomyelin and methylated acyl chains, that the hydrophobicity of the protein surface that interacts with the bilayer interstices will be even greater.

Progression through the stages of protein folding, when and where water is scarce, are dramatically slowed (Brooks 1998; Arumugam et al. 1996). Indeed, the concept of water and other protic solvents as catalysts for hydrogen bond exchange has been developed (Figure 3) (Xu 1996)(Xu and Cross 1999; Barron et al. 1997; Klibanov 1989). Furthermore, it has been established that the non-minimum energy conformations of a TM peptide can be kinetically trapped in the low dielectric environment of the membrane interior when water is scarce (Arumugam et al. 1996). This suggests that the low water content of the membrane protein environment maybe important for stabilizing the native protein conformation. Of course, for membrane proteins with three or more helices, some water may be present in the interior of the structure, and this could facilitate structural rearrangements, but the dominance of the hydrocarbon amino acid composition (Zhou and Cross 2013b) suggests that throughout the TM domain of membrane proteins, hydrogen-bond exchange will be slow.

C. Order Parameter, Dynamics and Phase Behavior

The thermotropic phase behavior of natural membranes results from different dynamic states, primarily in the fatty acyl chain environment that can be modulated by cells depending on the temperature of their environment, to achieve a liquid crystalline environment for most cellular membranes under physiological conditions. In the interfacial region, the dielectric and water concentration gradients induce significant ordering (higher than anywhere else in the membrane) of the chains through the hydrophobic effect (Tanford 1973). This behavior is manifest in the data from many physical methods, including the order profiles from predominantly ^{2}H NMR (Figure 4) (Seelig and Seelig 1974; Tieleman et al. 1997) and spin-label ESR in model and natural membranes (Marsh and Watts 1982; Fretten et al. 1980). The lipid-chain order profiles show a similar order parameter (measured from these different methods having different motional time-scales for anisotropic averaging) of around $|S| \sim 0.4$ over the lipid chains from the interface (glycerol region) to about C8 – 10, approximately located at the middle of the chains. Additionally, in the region near the ester linkages of the fatty acyl chain, Trp and Tyr residues intercalate into the acyl lipid chains for interactions with the carbonyl groups forming the so-called “life-belt” of integral membrane proteins (Killian and von Heijne 2000) (Figure 5).

From this more ordered region to the center of the membrane, lipid chain order reduces significantly with little anisotropy ($S \sim 0.1$) at the end of the chains. Correspondingly, chain dynamics increase significantly. The embedded protein thus senses a very wide range of chain order and dynamics along its surfaces exposed to the membrane lipids.

The phase behavior of water and lipids as a function of lipid composition, including headgroup, fatty acyl chain and sterol composition, as well as temperature, is complex (van Meer et al. 2008). The lyotropic behavior results from the amphiphilic character of the lipid molecules in an aqueous environment and the packing geometry of the polar and apolar regions of the amphiphiles. For a monolayer, if the lateral packing dimensions of the polar headgroup region and the fatty acyl chains are similar as in many diacyl phosphatidyl cholines, the monolayer is approximately planar. On the other hand for a monoacyl phosphatidyl choline, the monolayer displays positive curvature potentially leading to a normal hexagonal phase H_I or micellar structure with a single polar surface. If a less bulky headgroup is chosen, such as phosphatidyl ethanolamine, negative curvature results that could lead to cubic or reversed hexagonal phase HII or reversed micelles. These different phases represent extremes, but in biological systems the typical lipid phase is a bilayer with a hydrated polar surface and a characteristic hydrophobic thickness. However, when bilayers fuse, such as in endocytosis or viral budding, surfaces are generated that take on a structure that is more reminiscent of a cubic phase than a planar bilayer. Bilayer curvature has been shown to influence membrane protein function (Botelho et al. 2006). Even in typical

situations, the bilayer configuration can experience curvature influences, known as curvature frustration (Helfrich 1973; Marsh 2007; Gruner 1985). Such a feature is highly sensitive to the fatty acyl and headgroup composition of each monolayer.

The interactions that stabilize (e.g. hydrophobic interactions) and destabilize (eg: electrostatic repulsion in the headgroup region) bilayers operate in different planes of the lipid bilayer. Consequently, the forces are not distributed evenly throughout the lipid bilayer leading to a lateral pressure profile (Cantor 1999). This profile suggests a region of lipid repulsion in the headgroup from charge repulsion and in the acyl chain region from an entropic effect. However, at the interface between headgroups and acyl chains, a strong negative pressure or attractive interaction exists, presumably associated with the hydrophobic effect. Overall, the integral of the pressure profile sums to zero as long as the bilayer is not under tension, but local curvature and deformations can occur. This complexity in the pressure profile can, in turn, influence protein shape and function. For example, de Kruijff and Killian have argued convincingly that for KcsA, a bacterial K^+ channel, monomerization by alcohols, such as trifluoroethanol, is impeded by non-bilayer lipids that influence the lateral pressure on the protein (van den Brink-van der Laan et al. 2004; Raja et al. 2007). It was suggested that in forming tetramers, the shape of the channel in lipid bilayers becomes that of an hour-glass, while the monomer in trifluoroethanol is more cylindrical. Furthermore, in native plasma membranes, negative curvature is induced through a higher concentration of phosphatidylethanolamine in the inner bilayer leaflet. Membrane proteins can compensate for the negative curvature by the insertion of an amphipathic helix that expands the protein surface on the inner leaflet (Lee et al. 2005; Drin and Antonny 2010; Peter et al. 2004).

It has been suggested that native eukaryotic membranes have transient phase separations known as lipid domains (Simons and Sampaio 2011). Such assemblies of lipids and membrane proteins facilitate and organize essential cellular functional activities. Domains or “rafts” are known to have a high content of sphingomyelin and sterols such as cholesterol. The immiscibility of these membrane components with lipid components that form liquid-crystalline domains appears to be responsible for the formation of transient phase separations. The properties of these environments may be responsible for selective membrane protein affinity. Rafts have a substantially thicker hydrophobic domain, and are also significantly stiffer (liquid ordered). The latter is a result of the high cholesterol content and the influence of the sterol rings on the dynamics of the fatty acyl chains. Raft-like domains may also display differences in the lateral pressure profile and even more dramatic water concentration gradient. As an example, Influenza A buds through a raft-like domain (Scheiffele et al. 1997) in which hemagglutinin and neurominidase are highly soluble, but the M2 proton channel, which is a third essential transmembrane protein for the viral coat, is not highly soluble in this environment (Zhang et al. 2000). Indeed, it has now been shown that M2 is not sensitive to the anti-viral drug amantadine when it is solubilized in a high sphingomyelin and cholesterol bilayer (Cady et al. 2011), but is sensitive to amantadine in liquid crystalline lipid bilayers (Hu et al. 2007a; Hu et al. 2007b). As with many viral proteins, M2 has additional functions, one of them is associated with the budding process (Schroeder et al. 2005) (Figure 6). M2 has now been shown to cluster at the boundary of the raft and non-raft regions of the membrane where it is optimally positioned to influence membrane curvature for the budding process (Rossman et al. 2010; Rossman and Lamb 2011). The association of M2 with the “raft” boundary may be facilitated by its cholesterol binding affinity and the palmitoylation of Cys50 (Schroeder et al. 2005). Similarly, GPCRs are post-translationally modified in helix 8, which is located at the protein-lipid interface, and may have implications for localization of these proteins (Adams et al. 2011). However, it is clear that “raft-like” environments could potentially both organize functional activities, as well as sequester proteins through their membrane properties, leading to the conclusion

that protein function, and presumably structure, can potentially be influenced by solubilization in either a liquid-ordered or liquid-disordered environment.

III. Membrane Mimetics and Structural Characterization

A. Membrane protein structures are determined in membrane mimetic environments

Here, we briefly introduce the various membrane mimetic environments used for structural characterizations of membrane proteins. Some of the properties of these environments are compared to those of the native membrane protein environments, but the discussion of how this impacts specific protein structures will await a later section.

Since protein structures are determined by the totality of interactions within the protein and between the protein and its environment (Anfinsen 1973), it is important to consider the limitations of membrane mimetic environments used for samples to structurally characterize membrane proteins. In view of the complexity of the native membrane environment, there is a range of parameters to consider. These limitations may or may not lead to structural perturbations, since the membrane environment for a given protein is not static (Sanders and Mittendorf 2011). While many of the biophysical properties of synthetic bilayers and detergent micelles can be experimentally or computationally characterized, the properties of concentrated protein phases such as detergent based 3D crystals, and even lipid based 2D crystals, are much more difficult to assess. It is important to note that proteins in native membranes exist in a concentrated protein phase with as much as 35% of the cross-sectional area occupied by protein (Dupuy and Engelman 2008; Zhou 2009). This still implies that on average multiple annular rings of lipid molecules will surround each membrane protein or membrane protein complex. Furthermore, there is a great diversity of membrane proteins in native membranes suggesting that proteins of the same type are rarely in contact unless they are drawn to a particular membrane site for functional reasons, such as viral budding or cell division. The current structural techniques require a homogeneous preparation of the protein in a membrane mimetic environment that may differ from the native membrane. All of these membrane mimetic environments are different, and the structural issues that arise vary depending on the environment. Furthermore, the success of X-ray diffraction (XRD) methods that have lead to the deposition of so many structures (~90,000 in total and ~300 unique membrane proteins) into the Protein Data Bank (PDB) provides numerous opportunities to observe these crystalline environments, while relatively few solid state NMR structures and electron diffraction structures in lipid environments have been deposited.

Simple micellar systems are often used to effect protein extraction, solubilization and purification, with an increasing, but still not a sufficient range, of ionic and non-ionic detergents including in some cases, detergents that approximate lipid chemistry, such as cholesterol hemisuccinate and lyso-lipids (1, O-acyl-phospholipids) (Krueger-Koplin et al. 2004; Robinson et al. 2011). These mimetics are intended to maintain the protein in an isotropic environment and in a native-like form for structural studies. In some cases, protein folding from inclusion bodies following expression requires detergent mediation with the goal of achieving activity and functionality. Depending on the success of such refolding efforts, structural studies may have more or less success in producing the native-like, functionally competent structure.

3D crystal formation for XRD studies requires electrostatic crystal contacts to establish a crystal lattice. The lattice may take on the appearance of a bilayer arrangement for the proteins with all of the hydrophobic domains in a plane (Figure 7a) or a non-bilayer arrangement (Figure 7b&c), such that the hydrophobic domains do not form a sheet within the crystal lattice. Sometimes the lattice promotes oligomerization that is not present in the

native environment, for instance, when proteins pack in an antiparallel orientation (Figure 7a). Crystallization can be enhanced by the insertion of lysozyme, Fab fusions, mutagenesis to enhance protein stability in the crystal lattice or a combination of these changes (Bill et al. 2011). It is no accident that those proteins that have a high density in their native membrane, and indigenously form 2D arrays (bacteriorhodopsin) or are inherently rigid (light harvesting centers, bacterial antennae complexes), have had their structures solved first, with EM often giving initial, lower resolution structural information that can aid phasing for the analysis of higher resolution diffraction data. Similarly, rhodopsin, which is known to be more rigid than other class A, ligand binding GPCRs as a result of the bound inverse agonist, retinal, was crystallized (Palczewski et al. 2000) more readily than the β_2-adrenergic receptor (Rosenbaum et al. 2009), which has been stabilized through bound ligands, mutations and fusions.

Micelles, and low q (ratio of lipid to detergent) bicelles, typically separate proteins into monomeric or minimal oligomeric structures. However, these detergent structures are not inherently bilayers, but possess a single hydrophilic surface and, especially for micelles, it is a highly curved surface. While functional competence can be assessed for some proteins in these environments, vectorial transport activity to set up concentration gradients cannot be observed. The bicelles used for solution NMR typically have a preponderance of detergent versus lipid (q = 0.3). The low q bicelle environments are useful for isotropic NMR methods and they provide some lipids for the protein environment generating a better environment for the protein interface and amphipathic helices. Significant intermolecular dynamics are expected as these environments are less constraining than a bilayer, and it might be expected that some structural excursions and distortions of α-helical, but maybe not for the more rigid β-barrel proteins, would be likely.

Whilst bilayers could be argued to be a much more satisfactory environment for a membrane protein, selecting the lipid environment that best matches the membrane protein, and induces a native conformation, may not be straightforward. Many studies have used a single synthetic lipid composition that seems suitable for the protein structure, based on functional criteria. For both lower and higher resolution structural studies, high protein content and possibly 2D arrays of proteins can be induced for EM (Fujiyoshi 2011; Sabra et al. 1998) and solid state NMR investigations – these sample preparations may be observed at low temperature or at low hydration levels resulting in an environment that may be more rigid than the native environment.

The incompatibilities in the hydrophobic regions of the membrane mimetics for solution NMR spectroscopy compared with natural membranes are clear, given the complexity of the physicochemical nature of the hydrophobic core of membranes (see above). The hydrophobic dimension of a micelle or bicelle is highly variable, and therefore not very constraining for the transmembrane protein. Peptides have been shown to tilt in response to bilayer thickness, and so the implications from an environment that does not constrain helical tilt for structural integrity are clear. The extent to which proteins can accommodate bilayer properties, such as hydrophobic thickness, is probably dependent on the protein and has functional significance in laterally separated membrane regions (Kaiser et al. 2011; Phillips et al. 2009), which necessarily have different properties, despite being in diffusional equilibrium with the bulk phase – lateral separation cannot occur without such diffusion.

Lipid asymmetry is another membrane property that is difficult to reproduce in a membrane mimetic – this is closely controlled in natural membranes and has functional importance, with most detail known for the head-group interactions with proteins or the extracellular environment. The membrane surface is characterized by polar species that can include charged oligosaccharides and phosphate esters, as well as neutral or non-covalently bound

moieties. Most phospholipids are anionic or zwitterionic, and proteins have an asymmetric charge distribution (the positive inside rule, (von Heijne 1992)), and so specificity of electrostatic interactions would be most likely through cationic residues on the protein surface, as shown for bacteriorhodopsin – these charge interactions can promote 2D array formation (Sternberg et al. 1992; Sternberg et al. 1989; Sternberg et al. 1993; Negishi and Mitaku 2011). How this affects function for those proteins that show such charge sensitivity, as in the Na^+/K^+-ATPase, is less clear (Esmann et al. 1985). Allosteric mechanisms, as well as charge relay systems as with GPCRs, or ionic concentrations at the membrane surface, are certainly important, not least with ion channels.

B. Solution NMR – Micelles, Bicelles, etc

Here, through several examples we illustrate some of the complications that can arise in using micelles and low-q bicelles for solution NMR studies of membrane proteins. While the structural characterization of water soluble proteins in dilute aqueous solution was a significant advantage over XRD, such dilute systems achieved using detergent micelles or low q bicelles for membrane proteins do not appear to share similar advantages. Developing strategies for solubilizing membrane proteins with amphipols, nanodiscs and macrodiscs appear to hold more promise for future studies (Long et al. 2013).

Numerous membrane protein structures have been characterized in detergent micelles using solution NMR spectroscopy. There has been rapid development of this approach over the past two decades (Sanders and Mittendorf 2011; Tamm and Liang 2006; Kang and Li 2011). Typically, the application of solution NMR has focused on relatively small membrane proteins, although recent efforts characterized a GPCR structure aided by some additional restraints from a crystal structure (Gautier et al. 2010). The influence of the membrane environment can be anticipated to be greatest for membrane proteins with the smallest TM domain, as there will be fewer interhelical interactions between a two helix TM domain than between a multi helix bundle protein with lipid exposure per helix being greater for smaller helical bundles. Consequently, the small TM helical bundle proteins may be one of the most challenging classes of membrane proteins to characterize, partly explaining their lack of structures in the PDB. Improvements on the use of detergent micelles have led to the use of bicelles, and while bicelles that tumble rapidly enough for high resolution spectra are dominated by detergents, there is a substantial lipid content in these preparations. Nanodiscs have also been used for solubilizing membrane proteins for solution NMR (Nath et al. 2007; Etzkorn et al. 2013). These particles encapsulate with a membrane scaffolding protein a disc of lipid containing the TM protein of interest. Typically, the correlation time for these particles is long, but some success has been achieved (Raschle et al. 2010; Marassi et al. 2011; Hagn et al. 2013). Amphipols replace lipids with a synthetic polymer leading to some preliminary success for solution NMR spectroscopy of membrane proteins (Bazzacco et al. 2012), and synthetic polymers hold further promise as stable nanodisc forming agents (Orwick et al. 2012a; Orwick et al. 2012b).

(1). Single Hydrophilic Surface—A characteristic feature of micelles and low q bicelles is that they have a single hydrophilic surface compared to bilayers that have two distinct surfaces (for the length scale of a TM helix). An immediate consequence is that hydrophilic residues in the middle of a TM helix can have access to the hydrophilic surface without dragging the helix termini (with its charged and polar residues) through the hydrophobic domain. A histidine kinase receptor, ArcB (PDB: 2KSD), has two TM helices as characterized in LMPG micelles, one of which is a uniform, nearly linear, helical structure with no H/D exchange of the helical amide protons (Fig. 8a) (Maslennikov et al. 2010). However, the other TM helix has several hydrophilic residues that are oriented toward the surface of the micelle that would be the hydrophobic interstices of a native membrane

environment. This outward facing surface of the helix is almost entirely H/D exchangeable unlike the other helix. Normally such hydrophilic residues would be oriented toward the interior of the protein, which would be the helix-helix interface. It is likely that the hydrophophilic residues interacting with the micelle surface have drawn waters into the micelle facilitating H/D exchange. A consequence of this aberrant surface interaction is that there is no significant helix-helix interaction between these helices over the majority of the hydrophobic region.

Another example of the outward curvature of TM helices is the trimeric diacylglycerol kinase structure (DgkA; Figure 8d, PDB: 2KDC) (Van Horn et al. 2009). Here, there are no hydrophilic residues exposed to the surface, but the longest backbone amide hydrogen bonds are on the outer surface of such curved helices exposing the carbonyl partial charges to the micellar interfacial region that would, in the native environment, result in exposure of these partial charges to the lowest dielectric environment of the membrane. Another consequence of this curvature is that there are numerous cavities in this DgkA structure weakening the helix-helix interactions as in ArcB. Data from solid state NMR of lipid bilayer preparations suggest that the helices are not curved and have small tilt angles to the bilayer normal (Li et al. 2007). Such outward helical curvature is frequently, but not always observed with detergent micelle structures of TM proteins. The solution NMR structure of the drug resistant V27A M2 protein in DHPC micelles (PDB: 2KWX) has the hydrophilic residues protected from the hydrophobic environment and the helices do not have this outward curvature (Figure 8c) (Pielak and Chou 2010). Likewise, BNip3, an E1B interacting protein (PDB: 2KA2) has hydrophilic residues protected from the hydrophobic interstices by interhelical H-bonding (Figure 8b) (Sulistijo and Mackenzie 2009).

(2). Hydrophobic Dimension—The hydrophobic thickness of native membranes is carefully regulated *in vivo* and while that of detergent micelles is also well defined in the absence of a membrane protein (Lipfert et al. 2007), the hydrophobic dimension appears to change readily in response to an added protein. The pure detergent structures are oblate or prolate ellipsoids with hydrophobic dimensions that vary from 20 to 120Å, depending on the detergent. The variable hydrophobic thickness of a micelle may account for the short and long helices observed in the KdpD structure (PDB: 2KSF), another histidine kinase receptor characterized in LMPG micelles (Fig. 9a) (Maslennikov et al. 2010). Pure LMPG micelles have a minor axis for the hydrophobic dimension of 33-35Å (Lipfert et al. 2007), but the hydrophobic helices of KdpD range in length from 3.5 turns (19Å) to 5.5 turns (30Å). DHPC (dihexylphosphocholine) forms a prolate ellipsoid with the minor axis of 19-20Å and a major axis of 41-43Å (Lipfert et al. 2007). Surprisingly, the TM helices of the M2 proton channel solution NMR structure have a smaller tilt relative to the channel axis in DHPC micelles (PDB: 2RLF; Fig. 9d) (Schnell and Chou 2008) than in DOPC/DOPG bilayers (PDB: 2L0J; Fig. 9c) (Sharma et al. 2010) suggesting a greater hydrophobic thickness in micelles. In addition, the structure in DHPC micelles does not bind the antiviral drug amantadine in the pore where it binds when the protein is in liquid crystalline lipid bilayers (PDB: 2KQT; Fig. 9b) (Cady et al. 2010). This is likely due to tighter helix packing and closure of the pore opening at the Val27 secondary gate resulting from a smaller helix tilt angle in DHPC micelles than in lipid bilayers.

(3). Weak Hydrophobic Domain and Lateral Pressure Profile—While native membranes have low water permeability and H/D exchange of TM helix amides (Earnest et al. 1990), especially at the protein/fatty acyl chain interface, is exceptionally slow - the exchange rate in membrane mimetics is faster. In LMPG micelles KdpD, mentioned above, has three of its four TM helices undergoing H/D exchange (Fig. 9a), suggesting that water is penetrating into the micelle or that the helices are at the surface of the micelle and not penetrating through the center of the micelle as normally assumed. It is possible that the

hydrophilic residues interacting with the micelle surface are drawing waters into the micelle that induce H/D exchange, similar to that for ArcB (Fig. 8a). The debate over water penetration in detergent micelles is long-standing, with some contending that water penetrates deeply into the micelles (Menger and Boyer 1980; Turro and Okubo 1981), and others reporting that it is much more like lipid bilayers, where the hydrophobic core of the bilayer is essentially devoid of water (Dill et al. 1984; Kalyanasundaram and Thomas 1977; Podo et al. 1973). The observation that one of the KdpD (introduced above) helices does not undergo H/D exchange, suggests that there is a region of the micelle that is largely inaccessible to water, while the other three helices may be closer to the micelle surface. The lateral pressure profile for detergent micelles have been calculated displaying a broadened profile that implies a broadened hydrophobic/hydrophilic boundary compared to lipid bilayers (Nakamura et al. 2011). It is possible that this helps to account for the H/D exchange frequently observed in detergent micelle-solubilized membrane proteins. Cramer, in describing the lateral pressure profile for detergent micelles as being reduced when compared to lipid bilayers, suggests that sidechains and helices may have greater conformational heterogeneity in the micellar environment (Zhang et al. 2003).

(4). Monomeric Detergents—Monomeric concentrations of diacyl lipids in the presence of lipid bilayers is nanomolar or less, however monomeric concentrations of detergents for samples above the critical micelle concentration (CMC) is on the order of the CMC, which is frequently in the mM range, i.e. a monomer concentration difference between detergents and lipids is 10^6 or more (Cevc and Marsh 1987). Such monomeric detergent concentrations throughout the aqueous solution may destabilize water soluble domains of the proteins. Furthermore, monomeric detergents may bind in the pores and active sites of TM proteins, but for solution NMR there does not seem to be clear evidence for this behavior, since the locations of the detergents are not sufficiently defined to be reported with the protein structure in the PDB.

C. X-ray crystallography – Detergents, Organics and Lipids

In detergent based crystals there exist many differences between the environment generated for membrane proteins and that generated by native membranes. Here, we describe these differences in more detail and provide multiple structural examples. Once again it is important to recognize that despite the imperfections in the crystal environments, XRD has resulted in many structures that appear native-like from the perspective of the TM domain. We know much less at this time about the structure and dynamics of membrane proteins in the membrane interfacial region (Higman et al. 2011), a topic that is beyond the scope of this review. For the TM domain it appears that when crystal lattices take on a bilayer appearance (Schulz 2011) and when lipids are integrated into the membrane protein environment the probability for a native-like structure increases.

There have been numerous reviews of membrane protein crystal structures (Vinothkumar and Henderson 2010; Baker et al. 2010; McLuskey et al. 2010; Bill et al. 2011). Typically, XRD has focused on large TM proteins and on those proteins with the greatest stability, such as those with ligands in the TM domain or with proteins that occur at high concentration in native membranes. Most of the crystal structures have been achieved using detergents to model the membrane environment along with other additives to induce crystallization. Typically, functional assays are performed, but as with the preparations for solution NMR it is often not possible to perform these assays for detergent solubilized proteins involved in vectorial transport. Instead, these assays are performed in liposomes or planar bilayers and while they are important in that they validate the use of the protein construct, they do not validate the structure itself. Recently, to increase stability and reduce flexibility, considerable specific site mutagenesis has been employed and antibodies,

lysozyme or nanobodies are bound or inserted into the sequence to increase the interactions at the crystal lattice contacts, and consequently the functional assays even if not in the crystalline environment, are important. Often care needs to be taken to purify lipids with the protein. The ligand gated ion channel, GLIC (PDB: 3EAM; Figure 10a) has nearly a complete annulus of lipids formed around the protein structure (Baenziger and Corringer 2011). Yet even in this structure the pore is occupied by detergent molecules that may influence the dimensions of the pore. In other cases detergents are observed to fill what is presumed to be a lipid binding site, such as in the voltage gated sodium channel (PDB: 3RVY; Figure 10b) (Payandeh et al. 2011; Hunte and Richers 2008). Bicelles (Ujwal and Bowie 2011) and lipidic cubic phase preparations of monoolein and cholesterol (Cherezov et al. 2002), have also been used with some success to crystallize membrane proteins. Indeed, lipidic cubic phase preparations may be a good approach for crystallizing small helical bundles that are so difficult to crystallize with traditional approaches, but this has yet to be demonstrated (Caffrey 2010; Separovic et al. 2011).

(1). Crystal Contacts—Specific contacts between oligomeric states of membrane proteins in a crystal lattice are non-native and can shift the balance of interactions that stabilize a monomeric or oligomeric state of the protein. As noted earlier even membranes with the highest fraction of protein have more than 50% of the membrane surface occupied by lipids (Zhou 2009). The XRD structure of the M2 TM domain in the absence of bound drug (PDB 3BKD) had significant hydrophobic interactions between helices of adjacent tetramers as well as a salt bridge between arginine and glutamate of adjacent tetramers in the crystal lattice (Figure 7a) (Stouffer et al. 2008). This broke the four fold symmetry of this homo-tetramer. A second crystal structure of the M2 TM domain with amantadine present (PDB 3C9J) in the pore did not have such electrostatic crystal contacts between tetramers and the structure was nearly symmetric. Both of these structures differ dramatically from the structure obtained in lipid bilayers (PDB 2L0J; Fig. 9c) (Sharma et al. 2010) as well as the recent crystal structure of the thermally stabilized mutant G34A (PDB 3LBW) (Acharya et al. 2010). Also the first acid sensing ion channel structure (ASIC; PDB 2QTS; Figure 11a&c) has significant electrostatic interactions between trimers in the crystal lattice involving the TM helices of one trimer with the water soluble domain of another trimer (Gonzales et al. 2009). The resulting structural perturbation was almost entirely in the TM domain where the pseudo three-fold symmetry was lost, but in the water soluble domain where the tertiary and quarternary structure is more stable there was very little distortion. Yet another example, 5-lipoxygenase activating protein (FLAP), a trimeric protein (PDB 2Q7M; Figure 11b), also illustrates the instability of the tertiary structure in the TM domain (Gilbert et al. 2011). In the crystal lattice there is no resemblance of a planar hydrophobic environment and the terminal helix from one trimer forms a salt bridge with the same helix in a neighboring trimer as well as additional electrostatic interactions. The result is that the helix appears to be shifted by ~10Å out of what would be the hydrophobic environment of the membrane. This exemplifies how weak the interactions are that stabilize tertiary structure in the TM domain.

(2). Thin Hydrophobic Domain—The hydrophobic thickness of the detergent based crystal environment can lead to a hydrophobic mismatch and structural perturbations. The highly tilted and kinked helices of an ionophoric ATP receptor, P2x4 (PDB, 3I5D; Figure 12a) is such an example, where, as a result of the helical tilts and kinks the hydrophobic dimension is less than that for a native membrane (Kawate et al. 2009). Another example is a Zn^{2+} metalloprotease, the site 2 protease (PDB: 3B4R; Figure 12b) in which a short TM helix (4 turns) is terminated in a β-sheet that was described by the authors as being buried in the hydrophobic region (Feng et al. 2007). In the β-strands of β-barrels buried in the outer membranes of cells, the amide groups are all protected by H-bonds. Here, in the site 2

protease this is not the case and hence amides are exposed to the hydrophobic interstices, a result that is likely due to a thin hydrophobic membrane mimetic environment resulting in a conformation that is unlikely in a native environment.

(3). Weak Hydrophobic Domain—Extensive water penetration into the hydrophobic surface of membrane proteins in detergent based crystals is occasionally observed as shown in Fig. 13a where a large number of water molecules diffracted from the would be fatty acyl environment of the metal chelate transporter (PDB: 2NQ2) (Pinkett et al. 2007). The maltose uptaker (PDB: 3FH6; Figure 13b) has a pair of helices that have become dissociated from the rest of the protein and do not span the putative bilayer (Khare et al. 2009). Both the crystal structure (2ZUQ) and solution NMR structure (2K73) of DsbB, part of the disulfide bond synthesis apparatus, has numerous exposed hydrophilic and charged residues in what should be the membrane environment (Zhou et al. 2008; Inaba et al. 2009) (Fig. 13c&d). Some of these polar and charged residues are associated with an amphipathic helix that appears to be misplaced, being buried too deep in what would be the hydrophobic domain of the native membrane environment. FLAP also displays a weak hydrophobic environment, with waters dispersed throughout the hydrophobic region as well as the unstructured loop between helices 3 and 4 has been drawn into what should be a very low dielectric environment (Figure 11b). Furthermore, the hydrophilic C-terminal residues of a FLAP monomer from the neighboring trimer are also in a similar environment that should be very hydrophobic. The result of the crystal contacts and/or the weak hydrophobic environment is a perturbed structure.

(4). Lateral Pressure profile—While Zhang and colleagues describe the lateral pressure profile of detergent micelles as being reduced from that of bilayers (Zhang et al. 2003), they also suggested that a similar reduced profile is present in detergent-based crystal lattices. Furthermore, they suggest that sidechains and helices will have greater conformational heterogeneity making crystallization more challenging. These authors argue for the integration of lipids into crystallization trials as a way of ameliorating this problem. Indeed, different crystal forms can be obtained by lipid incorporation (Watts et al. 1993) and lipids promote crystallization in some cases (Gabrielsen et al. 2011). However, it may not be only conformational heterogeneity that results from a low lateral pressure profile. The TM domains of transporters, such as the YiiP Zn^{2+} transporter (PDB: 2QFI; Fig. 14a) are dramatically splayed to such an extent that lipids in native membranes might be able to diffuse into the pore of the transporter interfering with the functional mechanism (Lu and Fu 2007). This structure also shows relatively poor packing of the helices in each arm (i.e. each six-helix bundle) of the transporter based on the substantial cavities present (Fig. 14b) suggesting that the structure is perturbed. In comparison the structure of pyrophosphatase (PDB: 4A01) shows a very well packed helical bundle with few cavities between the helices (Lin et al. 2012). Also, Pebay-Peyroula suggests that the lateral pressure profile may facilitate the seal between the lipid and protein environments in the membrane that is essential for native functioning of the membrane (Pebay-Peyroula and Rosenbusch 2001). The opening of the large mechanosensitive channel (Steinbacher et al. 2007) may be facilitated by a decrease in the lateral pressure that under normal circumstances keeps the protein in a closed state (Pebay-Peyroula and Rosenbusch 2001), but can be opened by the addition of lyso-lipids (Grage et al. 2011). Potentially, a weakened lateral pressure profile in the crystal lattice also facilitated the splaying of the M2 protein TM helices shown in Fig. 7.

(5). Monomeric Detergents—As mentioned previously, monomeric detergent concentrations are very high compared to monomeric lipid concentrations. The first crystal structure of the M2 TM domain from Influenza A (PDB: 3BKD;(Stouffer et al. 2008)) has two octylglucoside molecules and a polyethyleneglycol molecule in the pore of this tetramer

splaying the C-terminal ends of the TM helices apart (Fig. 15a) (Cross et al. 2011). In addition, hydrogen bonds form between one of the glucoside moieties and a His residue essential for proton conductance. The first crystal structure of the acid sensing ion channel (ASIC, PDB: 3HGC; (Gonzales et al. 2009)) that displayed significant inter-trimer electrostatic interactions also has three detergent molecules bound in this trimeric structure, one of which was on the trimer axis and presumably in the channel (Fig. 15b). Multiple structures of the ligand gated ion channels, while having lipids bound to the lipid facing surface, have detergents bound in the pore appearing to widen the pore. The NCX sodium calcium exchanger (PDB: 3V5U; Fig. 15c) also has detergents bound in the structure such that two helices are splayed from the rest of the helical bundle (Liao et al. 2012).

D. Electron Diffraction – 2D crystals with lipid annulus

Electron diffraction and cryo-EM of individual particles, forms the basis for membrane protein structural determinations at relatively low resolution, especially in the absence of symmetry (Muller et al. 2011; Wisedchaisri et al. 2011). Diffraction methods can only be employed for regular arrays of proteins, either naturally occurring, as for bacteriorhodopsin (Henderson and Unwin 1975), or induced through protein density (rhodopsin, porins, etc), or through specific lipid interactions (Sabra et al. 1998). New smart materials for arranging protein in a regular scaffold are now proving useful in increasing particle density, and for selecting only active proteins (Selmi et al. 2011).

One advantage of these approaches is that bilayers of lipids can form the local environment for the protein, and so achieve a more physiological condition, although reconstitutions into these preparations for structural studies are seldom monitored for functional activity. The subtleties of the lipid interactions are lost however, since the technical challenge of array formation is paramount and the data content resolution low (> ~ 7Å in the plane of the membrane in the best cases).

In one case, bacteriorhodopsin, some of the rigid, diphytanyl phospholipids (10 per monomer, mainly PGP and PGS) have been visualized in the protein trimers from EM diffraction, with the lipids enclosed at the center of symmetry being best resolved (Reichow and Gonen 2009). The purple membrane is very rigid and the chain and protein dynamics are relatively slow (>ms), but differential dynamics have been observed as slow helix dynamics and faster (short) loop dynamics by inelastic neutron scattering and solid state NMR (Zaccai 2000; Kamihira and Watts 2006).

Importantly, these 2D crystals have the hydrophobic domain organized in a bilayer like arrangement and many of the perturbations seen in 3D crystals have been associated with non-bilayer like crystal lattices. For MGST1 (microsomal glutathione transferase 1), a MAPEG family protein, the electron diffraction structure shows each of the TM helices spanning the lipid bilayer in contrast to the 5-lipoxygenase XRD crystal structure (Fig. 11b) (Holm et al. 2002). Consequently, the hydrophobic environment in the 2D crystal is likely to be much more native-like than a 3D crystal structure from a detergent environment. However, 2D crystal contacts may be significant as the lipid is restricted in this high protein concentration environment to an annulus surrounding the protein. Furthermore, the lack of a bulk lipid environment may have an influence on the lateral pressure profile. Unfortunately, at this time there are relatively few electron diffraction structures available to further assess the strengths and weaknesses of this environment as a model for the native environment.

E. Solid State NMR – Lipid bilayers and Liposomes

There are two techniques for achieving membrane protein structural restraints from solid state NMR (ssNMR); Magic Angle sample Spinning (MAS) (Huang and McDermott 2008;

Andreas et al. 2010; Li et al. 2008; Lange et al. 2006; Higman et al. 2011) and oriented sample (OS) spectroscopy (Watts et al. 2004; Ketchem et al. 1993; Cross and Opella 1994; Fu and Cross 1999). Structural restraints have been obtained for numerous membrane proteins using these techniques, with most success being reported for small TM proteins, and assignments reported for selected regions of large proteins in bilayers. A variety of sample preparations including precipitants, microcrystals and liposomes can be used for MAS, but the recent success with liposomes, appears to be generating excellent spectral resolution (Shi and Ladizhansky 2012). Backbone isotropic chemical shifts provide torsion angle restraints and spin exchange experiments provide qualitative distance restraints similar to NOE distance restraints in solution NMR spectroscopy.

Samples for OS ssNMR are typically synthetic lipid bilayers aligned between thin glass slides in which the normal to the glass slide and lipid bilayers is aligned parallel to the magnetic field of the spectrometer. Alternatively, high q (typically q=5) bicelles that have a much greater lipid content than those used for solution NMR have had considerable success (De Angelis and Opella 2007; Cook and Opella 2011). OS ssNMR leads to the development of orientational restraints that provides a high resolution structure of the polypeptide backbone. Only for the monovalent cation channel, gramicidin A, has a complete structure including all of the sidechains been characterized by solid state NMR (Ketchem et al. 1997). However, as we have shown here, helical packing is of primary importance and cannot be assumed to be native-like in the presence of detergents. Moreover, the conformation of the sidechains facing the lipid environment are of less importance due to the non-specific and dynamic interface with the acyl chain evironment. Therefore, the backbone structure obtained in a lipid bilayer environment can be considered an important structural accomplishment with or without the sidechains. Such has been achieved for the M2 conductance domain (PDB 2L0J) (Sharma et al. 2010) and several other helical bundles (Verardi et al. 2011). In addition, conformational disorder in oligomeric states can be studied by ssNMR, where such disorder can be troublesome for diffraction (Su and Hong 2011).

Since the structures are obtained in a lipid bilayer environment, not only is the hydrophobicity and hydrophobic dimension approximately correct, but the lateral pressure profile is likely to be more native-like than in a detergent based environment, and crystal contacts are not an issue. The combination of MAS and OS restraints holds great promise for the future of ssNMR as it greatly reduces the number of distance restraints needed from MAS spectroscopy (Cady et al. 2010; Can et al. 2012; Murray et al. 2013; Nishimura 2002). Recently, a technique for obtaining orientational restraints from MAS has been published along with a structure of the 2 TM helix mercury transporter, MerF. This approach expands the sample preparation possibilities for solid state NMR while allowing for the possibility to obtain distance, torsion angle and orientational restraints all from the same sample (Das et al. 2012). Recently, this has led to an initial structure of a GPCR, CXCR1 (Park et al. 2012).

IV. Recognizing native-like TM domains of helical membrane proteins

Based on the biophysical properties of the membrane environment there are numerous qualitative assessments that can be made for assessing the native-like characteristics of a helical TM protein structure. Here, we provide several measures by which these structures can be evaluated.

A. Oligomeric State

Knowing the oligomeric state is important since monomer-monomer contacts protect some of the surface area that would otherwise be thought to be lipid facing. While for many proteins the oligomeric state will be known from biochemical studies, this is not always the

case. Surfaces involved in protein-protein interactions are typically more hydrophilic than those exposed to the fatty acyl environment. Such hydrophilicity derives from both sidechains and backbone sites (i.e. backbone atoms not shielded by bulky sidechains). Consequently, it is important to know the oligomeric state so as not to judge a somewhat hydrophilic surface as being exposed to the lipid environment. While glycine motifs, such as GxxxG are frequently observed within monomeric structures, they are not typical of oligomeric interfaces where Ala/Ser motifs are more common.

B. Surface Exposure of Charged and Polar Residues

Charged residues appear to be never exposed to the hydrophobic interstices, although they may be occasionally used to thin the hydrophobic environment surrounding the protein. A classic example of this situation is Ca^{2+} ATPase responsible for the transport of the divalent cation across the membrane (Sonntag et al. 2011). Hydrophilic residues, other than the occasional Ser, Cys or Thr, are infrequently exposed in the hydrophobic interstices emphasizing the fact that native membrane protein structure aims to match the very low dielectric environment of the membrane interior with a hydrocarbon surface. Any hydrophilic sites exposed in the hydrophobic core will decrease the stability of the structure and can be expected to be present only for a significant functional activity and even so these residues can be expected to be protected in some fashion from the low dielectric environment. Such shielding from the fatty acyl environment is frequently achieved by the strategic placement of a phenylalanine ring and in some cases a tyrosine ring. The limited exposure of hydrophilic sites to the membrane interior clearly rules out the possibility that mistic, a membrane self-integrating protein is a TM helical protein, since its structure (PDB: 1YGM) (Roosild et al. 2005; Jacso et al. 2012) has 12 charged residues exposed to what would be the hydrophobic membrane environment. Similarly, the recently characterized structure from hepatitis C virus, p7 by solution NMR has 18 arginine sidechains in this hexameric structure exposed to what would be the hydrophobic membrane environment and is therefore, unlikely to be a native-like structure.

C. Surface Exposure of Gly Residues

For multiple reasons Gly residues are rarely exposed to the membrane interstices (Dong et al. 2012) despite the fact that they have a greatly increased abundance in TM helices versus helices in water soluble proteins. Gly and Pro residues are known to be “helix breakers” in that they often terminate water soluble helices. However, in TM helices they are typically present in continuous helices and at other times they induce a modest kink in the TM helix. Helices are much more stable in the low dielectric environment of the membrane interstices that strengthens the intrahelical hydrogen bonds (Kim and Cross 2002; Page et al. 2008). Because of the scarcity of charged and polar residues, the tertiary structure is infrequently stabilized by interhelical hydrogen-bonds or other specific electrostatic interactions. This is not to say that such hydrogen-bonds never exist (Marius et al. 2012b; Dawson et al. 2002), but many, if not most helical interfaces are dominated by van der Waals interactions. Furthermore, if TM helices were entirely uniform and rigid, their packing interface with neighboring helices would be weak and consequently the tertiary structure would have poor stability. It is well known that glycine motifs facilitate helix-helix interactions (Javadpour et al. 1999; Russ and Engelman 2000; Kleiger et al. 2002) by permitting close approach of the helices, greater van der Waals contacts, and some specific weak electrostatic interactions, such as CαH hydrogen-bonds between the backbones of the interacting helices. It has been recently suggested that both Pro and Gly serve to destabilize TM helices by inducing kinks or bends in the helices that can increase the surface contact area between interacting helices and hence increase tertiary stability (Dong et al. 2012). On the hydrophobic surface of a membrane protein the lack of a significant sidechain (i.e. glycine) results in the exposure of the hydrophilic backbone atoms that leads to the destabilization of the membrane protein in

its lipid environment. Indeed, it was shown that examples from nearly half of the known TM folds do not show any conserved Gly residues exposed to the hydrophobic interstices of the lipid bilayer. Therefore, it would seem that glycine residues should appear on the protein interior where such locations can facilitate tertiary structural contacts increasing tertiary structural stability.

D. Interfacial Location or Tyr, Trp and Charged Residues

It has long been recognized that Tyr and Trp residues are frequently positioned at the hydrophilic/hydrophobic interface and for Trp very rarely, if ever exposed to the membrane interstices. Indeed, Trp appears to have a well defined preferential orientation with respect to the interfacial layer (de Planque et al. 2003). In addition, there is also a belt of charged residues at the interface that also helps to define the hydrophobic/hydrophilic interface for TM proteins.

E. Hydrophobic Dimension

The hydrophobic dimensions of native membranes can vary significantly and the hydrophobic dimension of membrane proteins may vary even more, but even so there should be a relatively close match between the hydrophobic dimension of the protein and its native environment. Structures with hydrophobic dimensions less than 25Å also appear to have additional concerns generated by this list of factors for assessing the native-like character of the TM structure. Indeed, positioning the bilayer onto a structure derived in an isotropic environment, can be challenging, and reference to homology modeling or other data may be required (Judge and Watts 2011; Zhou and Cross 2013a; Killian 1998).

F. Internal Cavities

Since helical TM proteins have relatively weak tertiary structural stability, it is necessary that cavities in the protein interior be minimized except where they are essential for functional activities. Such cavities are not likely to remain vacant, but while vacant they do not add to the tertiary structural stability. To optimize the structural stability numerous Gly residues are present for the primary purpose of increasing the surface contact between helices. Fenestrations to the lipid environment may also expose hydrophilic sites to the membrane interstices degrading structural stability. These cavities and fenestrations are sometimes occupied with detergent molecules as discussed above. In addition to the situation where the detergents have distorted the structure, there are also occasions where detergents are bound in sites where lipids may normally bind (Marius et al. 2012a; Stansfeld et al. 2013; Clayton et al. 2008).

V. Conclusions

Clearly, the structure of helical TM proteins can be influenced by their environment. Furthermore, membrane mimetics differ from the native membrane environment in a variety of biophysical parameters and therefore, differing sample conditions can lead to membrane protein structural perturbations. These parameters and conditions include crystal contacts, the single hydrophilic surface of micelles or bicelles, weak or thin hydrophobic environments, the presence of monomeric detergents, “trapped” lipids or other organics, and possibly the lateral pressure profile. Exactly which parameters lead to the perturbations observed is not always clear, because for each protein there appears to be multiple parameters or conditions that differ from the native membrane environment.

Equally important is the realization that the difference between the native membrane and membrane mimetic environments does not always lead to such perturbations, in fact the majority of structures appear to show native-like features based on the relatively crude

criteria described here. Furthermore, developments in sample preparation for solution NMR spectroscopy and for x-ray crystallography have the potential to lead to more native-like membrane mimetic environments and structures in the future. At the same time electron diffraction and solid state NMR are developing technologies that can be used to characterize structures in a lipid bilayer environment without detergents present. In studying membrane proteins in lipid environments, these techniques, as well as distance restraints from electron spin resonance (Boura et al. 2011; Jeschke 2012) can be used to validate membrane protein structures obtained from samples in less native-like environments. Computational methods, such as molecular dynamics calculations in a full atomistic model of a lipid bilayer can also provide important insights into the native-like character of a protein structure.

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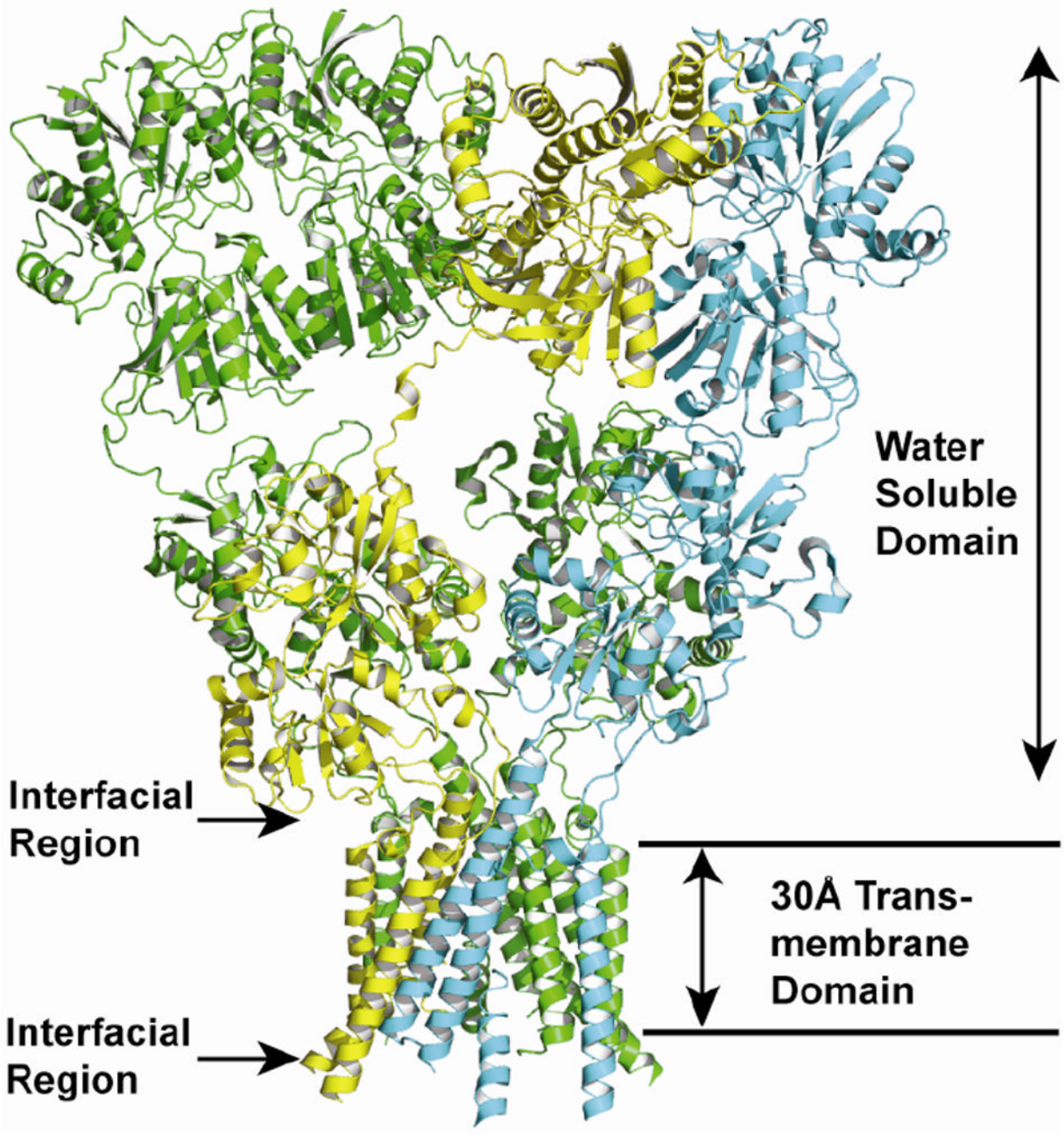

Fig. 1.

Membrane proteins may have significant extra-membranous portions that behave much like soluble proteins, and can receive signals from the extracellular environment and transmit them to the intracellular side through a membrane-embedded, close-packed α-helical, domain, as with an ionotropic glutamate receptor, [PDB 3KG2] (Sobolevsky et al. 2009). This review focuses on the native-like structure of the transmembrane domain of helical membrane proteins.

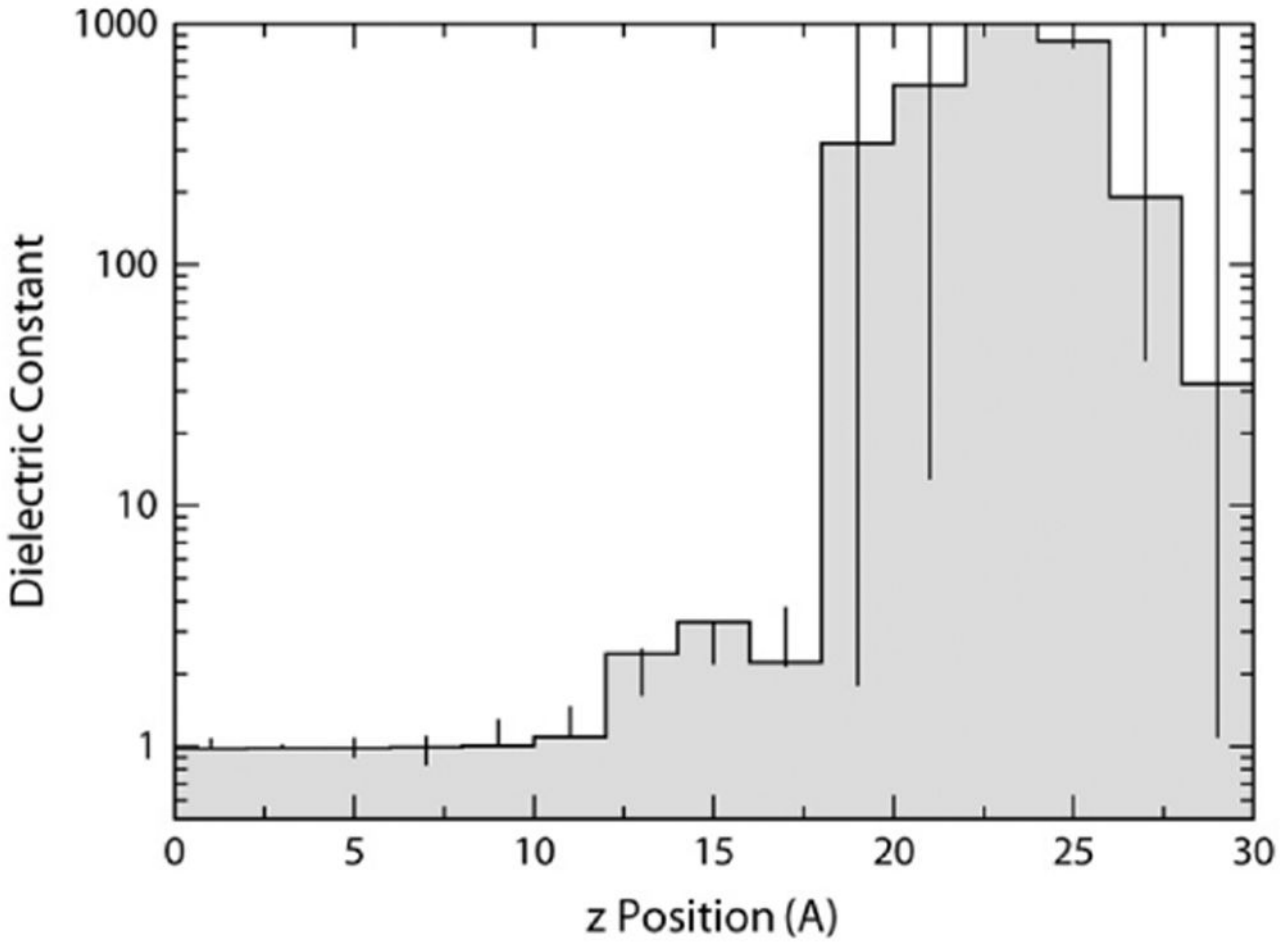

Fig. 2.

Computed dielectric constant as a function of distance from bilayer center (Z position) for a POPC bilayer. The average fatty acyl carbonyl position is about Z=16Å. The choline groups can extend to about Z=27Å. The vertical lines represent the error bars. Reproduced with permission from (Nymeyer and Zhou 2008).

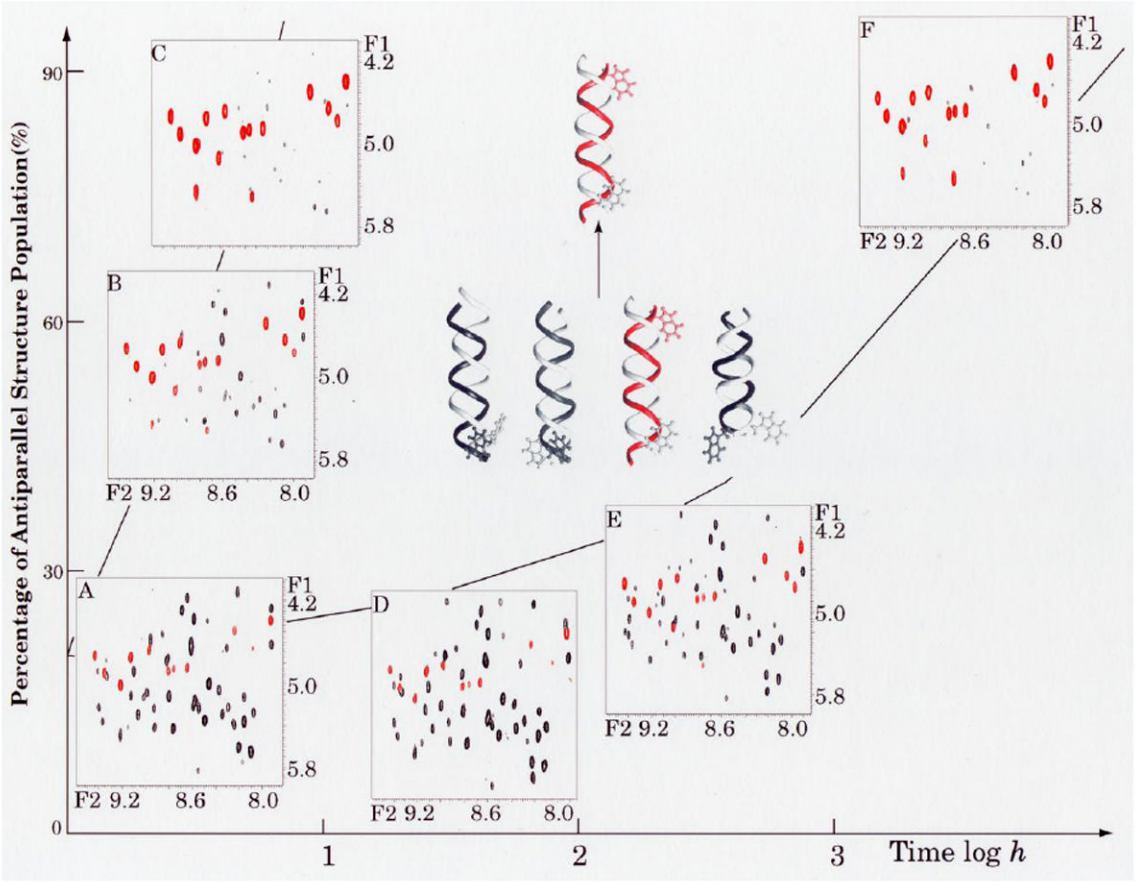

Fig. 3.

Conformational exchange for dimeric gramicidin A in isotropic solvents is enhanced by more than a 1000 fold by the addition of water (a protic solvent) that can facilitate hydrogen bond exchange. The conformational exchange is observed by GCOSY spectra of various double helical gramicidin A conformations in dioxane (a non-protic solvent). The red resonances highlight the stable state in this environment and its corresponding conformation is highlighted in red. a-c). a, d-f) In the presence of approximately 1% water in dioxane the conversion occurs in less than 1 h. This suggests that conformational interconversion in native membranes may be hindered by the sparsity of water in the membrane interstices. Reproduced with permission from (Xu and Cross 1999).

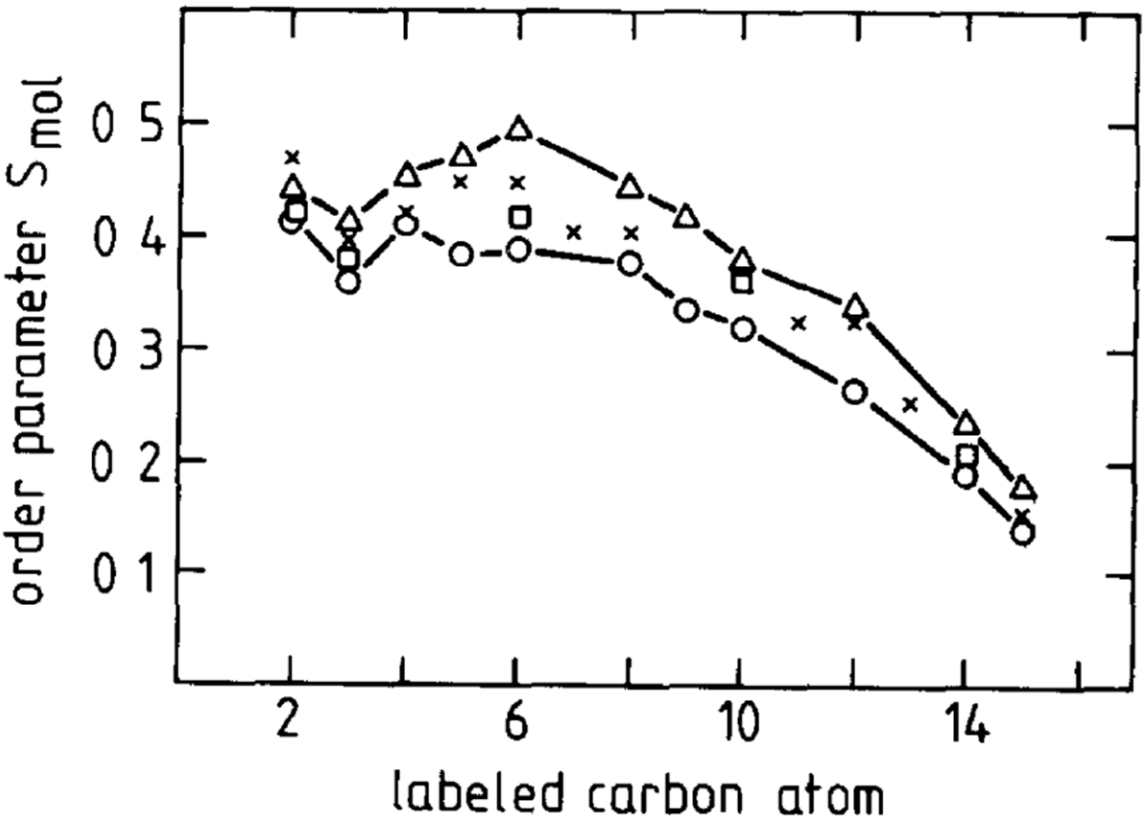

Fig. 4.

Lipid bilayers exhibit a large range of dynamics exemplified by normalized order parameters. The profiles for different bilayers and the variations of the molecular order parameter, S_{mol}, with the segment position. ○, 1,2-dipalmitoyl-sn-glycero-3-phosphocholine. △, 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine. □, 1,2-dipalmitoyl-sn-glycero-3-phosphoserine. ×, Acholeplasma laidlawii (Stockton et al 1977). Reproduced with permission from (Seelig and Browning 1978).

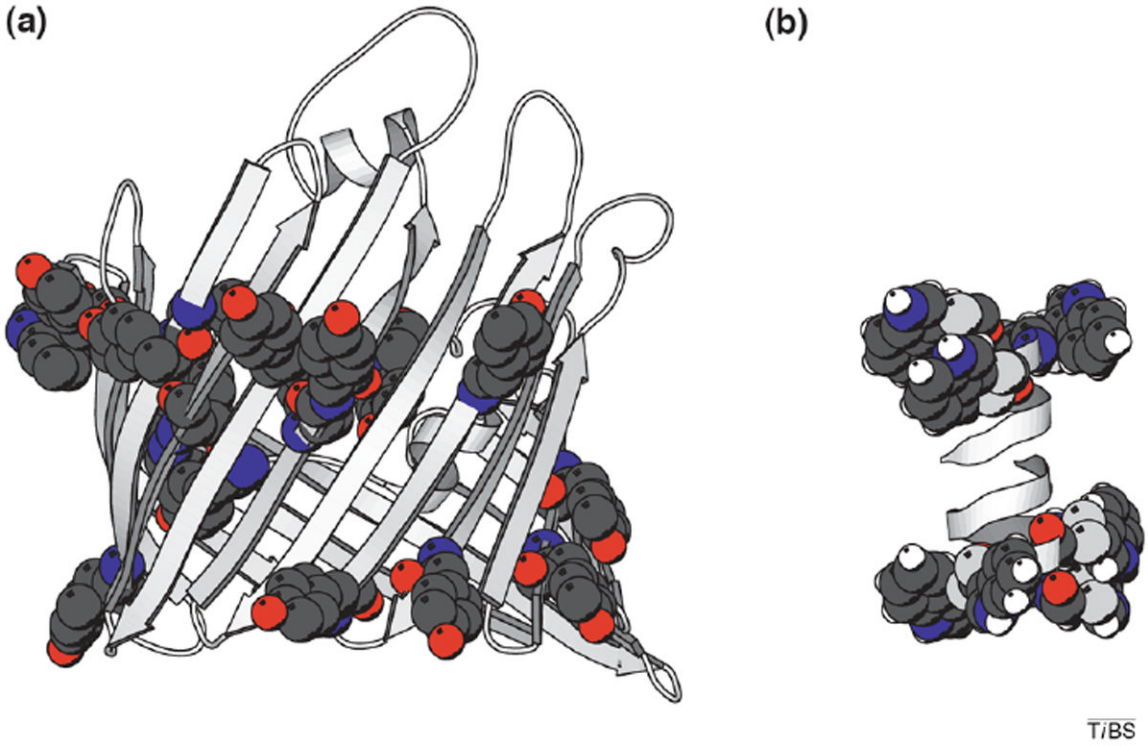

Fig. 5.

Key amino acids have preferential locations in membrane protein structures with respect to the lipid bilayer environment. For example, tyrosine and tryptophan sidechains interacting with the lipid interfacial regions from a porin (a) and gramicidin A (b). Reproduced with permission from Killian and von Heijne (2000).

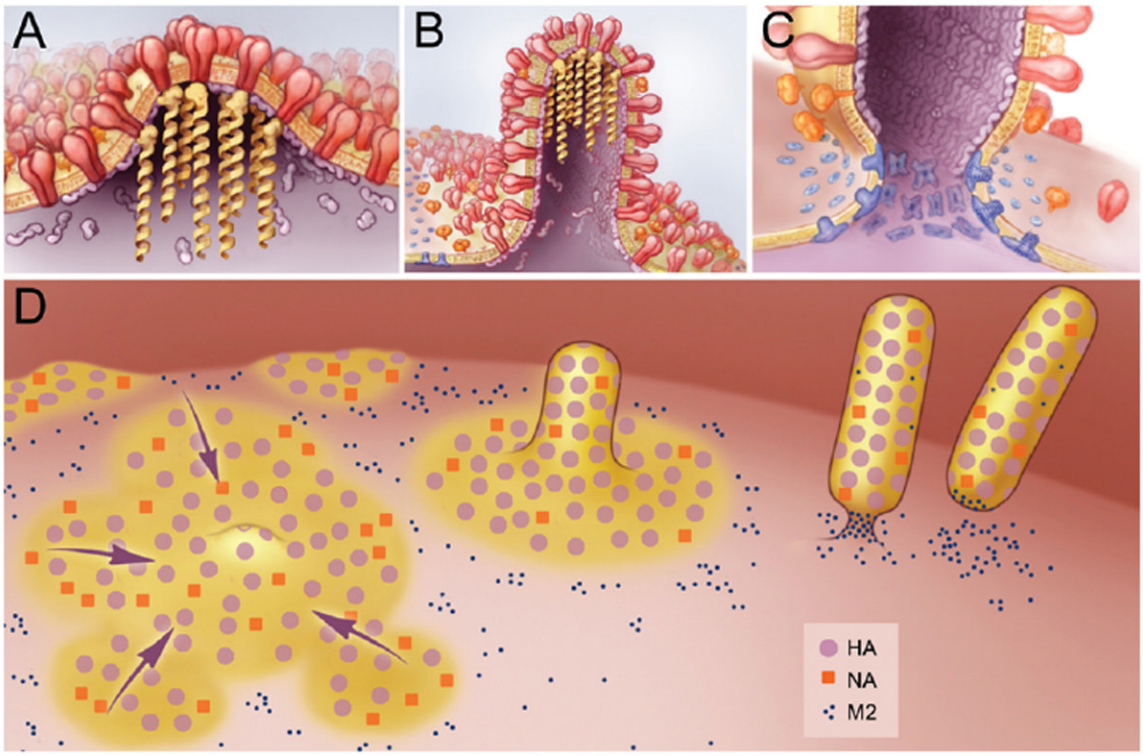

Fig. 6.

a) Viral budding of Influenza from a raft-like domain (yellow region in (d)). Hemagglutinin (HA) and Neuraminidase (NA) are highly soluble in the raft-like domain and have limited solubility in the pink liquid crystalline domain of the membrane; b) Elongation of the budding virus; c) membrane scission facilitated by M2 (blue) that has low solubility in the raft-like domain and functions in liquid crystalline domains; d) M2 (blue dots) is thought to associate with the raft/non-raft border, potentially by its affinity for cholesterol. It is therefore clustered in the non-raft region (pink) at the neck of the viral bud (c) where it induces curvature in the liquid crystalline environment via its amphipathic helices on the inner surface of the cellular membrane. Reproduced with permission from Rossman and Lamb (Rossman and Lamb 2011).

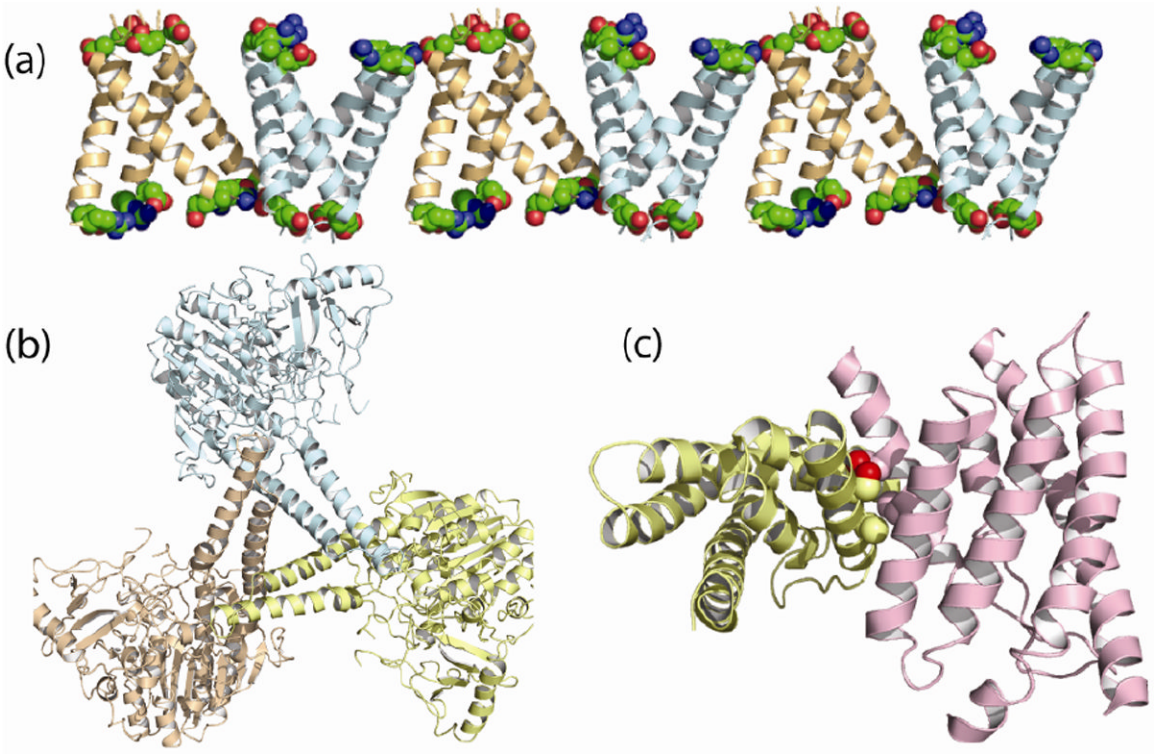

Fig. 7.

Bilayer and non-bilayer lattices in membrane protein crystals. a) Despite the bilayer lattice for the Influenza A M2 protein (3BKD) significant electrostatic interactions (space-filling Arg and Glu residues) between antiparallel tetramers appear to distort the tetramer helices (Stouffer et al. 2008). b) The non-bilayer lattice of estrone sulfatase (1P49) showing three monomers with nearly orthogonal TM helices and significant inter-monomer interactions (Hernandez-Guzman et al. 2003). c) The energy coupling factor-type riboflavin transporter (3P5N) also forms a lattice in which the TM domain of one protein is rotated by ~90° with respect to its neighbor and inter-protein interactions include helices that pack together via an Ala motif (Zhang et al. 2010).

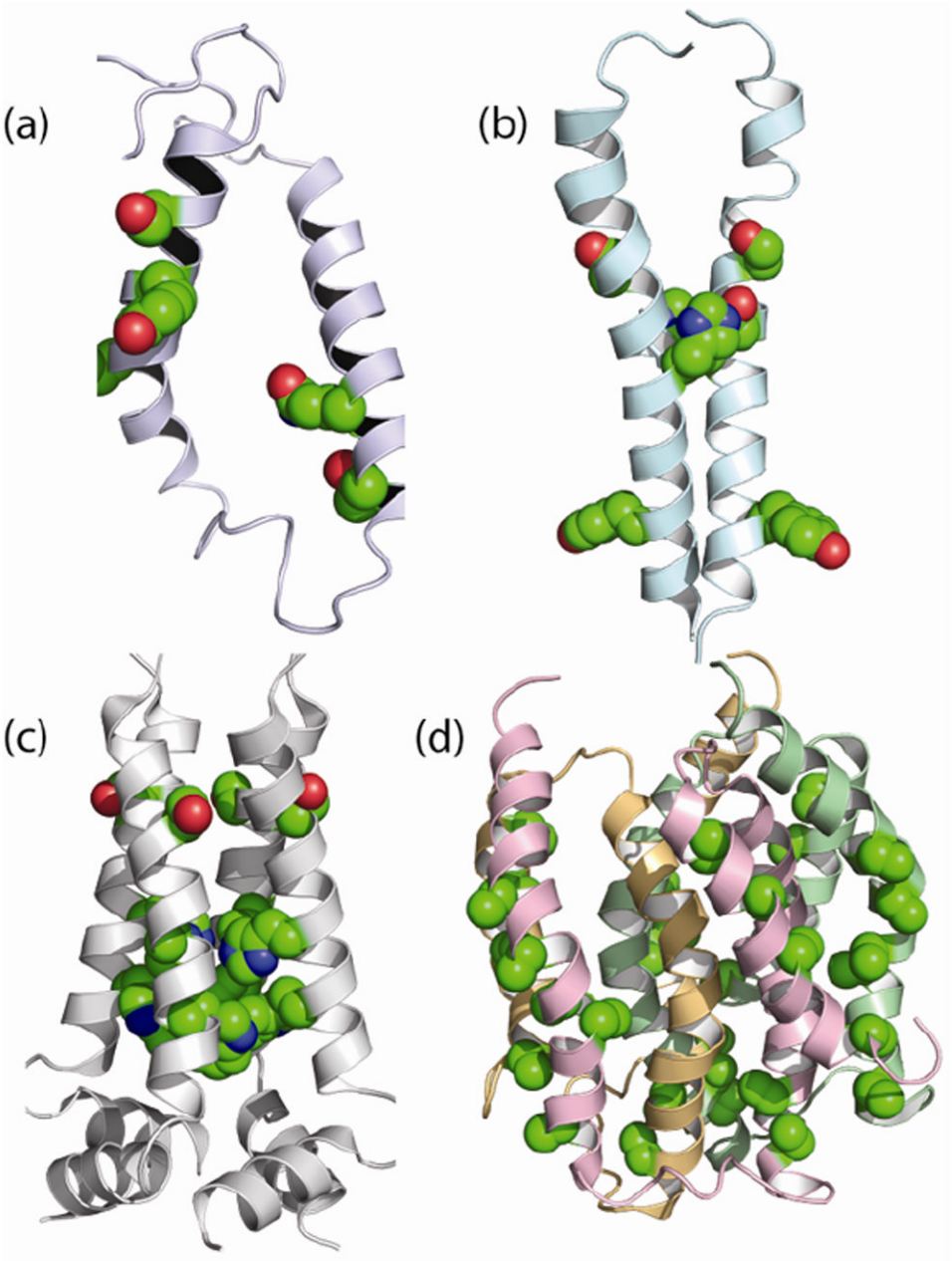

Fig. 8.

A few solution NMR structures of α-helical membrane proteins. a-c) Space filling depiction of the polar residues. a) Histidine kinase receptor, ArcB (2KSD) displaying hydrophilic residues facing the hydrophobic environment and outwardly curved helices, potentially following the inner surface of the micelle. b) The dimer of BCL2/Adenovirus E1B interacting protein 3 (2KA2) shows a pair of interhelical hydrogen bonds between His and Ser residues. c) The tetramer of the drug resistant V27A M2 protein (2KWX) shows His and Trp residues buried near the pseudo four-fold symmetry axis. Ser residues are near the would be membrane interfacial region. d) The trimeric structure of diacyl glycerol kinase (2KDC) displaying outward curved helices and no apparent use of the Ala motifs (Ala Ca and Cb shown in space-filling view) for helix-helix packing.

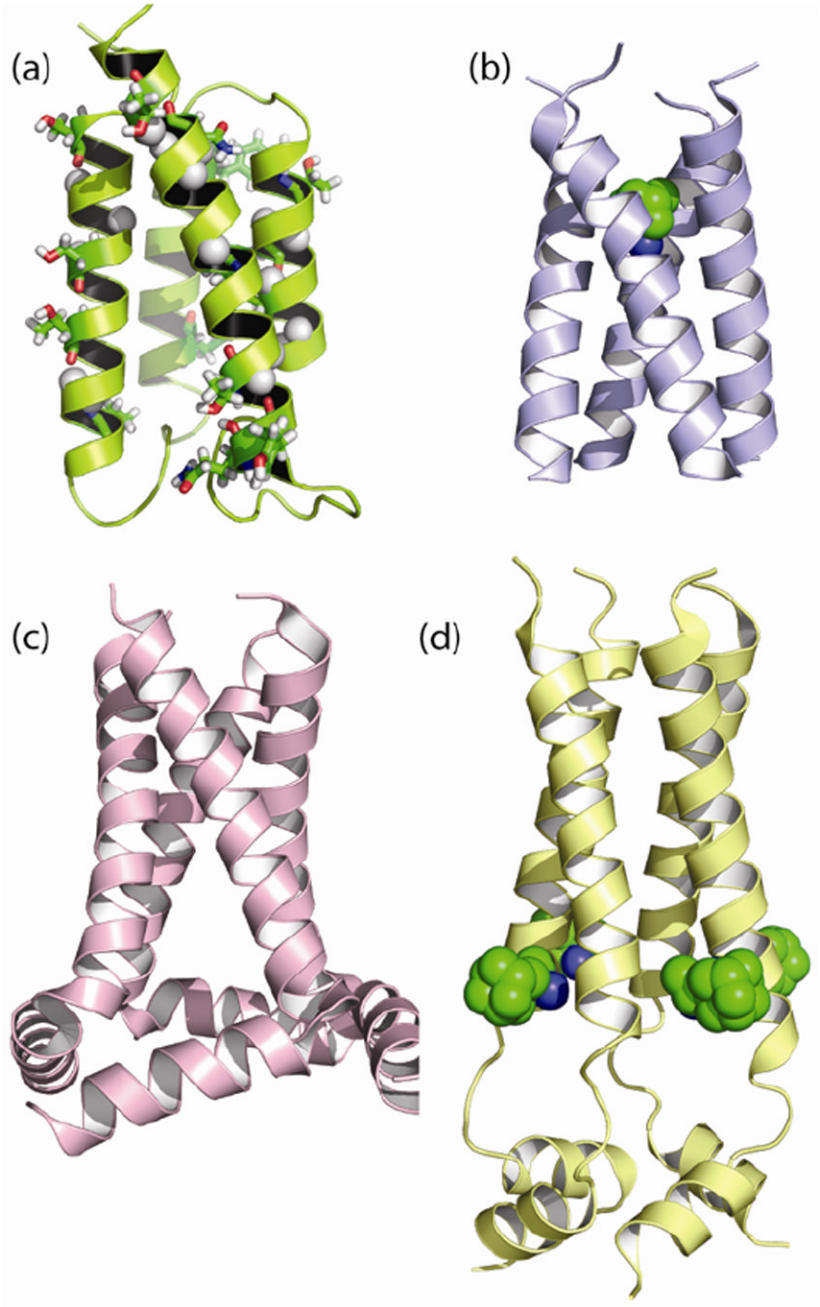

Fig. 9.

Hydrophobic match of the membrane environment with the protein TM domain. a) The histidine kinase receptor, KdpD (2KSF) displays a short helix as well as numerous hydrophilic residues on the external surface of the helical bundle leading to considerable H/D exchange (white space filling amide protons indicate sites that H/D exchange). b-d) Influenza A M2 protein structures. b) The TM domain characterized in lipid bilayers with amantadine bound in the pore (2KQT) displaying helical lengths that span the hydrophobic region of the membrane. c) The conductance domain of M2 (residues 22-62; 2L0J) displaying very similar helical tilt angles to that of 2KQT. d) The M2 conductance domain

in DHPC micelles (2RLF) displaying helices with a small tilt angle and drug (green space filling) on the external surface of the protein.

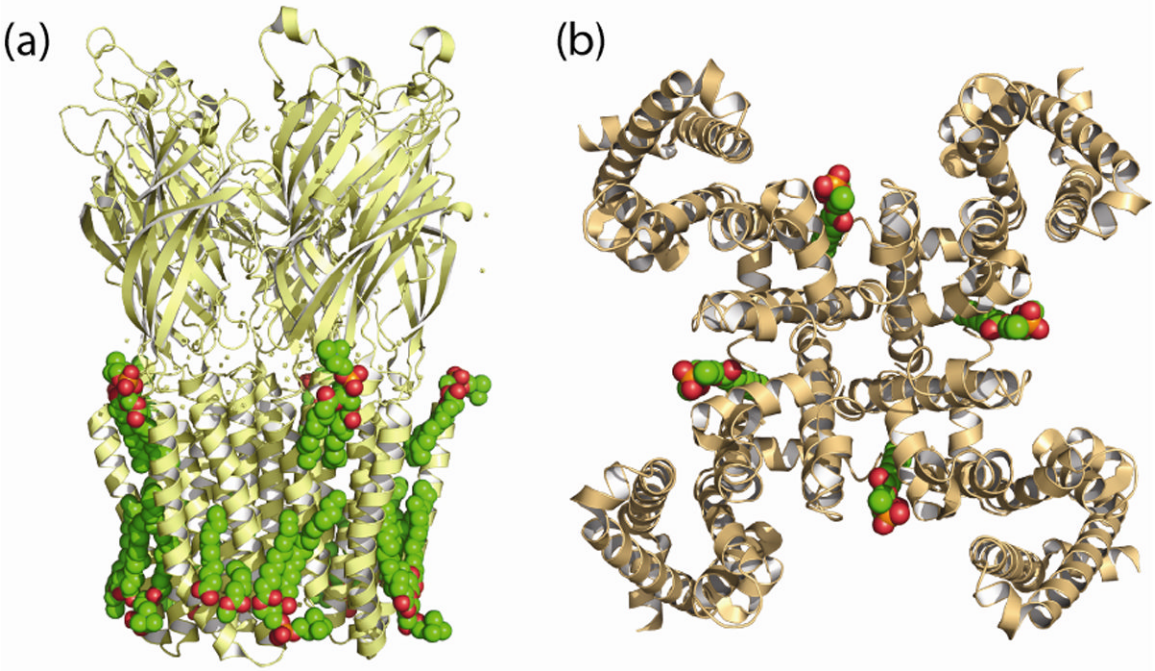

Fig. 10.

Native-like bound lipids and detergents in the crystal lattice shown with space filling atoms. a) In the ligand gated ion channel (PDB: 3EAM) a large number of diacyl lipids diffract in an annulus around the protein. b) For the voltage gated Na^+ channel (3RVY) a monoacyl detergent is bound per monomer in a crevice at the monomer junctions, thought to be a lipid binding site.

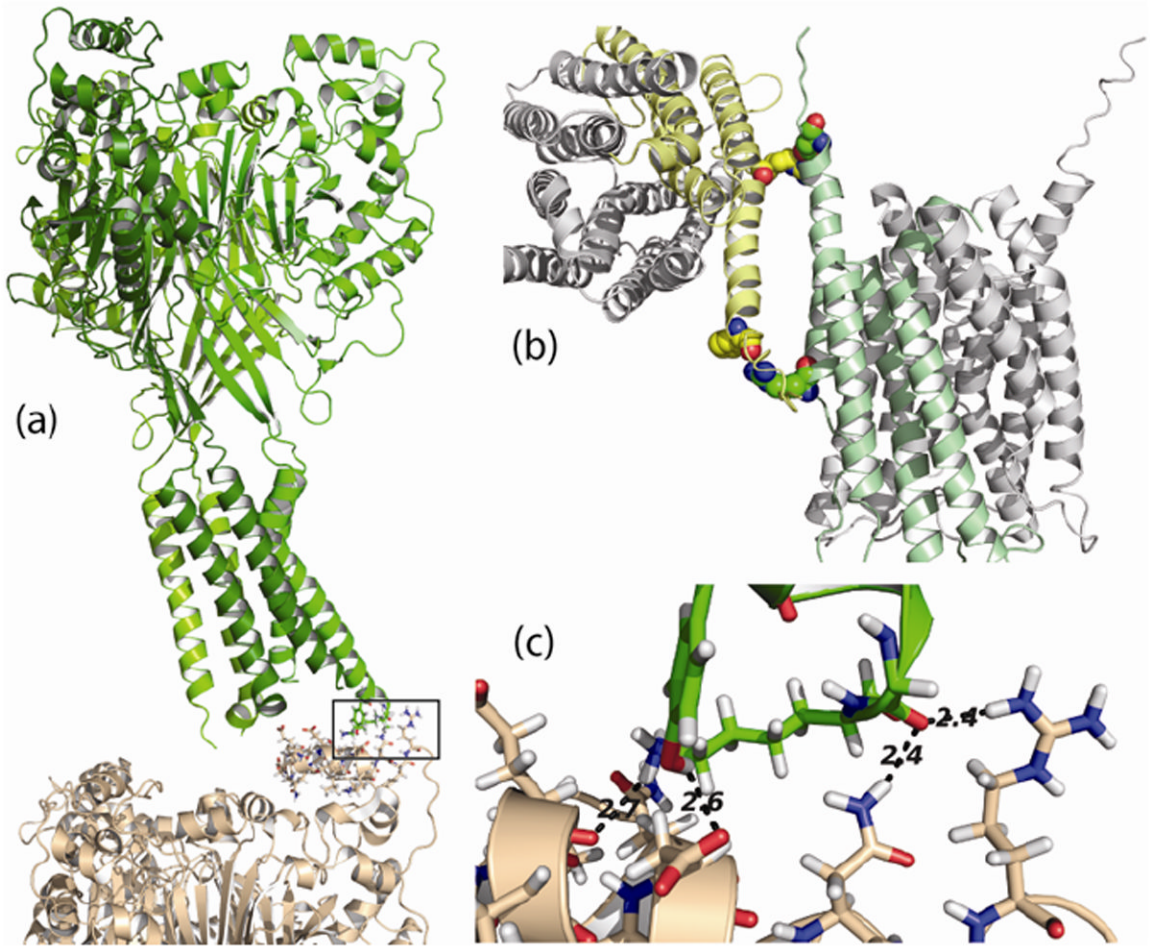

Fig. 11.

Protein-protein crystal contacts can lead to structural perturbations. a) The acid sensing ion channel (ASIC; 2QTS) has a three-fold pseudo-symmetric water soluble domain and a distorted asymmetric TM domain. The contacts between monomers are boxed and highlighted in (c). b) 5-lipoxygenase (2Q7M) is also a trimer and one of the four helices forms crystal contacts with a neighboring trimer leading to what appears to be a shift in the TM helix of nearly 10Å out of the hydrophobic environment and for a hydrophilic interhelical loop to penetrate nearly 10Å into the membrane.

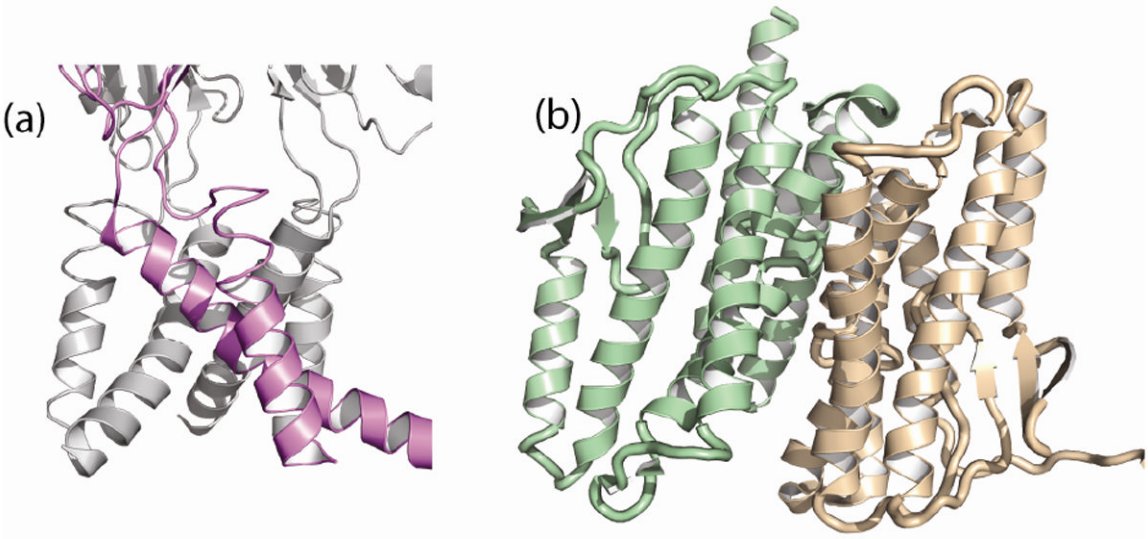

Fig. 12.

Thin hydrophobic environments can lead to excessively tilted helices and even disrupted helical structures. a) The P2X4 structure (3I5D) shows kinked and highly tilted helices resulting in a hydrophobic dimension that is less than 20Å thick and fenestrations into the pore from the fatty acyl environment. b) The site 2 protease (3B4R) displays TM helices that are interrupted by short β-strand segments that expose polar backbone sites to the lipid interstices.

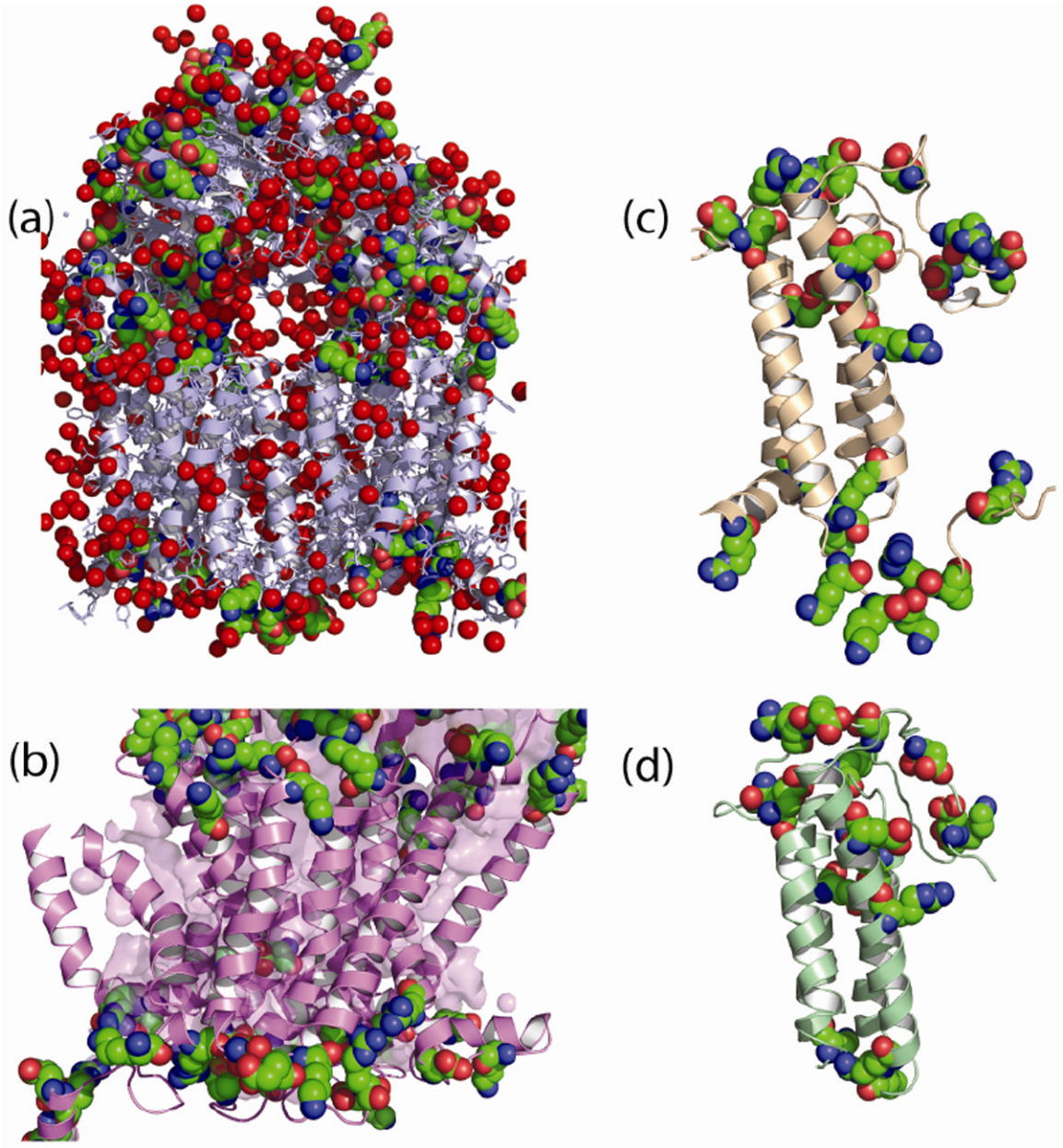

Fig. 13.

Weak hydrophobic environments are suggested by the presence of water and polar groups that appear in the hydrophobic domain of the membrane. The charged residues are shown as spheres. a) The metal chelate transporter (2NQ2) has a large number of water molecules (additional red spheres) crystallized in the vicinity of the hydrophobic helices in a structure that otherwise appears to have a native-like TM domain. b) The maltose transporter (3FH6) has a pair of incomplete helices that do not span the membrane – in a native environment these helices would not be disordered and would span the hydrophobic dimension. c&d) The disulfide bond forming protein DsbB has been characterized by solution NMR (c: 2K73) and XRD (d: 2ZUQ) showing the exposure of hydrophilic residues to the would be bilayer interior.

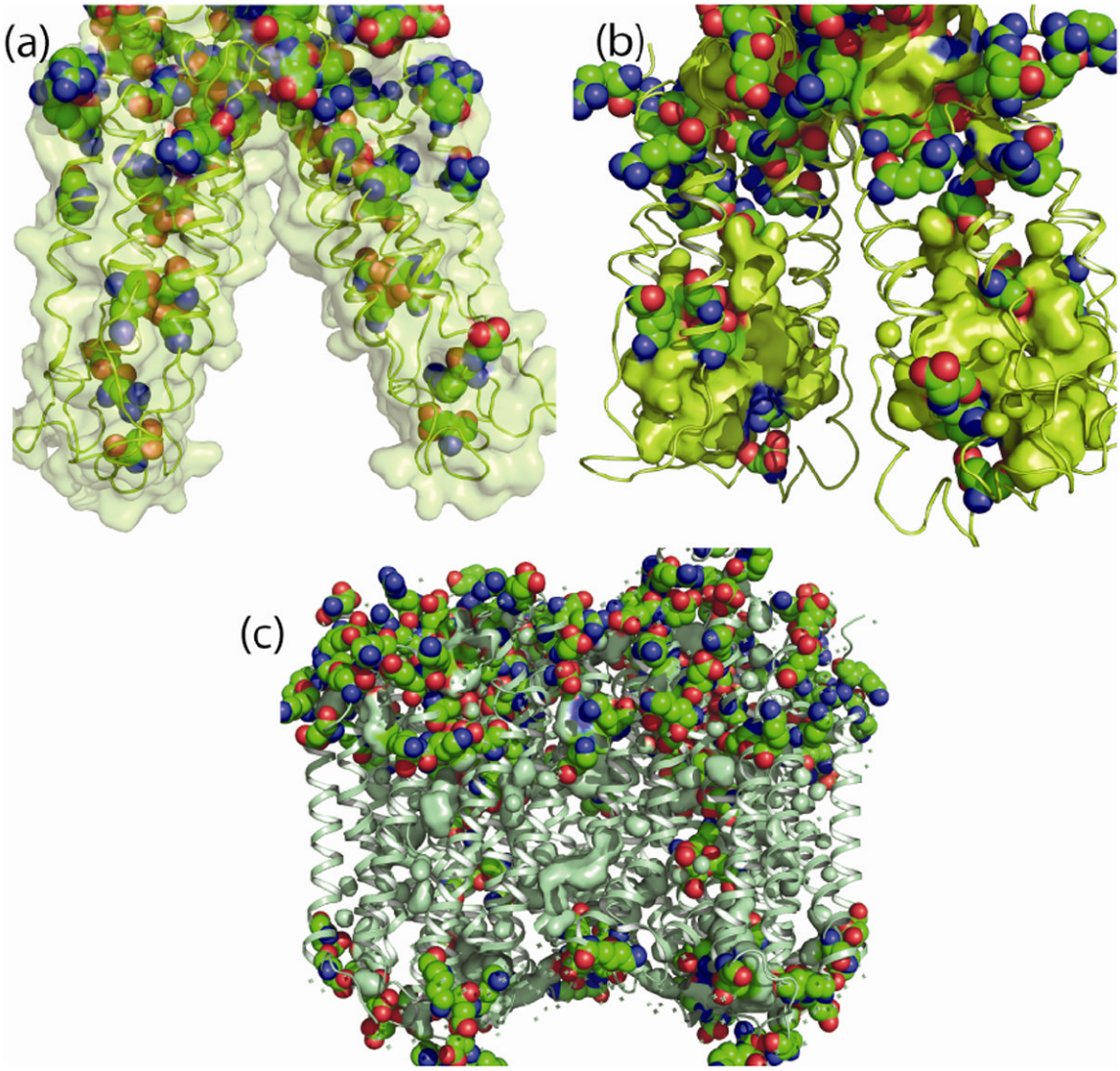

Fig. 14.

Potential Effects of the Lateral Pressure Profile. The atoms of the charged residues are displayed as spheres. a&b) The Zinc transporter, YiiP (2QFI) has two 6 helix bundles splayed when viewed orthogonal to the plane containing the bundles (a) such that lipids could diffuse into the structure. b) When viewed at an oblique angle the cavities within the bundles are substantial, especially in comparison to (c) the well packed helices of the pyrophosphatase (4A01) where again the cavities in the structure are displayed, but are much smaller.

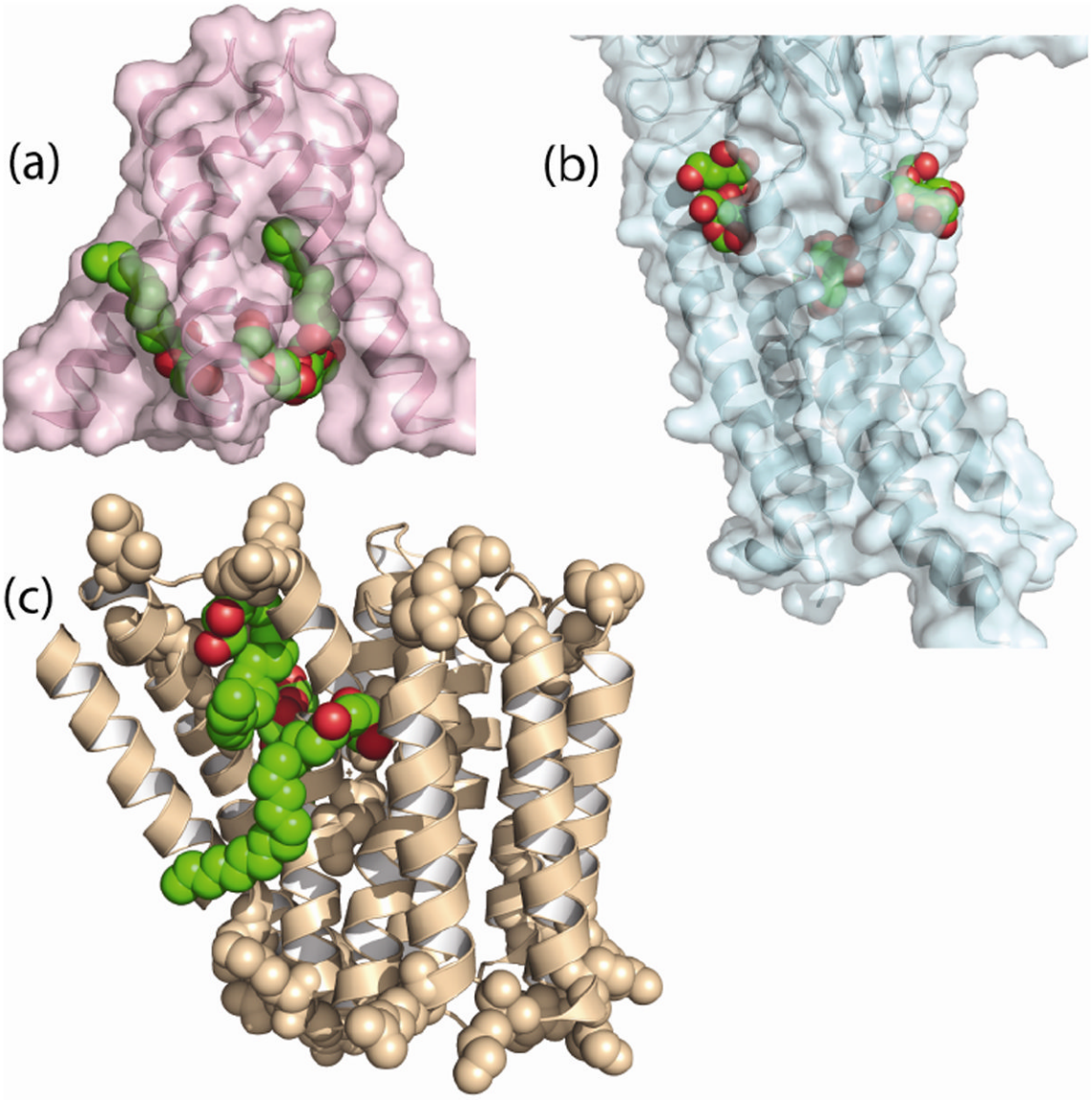

Fig. 15.

Detergent molecules embedded in the protein structures. Colored spheres represent the detergent molecules. a) The M2 proton channel (3BKD) with two octylglucoside and a polyethylene glycol in the pore of the structure and between the tetrameric helices. b) The acid sensing ion channel (2QTS) that displays an asymmetric trimeric TM domain has three detergent molecules embedded in the structure, one of which is on the trimeric axis. c) The sodium calcium exchanger (3V5U) has several detergent molecules embedded within the structure.