Chapter I

Introduction
Chapter 1. Introduction

1.1 Lipid-protein interactions in biological membranes

(i) The biological membrane provides a unique environment for protein function and folding

Biological membranes are complex assemblies of lipids and proteins that allow cellular compartmentalization and act as the interface through which cells communicate with each other and with the external milieu. Biological membranes are formed and maintained due to the hydrophobic effect (Tanford, 1978), an inherent property of the constituent lipids. Biomembranes are thus non-covalent assemblies that are organized and maintained due to the inherent thermodynamics of the system. They form the basis for cellular compartmentalization in biological systems and act as dynamic barriers between cells and their environment. Our view of biological membranes has evolved considerably from the basic idea of the membrane as a stable barrier between aqueous compartments (Stoeckenius and Engelman, 1969) to the cell membrane as a functionally active dynamic ‘border security system’ (Bowie, 2005) that plays an important role in cell function and death. The fluid mosaic model (Singer and Nicolson, 1972) was the first model to consider the inherent fluidity of the membrane, and visualized membranes as mosaics of freely diffusing lipid and protein. While this picture continues to be relevant today more than thirty years after it was first described, current membrane models recognize and give importance to lateral heterogeneity in membrane structure on a nanometer scale and in the distribution and lateral mobility of proteins within the membrane (Jacobson et al., 1995; Vereb et al., 2003).

The biological membrane provides a unique environment for protein function and folding. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of olive oil (Cone, 1972; Poo and Cone, 1974; Edidin, 2003a). In addition, membranes exhibit a considerable degree of anisotropy along the axis perpendicular to the bilayer. While the center of the bilayer is nearly isotropic, the upper portion, only a few angstroms away toward the membrane surface, is highly ordered
Properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of solvent penetration vary in a depth-dependent manner in the membrane. It is important to recognize that the structure of a functional membrane varies considerably from structures obtained for model membranes by crystallography since fluctuations and dynamics are an essential aspect of bilayer structure (Wiener and White, 1991; Nagle and Tristram-Nagle, 2000). The ‘structure’ of a fluid bilayer is therefore operationally defined as a time-averaged spatial (Gaussian) distribution of the principal structural groups, viz., phosphates, carbonyls, and methylenes (Wiener and White, 1992). These distributions are representations of thermal motion, which is a fundamental feature of fluid bilayers.

A striking feature of this structure is that the membrane interfacial region accounts for ~50% of the thermal thickness of the bilayer (White and Wimley, 1994). The interface is a region of ‘tumultuous chemical heterogeneity’ composed of lipid headgroup, water and portions of the acyl chain (Wiener and White, 1992). It is characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the relatively isotropic hydrocarbon-like interior of the membrane (Seelig 1977; Ashcroft et al., 1981; Perochon et al., 1992; Venable et al., 1993; White and Wimley, 1994). The membrane interface is now recognized as the most important region so far as the dynamics and function of the membrane is concerned (Chattopadhyay, 2003). The anisotropy of the membrane environment (chemical as well as motional; also see Figure I.1) has direct implications on the sequence, structure and function of proteins embedded within the membrane.
Figure I.1 (a) A schematic representation of the membrane showing the various regions of the bilayer displaying motional anisotropy. The membrane lipids shown have two hydrophobic tails with a phosphatidylcholine (PC) headgroup. The preferred locations of various amino acids present in a membrane-spanning transmembrane domain are also shown. It should be noted that the tryptophan residues are localized in the membrane interface region, a region characterized by unique organization, dynamics, hydration and functionality. See text for other details (from Raghuraman et al., 2005). In addition, known structures of membrane proteins show that tryptophan residues are clustered at the putative transmembrane boundary. Representations of (b) the crystal structure of the potassium channel KcsA (Doyle et al., 1998; PDB code 1BLB) and (c) the high resolution solid state NMR structure of the gramicidin ion channel (Ketcham et al., 1997; PDB code 1MAG) are shown highlighting the position of tryptophan residues (color code: gray, backbone; blue, tryptophan). All structures were made using RASMOL ver. 2.7.2.1 (Sayle and Milner-White, 1995) using the given PDB coordinates.
The membrane environment influences sequence, structure, and function of membrane proteins

As a result of lipid-protein and protein-protein interactions, the biological membrane constitutes the site of many important cellular functions such as energy metabolism, muscle contraction, nutrient absorption, signal transduction, ion transport, cell-cell contact and recognition (Shai, 2001). However, our understanding of these processes at the molecular level is limited by the lack of high-resolution three-dimensional structures of membrane-bound proteins and peptides (see later). This is in spite of the fact that about 30-40% of all proteins are integral membrane proteins (Smith et al., 2001). Interestingly, membrane proteins, G-protein coupled receptors in particular, constitute drug targets for 50% of recently released drugs, and 25 of the top 100 best-selling drugs (Klabunde and Hessle, 2002). Knowledge of the structure and organization of membrane proteins and an understanding of the influence of the membrane environment on membrane proteins therefore represents a major step toward understanding membrane protein function.

Over the past few years, bound lipid molecules have been resolved in a few high-resolution crystal structures of membrane proteins (Fyfe et al., 2001; Lee, 2003; Palsdottir and Hunte, 2004). Such bound lipids have been described both in annular sites in hydrophobic grooves on the protein surface and at non-annular sites between monomers of multimeric proteins (Luecke et al., 1999). Annular lipids form the first lipid shell around the transmembrane region of the protein and generally interact non-specifically with the protein. The surface presented by transmembrane proteins is rigid and rough as compared to the bulk lipid phase. The dynamic properties of annular lipids are therefore distinct from the bulk, since these lipids are distorted to maximize contact with the surface of the protein (also see Section I.3(i)). Non-annular lipids, on the other hand, bind at specific sites in the transmembrane region of the protein and do not exchange with the bulk lipid. Such non-annular binding sites have been described for the nicotinic acetylcholine receptor (nAChR) (Jones and Mcnamee, 1988). A minimum lipid-protein ratio has been shown to be essential to support activity of nAChR (Jones et al., 1988), and lipid-protein ratios below this minimum, result in irreversible inactivation of the protein. Importantly, this minimum ratio
corresponds to a single shell of lipids around the perimeter of the protein (the annular lipids). In addition, for several proteins specific lipids are required to support protein activity. For example, the nicotinic acetylcholine receptor requires the presence of both sterol and negatively charged lipids for ion gating activity (Fong and McNamee, 1986) and D-β-hydroxybutyrate dehydrogenase has an absolute requirement of phosphatidylcholine for activity (Sandermann et al., 1986) but not lipid binding. Cholesterol has been found to be an important modulator of membrane protein function (Burger et al., 2000; Pucadyil and Chattopadhyay, 2004a) both due to its effects on the bulk membrane phase (Sankaram and Thompson, 1991; Needham and Nunn, 1990; Needham, 1995) and due to specific interactions with the protein (Jones and McNamee, 1988; Pucadyil et al., 2005).

Membrane-spanning proteins have distinct stretches of hydrophobic amino acids that form membrane-spanning and membrane interface flanking domains (Figure I.1). Thus membrane anisotropy involves sequence anisotropy of membrane spanning domains. Statistical studies of sequence databases and available crystal structures of integral membrane proteins show that hydrophobic amino acids such as Ile, Leu, Val, and Phe are present in the transmembrane region of membrane proteins (Landolt-Marticorena et al., 1993; Reithmeier, 1995; Ulmschneider and Sansom, 2001; Adamian et al., 2005). Charged residues, in particular positively charged residues such as Lys and Arg are found at the membrane interface particularly at the cis (intracellular) side of the membrane (von Heijne, 1986). This 'positive inside rule' is most probably related to the asymmetric distribution of negatively charged lipids on the cis side of the membrane. In addition, aromatic amino acids in particular tryptophan residues, are not uniformly distributed and tend to be preferentially clustered at the membrane interface, possibly because they are involved in hydrogen bonding (Ippolito et al., 1990) with the lipid carbonyl groups or interfacial water molecules (see Figure I.1). Interestingly, membrane proteins are reported to have a significantly higher tryptophan content than soluble proteins (Schiffer et al., 1992). Recent crystal structures of membrane proteins such as the potassium channel (Doyle et al., 1998), bacteriorhodopsin (Luecke et al., 1999), maltoporin (Schirmer et al., 1995) and others have shown that most tryptophans are located in a saddle-like 'aromatic belt' around the
membrane interfacial region. Furthermore, for transmembrane peptides and proteins, tryptophan has been found to be an efficient anchor at the membrane interface (Schiffer et al., 1992; de Planque et al., 1998) and defines the hydrophobic length of transmembrane helices (Demmers et al., 2001). Importantly, the role of tryptophan residues in maintaining the structure and function of membrane proteins is exemplified by the fact that substitution, deletion or modification of tryptophans often results in reduction or loss of protein functionality (Becker et al., 1991; Fonseca et al., 1992; Miller and Falke, 2004; Sobko et al., 2004; Draheim et al., 2005; also see Section 1.2(ii)).

The preferential clustering of tryptophan residues at the membrane interface in membrane proteins and peptides is related to the energetics of interfacial partitioning of amino acids and the unique molecular structure of tryptophan (see later). White and co-workers showed in a series of elegant experiments that the energetics of partitioning of amino acids (as guest residues in unstructured pentapeptides) to a membrane phase is distinct from partitioning to a bulk hydrophobic phase such as octanol (White and Wimley, 1999). The scale based on partitioning from water to a bulk octanol phase measures the free energy of transfer of a residue from water to the bilayer hydrocarbon core (Wimley et al., 1996). The interface scale (based on partitioning to a membrane phase), however measures the free energy of transfer of a residue within an unfolded polypeptide chain from water to a membrane bilayer interface (Wimley and White, 1996). Both scales in combination therefore, identify those regions of a peptide chain most likely to associate with membranes, and establish a hydrophobicity scale (Wimley-White scale) that can be used to predict transmembrane regions in protein sequences (Jayasinghe et al., 2001). Importantly, both scales provide a measure of the significant unfavorable free energy change involved in partitioning non-hydrogen bonded peptide bonds to the membrane phase, bringing into focus the coupling of folding and membrane partitioning in membrane protein biosynthesis (White et al., 2001). In fact, the membrane interface has been referred to as a catalyst for the formation of secondary structure by peptides (White et al., 2001). Interestingly, it has very recently been reported that such an 'in vitro' hydrophobicity scale
is relevant to the stability of transmembrane helices in the ER membrane during Sec61 translocon mediated insertion (Hessa et al., 2005).

(iii) Tryptophan at the membrane interface

As mentioned earlier, tryptophan residues are not uniformly distributed in membrane proteins and tend to be preferentially clustered at the membrane interface. According to the hydrophobicity scale discussed above, the experimentally determined interfacial hydrophobicity of tryptophan is the highest among the naturally occurring amino acid residues (Wimley and White, 1996). While tryptophan has the polar $-\text{NH}$ group which is capable of forming hydrogen bonds, it also has the largest nonpolar accessible surface area among the naturally occurring amino acids (Chothia, 1976). Due to its aromaticity, the tryptophan residue is capable of $\pi-\pi$ interactions and weakly polar interactions (Burley and Petsko, 1985, 1988). This amphipathic character of tryptophan gives rise to its unique hydrogen bonding property and ability to function through long range electrostatic interaction (Fonseca et al., 1992; Andersen et al., 1998). The preferential location of tryptophan residues at the membrane interface is thought to be due to the aromaticity of the indole moiety and the overall amphipathic nature of tryptophan (Yau et al., 1998). Interestingly, tryptophan has very recently been shown to interact specifically with certain phospholipids (Popova and Hincha, 2004).

Knowledge of membrane protein structure is key to understanding membrane function. Since the elucidation of the first membrane protein structure ~20 years ago (Deisenhofer et al., 1985), there have been several reports of high-resolution crystallographic structures of physiologically relevant membrane proteins (Doyle et al., 1998; Sui et al., 2001; Jiang et al., 2003; Abramson et al., 2003).* However, despite these successes and predictions of an explosion of membrane protein structures in the future (Bowie, 2000; White, 2004), information obtained from crystallographic data is necessarily

* for a complete list of known membrane protein structures see blanco.biomol.ucla.edu/Membrane_Proteins_xtal.html
The utility of such static membrane protein structures in the interpretation of functional mechanisms is limited (Miller, 2003; Jackson, 2004; Bezanilla, 2005) by the inherent disorder and fluctuation of the membrane environment.

For this reason, spectroscopic approaches in general and fluorescence spectroscopic approaches in particular have become very useful for analyses of membrane protein dynamics (Chattopadhyay and Raghuraman, 2004). Fluorescence spectroscopy is widely used in the analysis of membrane protein structure and function. The advantages of using fluorescence are intrinsic sensitivity, suitable time scale, non-invasive nature, and minimum perturbation (Lakowicz, 1981; Chattopadhyay, 1992; Stubbs and Williams, 1992; Mukherjee and Chattopadhyay, 1995; Chattopadhyay, 2003). In addition, the ability to incorporate fluorophores in a site specific manner makes fluorescence approaches very powerful (Cohen et al., 2002a; Clark et al., 2003; Powl et al., 2005).

Tryptophan residues serve as useful site-specific fluorescence probes for protein structure and dynamics (Eftink, 1991c) and are generally present at about 1 mol% in proteins (Lakowicz, 1999). The well documented sensitivity of tryptophan fluorescence to environmental factors such as polarity and mobility makes tryptophan fluorescence a valuable tool in studies of protein structure and dynamics by providing specific and sensitive information of protein structure and its interactions (Kirby and Steiner, 1970; Beechem and Brand, 1985; Eftink, 1991c; Lakowicz, 1999). The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analysis as apparent from the fact that ~300 papers utilizing tryptophan fluorescence in proteins are published per year (Vivian and Callis, 2001). The non-random distribution of tryptophan residues at the membrane interface, is an added advantage to use tryptophan fluorescence to study membrane protein structure and dynamics (Chattopadhyay et al., 1997; Williamson et al., 2002; Raghuraman and Chattopadhyay, 2004b; Rawat et al., 2004). This thesis is focused on understanding the influence of the lipid environment on membrane protein conformation and organization. I have utilized the environmentally sensitive fluorescence of the functionally important tryptophan residues of the gramicidin ion channel to monitor the lipid-protein interactions.
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of this model ion channel. I have used novel fluorescence approaches such as wavelength-selective fluorescence, and other sensitive fluorescence and CD spectroscopic approaches to address this issue. The following sections of this chapter will focus on the model ion channel gramicidin, which represents a paradigm for transmembrane proteins whose function and conformation is intimately linked to the membrane environment and on a particular type of lipid-protein interaction, hydrophobic mismatch.

1.2 Gramicidin: a model ion channel

Ion channels are transmembrane proteins that regulate ionic permeability in cell membranes. They represent an important class of molecules due to their ability to serve as key elements in signaling and sensing pathways and to connect the inside of the cell to its outside in a selective fashion. They are crucial for normal functioning of cells and defective ion channels are implicated in a number of diseases collectively known as ‘channelopathies’ (Cooper and Jan, 1999; Jentsch et al., 2004). Mutations in ion channels underlie diseases such as cystic fibrosis (Stutts et al., 1995), renal disorders, certain types of hypertension, osteoporosis (Kornak et al., 2001), and conditions such as deafness (Kharkovets et al., 2000) and reduced sleep (Cirelli et al., 2005). The recent successes in crystallographic analyses of ion channels starting with the KcsA potassium channel (Doyle et al., 1998), the chloride channel (Dutzler et al., 2002) and more recently the voltage gated potassium channel (Jiang et al., 2003) have provided exciting molecular insights to ion channel function and structure (MacKinnon, 2004). However, it is becoming increasingly clear that static crystallographic structures of membrane proteins may not always provide accurate representations of channel function (Gonzalez et al., 2005; Bezanilla, 2005). Due to this ambiguity in the structural analysis of ion channel function, simple models of ion channels continue to provide useful information to understand and characterize more complex systems (Miloshevsky and Jordan, 2004a).
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The linear gramicidins are a family of prototypical channel formers that have been extensively used to study organization, dynamics and function of membrane-spanning channels (Koeppe and Andersen, 1996; Miloshevsky and Jordan, 2004a). Gramicidins are linear pentadecapeptide antibiotics with a molecular weight of ~ 1900. They are produced by the soil bacterium Bacillus brevis, and consist of alternating L- and D-amino acids (Sarges and Witkop, 1965). They form well-defined cation-selective ion channels in model membranes (Hladky and Haydon, 1972) with conductance of the order of $10^7$ ions per second. Due to their small size, ready availability and the relative ease with which chemical modifications can be performed, gramicidins serve as excellent models for transmembrane channels. The natural mixture of gramicidins, often denoted as gramicidin A' (termed gramicidin D (after René Dubos, who originally discovered gramicidin) in older literature), consists of ~ 85% of gramicidin A, which has four tryptophan residues at positions 9, 11, 13 and 15 (see Figure 1.2). Gramicidin A' is readily available commercially and is fluorescent, due to the presence of tryptophan residues (Mukherjee and Chattopadhyay, 1994). It has one of the most hydrophobic sequences known (Segrest and Feldman, 1974).

HCO-L-Val$^1$-Gly$^2$-L-Ala$^3$-d-Leu$^4$-L-Ala$^5$-
d-Val$^6$-L-Val$^7$-d-Val$^8$-L-Trp$^9$-d-Leu$^{10}$-L-Trp$^{11}$-
d-Leu$^{12}$-L-Trp$^{13}$-d-Leu$^{14}$-L-Trp$^{15}$-NHCH$_2$CH$_2$OH

Figure 1.2 Amino acid sequence of gramicidin A. Note that all amino acid side chains are either hydrophobic (Ala, Leu, Val) or amphipathic (Trp). In addition, the -NH$_2$ and -COOH termini are blocked making the sequence unusually hydrophobic. Alternating d-amino acid residues are shown in blue.

Gramicidins were first identified as an antibiotic produced by a culture of Bacillus brevis isolated from soil samples by Dubos (Dubos and Cattaneo, 1939; Hotchkiss and Dubos, 1940). Gramicidin was the first antibiotic to be used clinically (in 1939) and its success in clinical settings stimulated research into the practical application of other drugs such as penicillin (Moberg and Cohn, 1991). Gramicidin is produced during sporulation shortly after the production of another antibiotic, tryrocidine (Killian, 1992). The concentration of gramicidin in the bacterial cell is known to reach up to 1 M during
sporulation. Its function in *B. brevis* is not known, but it is believed to play a role in gene regulation during the shift from vegetative growth to sporulation and has been shown to inhibit *Escherichia coli* RNA polymerase (Killian, 1992).

Interestingly, the amino acid sequence of gramicidin consists of alternating L- and D- amino acids (Sarges and Witkop, 1965), in sharp contrast to most proteins which contain exclusively L- amino acids (Mitchell and Smith, 2003). Gramicidin is synthesized by non-ribosomal multienzyme complexes, and the conversion of L- to D- amino acids takes place during biosynthesis. As mentioned earlier, the natural mixture of gramicidins contains predominantly gramicidin A (85%). The other peptides, gramicidin B and C differ in the nature of the aromatic residue at position 11, where Trp (in A) is replaced by Phe and Tyr, respectively. Further, in about 5 to 20% molecules Val at position 1 is replaced by Ile. In spite of the alternating sequence of L-D chirality generally not encountered in naturally occurring peptides and proteins, gramicidin represents a useful model for realistic determination of conformational preference of proteins in a membrane environment. This is due to the fact that the dihedral angle combinations generated in the conformation space by various gramicidin conformations are 'allowed' according to the Ramachandran plot (Andersen et al., 1996). The gramicidin channel is formed by the head-to-head dimerization of β (π LD) helices. The π LD helix was proposed by Urry (1971) on the basis of molecular modeling, in which consecutive peptide carbonyl groups alternate direction with respect to the helix axis. The nomenclature of these structures was later changed to β-helices to better reflect the type of hydrogen-bonding present, which is in fact the same as that of β-sheets (Venkatachalam and Urry, 1983). Due to alternating D and L residues, all side chains project from the same side of the peptide strand, which becomes the outside when the sheet is rolled into a helix. The inner wall of the gramicidin channel is therefore lined by the polar groups of the peptide backbone, while the side chains project from the exterior surface (See Figure 1.3a).
The structural features that make gramicidin channels so unique raise the question whether gramicidin channels are appropriate models for "real" ion channels. Fortunately, the elucidation of the crystal structure of the *Streptomyces lividans* K+ channel (KcsA) (Doyle *et al.*, 1998) in molecular detail has provided an opportunity to evaluate the features of the gramicidin channel that are shared with other channels from natural sources. This has led to the rather interesting finding that gramicidin channels share important structural features with "real" ion channels (Chattopadhyay and Kelkar, 2005). An essential feature of ion channels is the ability to select for specific ions using a selectivity filter.

**Figure I.3**

(a) Top view of the gramicidin channel as a space-filling model (color code: white, carbon atoms; blue, nitrogen atoms; red, oxygen atoms) made as in Figure I.1. Note how the alternating L-D arrangement allows all amino acid side chains to project outward from the channel lumen and the channel lumen is lined by the peptide backbone.

(b) Structure of the selectivity filter (residues 75-79) of the KcsA K+ channel. Two subunits are shown in ball-and-stick representation made as in Figure I.1 (color code: same as in (a)). Note how all amino acid side chains extend outwards from the selectivity filter.
Remarkably, both the gramicidin channel and the selectivity filter of the KcsA $K^+$ channel are lined by the polar carbonyls of the peptide backbone and ion selectivity in both cases arises due to backbone interactions with ions (Wallace, 1999, 2000). While in the gramicidin channel such interactions are possible due to alternating L-D chirality, KcsA utilizes two highly conserved glycine residues in the selectivity filter (See Figure I.3b). The choice of glycine is obvious since it is achiral, and has a larger allowed region in the Ramachandran plot, and so effectively it can behave as a D-amino acid. A very recent report (Valiyaveetil et al., 2004) confirms and extends this notion by pointing out that the two absolutely conserved glycine residues in the KcsA $K^+$ channel, which are essential in the $K^+$-selectivity filter, serve as surrogate D-amino acids, reminiscent of the D-amino acids found in the gramicidin channel.

(i)  
**A testing ground for structure-function relationships**

Gramicidin forms well-defined ion channels in membrane environments and induces rectangular current steps of defined amplitudes in planar lipid bilayers (Hladky and Haydon, 1972). The ability to synthesize gramicidin incorporating specific modifications has greatly enhanced the utility of gramicidin as a model ion channel to understand specific structure function relationships (Greathouse et al., 1999). This includes various isotope labeled gramicidins which have greatly advanced NMR studies of this peptide to study specific backbone and side chain conformations (Ketcham et al., 1993; Hu and Cross, 1995; Koeppel et al., 2003). Single channel measurements of gramicidin activity have become possible by the use of a modification of the patch clamp method called the 'bilayer punch' method (Andersen, 1983). In addition, hybrid channel formation (using gramicidin analogues that form functionally distinct channels) can be used to test the structural equivalence of different gramicidin analogues (Durkin et al., 1990, 1992). Using negatively charged analogues of gramicidin (Bamberg et al., 1977) that form distinct hybrid channels with native gramicidin it was shown that the functional channel is formed by a transmembrane dimer with no lateral associations (Cifu et al., 1992). The
transmembrane dimer is formed by the head-to-head (transmembrane) association of $\beta^{63}$ monomers (O'Connell et al., 1990). Surprisingly, even though the sequence of gramicidin is very hydrophobic, it is relatively impermeant in membrane bilayers (i.e., it does not flip to the other membrane leaflet), and very low channel activity is seen when gramicidin is added asymmetrically to membranes bilayers (black lipid membranes) (O'Conell et al., 1990). This is probably due to the high tryptophan content at the C-terminus (see Fig. 1.2) that would prevent transmembrane flipping. As discussed earlier, tryptophan is an amphipathic amino acid (see Section I.1(iii)) with a marked preference for the membrane interface. The burial of tryptophan residues in the hydrophobic core of the membrane is therefore relatively unfavorable (Yau et al., 1998). Interestingly, theoretical calculations predict a large free energy barrier of $\sim 35$ kcal/mol for the vertical insertion of gramicidin dimers in the channel form into a membrane bilayer (Bransburg-Zabary et al., 2002).

Detailed analysis of the contributions of specific amino acids to channel function has been possible due to a synergistic combination of functional and spectroscopic studies. The C-terminal tryptophan residues play an important role in channel conductance and gramicidin structure (discussed in detail in Section (ii)). While the tryptophan residues are known to be important for channel function and structure, recently the intervening D-Leu ‘spacer’ residues (residues 10, 12, and 14) have been implicated in maintaining ion conductance (Jude et al., 1999a; Koeppe et al., 2000). Semi-conservative substitutions of the D-Leu residues with residues such as D-Ala, D-Val or D-Ile were found to have surprisingly large effects on channel structure and function. All the substituted analogues were found to occur in heterogeneous conformations (both double and single stranded) in membranes and had significantly lower conductance as compared to native gramicidin (Jude et al., 1999a). In view of the importance of the orientation of tryptophan dipoles in channel conductance, these results point to a complex interaction between the aromatic and aliphatic side chains that can influence backbone folding and ion entry. In addition, D-Leu-10 stabilizes optimum orientations of Trp-9 in membrane bilayers and therefore substitutions at this position alter channel conductance without affecting the basic fold of the peptide backbone (Koeppe et al., 2000).
While C-terminal residues play important roles in ion entry and channel conductance, the residues at the N-terminal junction influence the formation of the transbilayer channel. Using simultaneous conductance and fluorescence measurements, Veatch et al. (1975) showed that the dimerization constant of gramicidin in black lipid membranes is $\sim 2 \times 10^{13} \text{ mol}^{-1} \text{cm}^2$. The dimeric channel is stabilized by at least six hydrogen bonds at the N-termini (O’Connell et al., 1990) and channel gating is thought to occur when at least two hydrogen bonds are broken (Miloshevsky and Jordan, 2004b). Substitutions or deletions at N-terminal positions therefore interfere with the formation of the conducting dimer (Durkin et al., 1993; Jude et al., 1999b). Introduction of a dipolar F$_6$Val-1 at the N-terminus leads to the formation of homodimeric channels with reduced conductance and lifetime as compared to gramicidin A channels (Durkin et al., 1990). However, heterodimers of this analogue and gramicidin A were found to have much lower conductance than either homodimer rather than the expected intermediate conductance. The heterodimeric channels were found to show voltage dependent transitions from a low to a high conductance state and are therefore voltage gated (Oiki et al., 1995). Interestingly, voltage gating for this heterodimeric channel occurs even though there is no fixed charge, raising questions for voltage gating of channels in general (such as in voltage gated potassium channels).

(ii) Tryptophans in ion channel function

The C-terminal tryptophan residues of gramicidin have been shown to be an integral component of channel activity and the ‘thermodynamically preferred’ structure of gramicidin in membrane environments. Gramicidin is a very hydrophobic peptide and the four C-terminal tryptophan residues (in gramicidin A) are the only amphipathic moieties in the sequence. As discussed previously, tryptophan has a distinctive amino acid side chain with unique molecular properties among the naturally occurring amino acids (see Section 1.1 (iii)). The membrane interface seeking properties of tryptophan and the oriented dipole moments of the tryptophan side chain influence gramicidin structure and function.
Replacement of gramicidin tryptophans with other aromatic side chains such as napthylalanine, phenylalanines or tyrosine (Prasad et al., 1983; Daumas et al., 1989; Becker et al., 1991; Fonseca et al., 1992) has been found to reduce gramicidin channel activity. In addition, photolysis of tryptophan by ultraviolet irradiation (Busath and Waldbillig, 1983; Jones et al., 1986; Busath and Hayon, 1988; Strässle et al., 1989) or chemical modification by an oxidizing agent such as N-bromosuccinimide (Sobko et al., 2004) leads to reduction in cation conductivity.

Trp → Phe substitutions represent an interesting case since they are semi-conservative. While both Trp and Phe are aromatic, Phe is nonpolar and does not function as a hydrogen bond donor like Trp (through the indole -NH). Importantly, while Phe does not have a dipole moment, Trp has a dipole moment of ~2D (Weiler-Feilchenfeld et al., 1970). In a careful analysis of cumulative Trp → Phe substitutions, Andersen and co-workers showed that the single channel conductance of substituted gramicidins decreases as a function of the number of Trp residues substituted by Phe (Becker et al., 1991). In addition, the specific position of the Phe substitution is important for the effect on channel conduction (but not channel lifetime). Specifically, for monosubstitutions channel conductance decreases as Phe is moved from position 15 to 9 (and vice versa for the single tryptophan of trisubstituted analogues). This suggests that the predominant effect of Trp → Phe substitutions is the removal of electrostatic interactions between the indole dipole and the permeant ion. In addition, orientation and mobility of gramicidin tryptophans (and hence, indole dipole moments) in membrane environments would be an important structural feature of gramicidin.

If long-range electrostatic interactions are important for gramicidin conduction, Trp modifications that modulate the indole dipole moment without affecting the hydrogen bonding ability of tryptophan should alter channel conductance. Fluorinated tryptophans represent a useful class of Trp analogues, that can be used to alter the spectral properties of tryptophan (Ross et al., 1990) and the magnitude of the indole dipole moment (Weiler-Feilchenfeld et al., 1970). In 5F-Trp, fluorination at position 5 in the indole side chain results in an increased dipole moment of 3.6 D (as compared 2.1 D for indole) with a
similar orientation as the dipole of indole (Weiler-Feilchenfeld et al., 1970). Since the removal of a dipole due to Trp → Phe substitution decreases channel conductance, 5F-Trp analogues should exhibit increased conductance as compared to native gramicidin (Andersen et al., 1998). This was shown to be the case for a gramicidin analogue with 5F-Trp at position 11 (Andersen et al., 1998). Significantly, a doubly substituted analogue (5F-Trp-9 and Phe-11) was found to have increased conductance as compared to an analogue with a single Trp → Phe (Phe-11) substitution. In addition, the specific position of fluorination (i.e., 6, 5, or 4F-Trp) which would alter the orientation and magnitude of the indole dipole moment has been shown to influence the effect of the substitution on channel activity (Anderson et al., 2001). Importantly, solid-state NMR shows that fluorination of Trp side chains has little effect on side-chain positions (Cotten et al., 1999).

As mentioned earlier, side chain orientations of gramicidin tryptophan residues would play an important role in channel activity. The additive effect of replacing tryptophan residues by Phe on channel activity suggests that the orientation of the dipole moment of each tryptophan is similar. NMR analysis of specific indole ring geometry has shown this to be true in general (Hu et al., 1993; Koeppe et al., 2003), although the orientation of Trp-9 remains contentious (Hu and Cross, 1995; Allen et al., 2003; see later). Interestingly, the indole rings of gramicidin tryptophans have been shown to be relatively immobile (Hu et al., 1993; Mukherjee and Chattopadhyay, 1994; Chiu et al., 1999; Koeppe et al., 2003; Rawat et al., 2004), possibly due to hydrogen bonding with lipid carbonyl oxygens (Woolf and Roux, 1996). Molecular dynamics simulations have shown this to be particularly true for Trp-13 which forms a long-lasting hydrogen bond to a phospholipid oxygen (Chiu et al., 1999). However, it has been suggested that Trp-9 is relatively more mobile than the other Trp side chains and high-resolution structures differ in the orientation of Trp-9 relative to Trp-15 (Ketchem et al., 1993; Townsley et al., 2001). Calculations suggest that changes in the rotameric state of Trp-9 could give rise to energy differences of the order of ~1 kcal/mol for an ion in the pore (Allen et al., 2003). Knowledge of the precise orientation of Trp side chains is important since the rate of ion movement depends
on the energetics of ion-channel interactions and a ~1.3 kcal/mol difference in energy could result in a 10-fold change in ion flux.

Considering that the β6.3 helix would have 6.3 residues per turn, any residue (residue i) would be spatially close to the seventh residue (i + 6) in the sequence. An interaction between the Trp-9 and Trp-15 indole groups is therefore possible due to their close proximity in space. However, high resolution solution NMR structures of gramicidin in sodium dodecyl sulphate (SDS) micelles showed that Trp-9 and Trp-15 do not interact through space (Arseniev et al., 1985; Townsley et al., 2001) while solid-state NMR (in oriented DMPC bilayers) has shown the Trp-9 and Trp-15 indole rings to be stacked (Ketchem et al., 1993; Hu and Cross, 1995). In addition, fluorescence spectroscopy suggested the presence of aromatic-aromatic stacking interactions in membrane environments on the nanosecond timescale (Mukherjee and Chattopadhyay, 1994). Importantly recent fluorescence spectroscopy results indicate that the stacking interactions of Trp-9 and Trp-15 may be dependent on the curvature of the host assembly (S.S. Rawat, D.A. Kelkar, and A. Chattopadhyay, manuscript submitted). Therefore, relatively planar ‘rod-shaped’ micelles or oriented bilayers (as used in solid state NMR) might favor aromatic-aromatic stacking interactions of Trp-9 and Trp-15 while in highly curved spherical SDS micelles orientations of Trp-9 away from Trp-15 are preferred. A recent report compared the conformations of Trp-9 during molecular dynamics simulation in DMPC, when the high resolution NMR structures of gramicidin reported from SDS micelles and DMPC bilayers were used as the starting conformations (Allen et al., 2003). The side chain of Trp-9 was found to be more mobile than Trp-11, 13, or 15 and undergoes spontaneous transitions between orientations of the DMPC (stacked) and SDS (non-stacked) structures. Importantly, it was suggested that a mixture of rotameric states for Trp-9 would better reproduce experimental results. A mixture of rotameric states with 80% of the SDS rotamer and 20% of the DMPC rotamer was found to best reproduce NMR side chain observables.

Keeping in mind the interfacial propensity of tryptophan residues, and the overall hydrophobicity of the gramicidin sequence, Trp → Phe conversions should result in altered
interactions with the membrane environment and therefore possible changes in backbone structure. It has been shown that the gramicidin analogue in which all four tryptophan residues are replaced by phenylalanines appears to preferentially adopt the alternate antiparallel double stranded helical dimer conformation (Cotten et al., 1997; Salom et al., 1998). In the absence of any tryptophan residues, the double stranded helical dimer non-channel conformation becomes the energetically favored state in the membrane. However, the naturally occurring variants of gramicidin, gramicidin B and C (with Trp → Phe, Tyr substitutions at position 11, respectively) have been shown to be structurally similar to gramicidin A (Townsley et al., 2001). The mono-substituted Trp → Phe analogues (at positions 11, 13, 15) have also been shown to have backbone conformations close to the native sequence (Jordan et al., 2005).

It should be noted that functional studies using heterodimer channel formation have indicated that mutant gramicidin sequences with variable Trp → Phe replacements form structurally equivalent channels with native gramicidin (Becker et al., 1991). However, functional approaches selectively study only that fraction of the peptide population that forms functional channels. It is possible that conducting channels formed by altered sequences would be structurally similar to native channels. This may not, however, reflect the average conformation of the altered sequence in membranes. Channel-forming potency is a measure of the gramicidin concentration (in the aqueous solution) required for a given channel appearance rate (Becker et al., 1991) and would therefore account for partitioning of the membrane adsorbed gramicidin among various conformers. While the channels formed by the tri-substituted analogues are qualitatively similar to native channels, channel-forming potency is found to decrease twenty-fold. This suggests the possibility of a population of ‘non-channel’ conformations in the membrane in addition to the subset that forms conducting channels.
(iii) *Membrane deformation and ion channel function: gramicidin as a 'molecular force transducer'*

Gramicidin channel gating is known to occur by a well-defined conformational change, viz., the formation and dissociation of a transmembrane dimer (O'Connell *et al.*, 1990; See Figure 1.4). The conformational change involves the breaking of hydrogen bonds at the monomer interface and lateral movement of the monomer (Miloshevsky and Jordan, 2004b). Such a conformational change implies changes at the lipid-protein interface and would be coupled to modifications in the immediate lipid environment. The effective hydrophobic thickness of the functional gramicidin dimer is considered to be ~22 Å (Elliott *et al.*, 1983), based on the dependence of mean channel lifetime on the hydrocarbon thickness (as determined by capacitance measurements) of the supporting membrane. In membranes with increased surface tension or increased membrane thickness, there is a sharp reduction in mean channel lifetime. Since the hydrophobic thickness of gramicidin is less than the thickness of the membrane used for functional measurements (Lundbæk *et al.*, 1996), gramicidin channel formation is almost always associated with a reversible local membrane deformation (Huang, 1986; Helfrich and Jakobsson, 1990). The energetic cost of membrane deformation associated with channel formation is therefore a primary factor governing gramicidin channel function.

*Figure I.4* Schematic representation of gramicidin channel gating by the formation and dissociation of transmembrane dimers. Average membrane thickness is more than the length of the functional dimer. Channel formation is therefore associated with membrane deformation. Membrane lipids are approximated as springs and membrane deformation is described by a spring constant that includes deformation due to compression/expansion, distortion and increased monolayer curvature (from Andersen *et al.*, 1999).
Modification of the material properties of the membrane (such as monolayer curvature, thickness and membrane stiffness) would affect membrane deformation and therefore gramicidin channel lifetime (i.e., stability of the functional dimer). Monolayer membrane curvature can be modulated by the addition of agents such as detergents and lysophospholipids. It has been shown that increased gramicidin channel lifetimes correlate with an increase in positive membrane curvature due to the addition of non-physiological detergents (Lundbæk et al., 2004), lysophospholipids (Lundbæk and Andersen, 1996) and lipids such as DOPS (dioleoyl-sn-glycero-3-phosphoserine) under conditions of reduced electrostatic repulsion (Lundbæk et al., 1997). In addition, when gramicidin analogues of reduced length are used, the potency of lysophospholipids to increase channel activity (as a result of increased gramicidin dimerization) is enhanced (Lundbæk and Andersen, 1996).

It has been well established that membrane material properties are extremely sensitive to cholesterol content (Needham and Nunn, 1990). Cholesterol (at high mol%) increases bilayer cohesion and reduces membrane compressibility (Needham, 1995) therefore increasing the free energy cost of membrane deformation (Lundbæk et al., 2003). In addition, cholesterol is known to increase membrane thickness (Nezil and Bloom, 1992). As a result of these effects of cholesterol on membrane properties, cholesterol is found to decrease gramicidin channel activity (Elliott et al., 1985; Schagina et al., 1989, 1992; Lundbæk et al., 1996). Gramicidin activity has been found to reduce in membranes of increased thickness (Elliott et al., 1983) however, this is also associated with a conformational change to non-conducting dimers (Mobashery et al., 1997; also see Chapter V). In addition, mini-gramicidins (with 11 amino acids instead of 15) have been shown to function better in shorter membranes as compared to gramicidin A (Arndt et al., 2001).

The energetic cost of membrane deformation ($\Delta G_{\text{def}}^*$) is associated with the material properties of the membrane bilayer. The membrane bilayer is considered to be a smectic liquid crystal and membrane deformation can be treated according to the theory of elastic liquid crystals (Huang, 1986). The free energy cost of membrane deformation is considered to be the sum of three components: compression/expansion, splay distortion, and surface tension. Gramicidin can be utilized as a 'molecular force transducer' such that changes in
channel lifetime (a measure of the stability of the dimer vs. monomer conformation) are related to changes in the ability of the membrane to deform due to altered stiffness and/or thickness (Lundbæk et al., 1996; Andersen et al., 1999). When a thick membrane deforms locally to accommodate the dimeric channel, the force resulting from the membrane deformation reduces the stability of the dimer (measured as the channel lifetime; Elliott et al., 1983). The energetic cost of this deformation has been quantified in terms of a phenomenological spring constant that accounts for all three interdependent modes of membrane deformation and scales according to the size of the protein (Andersen et al., 1999). Interestingly, deformation energies calculated this way, could be as large as 2-4 kcal/mol, comparable to the effects of point mutations on protein function (Nielsen et al., 1998). The dependence of channel dimerization on the free energy of membrane deformation has been utilized to obtain a measure of the energetic cost ($\Delta G_{\text{det}}$) of membrane deformation (Lundbæk and Andersen, 1999).

Experimental values of channel lifetimes have been analyzed this way to predict the spring constant of the host membrane (Lundbæk and Andersen, 1999) and have been shown to yield spring constants similar to experimentally observed spring constants. Gramicidin channel activity can therefore be used as a reporter to delineate the effect of membrane-active compounds and treatments on membrane material properties and predict how such treatments may influence membrane protein function under physiological conditions. For example, gramicidin has been utilized as a molecular force transducer to delineate the mechanism of action of genistein, an isoflavonoid frequently used as a tool to alter ion channel function (Hwang et al., 2003). Genistein is utilized as a generic tyrosine kinase inhibitor and is thought to alter the function of a range of ion channels due to inhibition of phosphorylation. However, genistein was found to inhibit gramicidin channel activity due to its affect on bilayer elastic properties as a result of membrane partitioning (Hwang et al., 2003) ruling out a general role for tyrosine kinase inhibitions in ion channel function. Such bilayer mediated mechanisms for the action of modulators of membrane protein function are being increasingly recognized (Suchyna et al., 2004; Garcia, 2004). Relatively simple model systems such as the gramicidin ion channel promise to be of
increasing importance to understand the functioning and modulation of complex physiological ion channels.

I.3 Hydrophobic mismatch in membrane protein function and structure

The hydrophobic thickness of the membrane is a fundamental property that has a profound effect on transmembrane protein structure and function (Lee, 2004; Jensen and Mouritsen, 2004). Hydrophobic mismatch is a specific case of lipid-protein interaction that occurs when the hydrophobic thickness of the transmembrane region of a membrane protein does not match the unperturbed hydrophobic thickness of the membrane in which it resides. Such mismatch has obvious energetic consequences due to the juxtaposition of hydrophobic regions of the membrane with polar and/or charged regions of the membrane protein. While many lipid-protein interactions involve interaction of specific residues of membrane proteins with specific lipid headgroups (such as negatively charged lipids), hydrophobic mismatch is dependent on the hydrophobic thickness of the membrane bilayer, specifically of the annular lipids and the hydrophobic surface of the protein in contact with the membrane. Mismatch is therefore a local interaction that causes local perturbations in the membrane and may be linked to lateral heterogeneity in the membrane (Jacobson et al., 1995; Vereb et al., 2003). As discussed previously, the membrane/water interface of a transmembrane protein is defined by aromatic residues such as tryptophan and charged residues such as lysine and arginine that would anchor the protein at the membrane interface. The driving force for mismatch adaptation is the energetically unfavorable exposure of these residues to the membrane interior (Wimley and White, 1996) in cases where the protein is shorter than the host membrane i.e., negative mismatch, and the exposure of hydrophobic residues of the transmembrane region of the protein to the relatively polar membrane interface, when the protein is longer than the host membrane i.e., positive mismatch. Adaptation to mismatch can occur by several mechanisms (see Figures I.5 and I.6) that may involve changes in the thickness of the membrane by
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Figure 1.5 Schematic representation of lipid adaptation in hydrophobic mismatch. A membrane protein of hydrophobic thickness $d_p$ is incorporated in a lipid bilayer of hydrophobic thickness $d_L$, which may be (a) smaller or (b) larger than $d_p$. (a) When $d_L < d_p$, annular lipids stretch to match the hydrophobic thickness of the protein, leading to a reduction in cross-sectional area (right panel). (b) When $d_L > d_p$, annular lipids compress to match the hydrophobic thickness of the protein, leading to an increase in cross-sectional area (right panel). Importantly, the influence of the protein extends over a few lipid layers (Jähnig, 1981). (Adapted and modified from Fattal and Ben-Shaul, 1993; Dumas et al., 1999).

Figure 1.6 Schematic representation of possible adaptations to hydrophobic mismatch. (a) When the transmembrane domain of the protein is longer and (b) shorter than the hydrophobic thickness of the membrane. (Adapted and modified from Killian, 2003).

compression or stretching of annular lipids (Mouritsen and Bloom, 1984), or through changes in membrane protein conformation (helix pitch, transmembrane orientation),
organization by lateral aggregation, and possible exclusion from the membrane (Killian, 2003).

(i) Adaptation to hydrophobic mismatch: local deformation of the membrane bilayer

Adaptation to hydrophobic mismatch has been described in terms of a ‘mattress model’ by Mouritsen and Bloom (1984). The basic idea underlying the mattress model is that any modification of the sharp chain melting phase transition of lipid bilayers by the inclusion of proteins is a direct result of adaptation to hydrophobic mismatch that would occur on either side of the phase transition (since phase transition involves a large change (~30%) in the hydrophobic thickness of the membrane; Sperotto and Mouritsen, 1988). In this model, adaptation to hydrophobic mismatch is modeled as a change in thickness of the annular lipid layer as a result of compression or stretching of acyl chains, which leads to a shift in the phase-transition temperature ($\Delta T$), relative to the pure lipid bilayer transition temperature ($T_m$). The magnitude of $\Delta T$ is related to the magnitude of the mismatch. Thus, short proteins in a long bilayer lead to fluidization and a decrease in $T_m$ due to compression. However, a long protein in a short bilayer would cause stretching of annular lipids leading to a shift towards a more gel-like phase, and an increase in $T_m$.

Fattal and Ben-Shaul (1993) characterized lipid-protein interactions and perturbations due to mismatch in terms of lipid deformation free energy change ($\Delta F$) represented as a sum of hydrophobic core (lipid chain) and interfacial contributions. Importantly, this model assumes that protein-induced deformations persist in the membrane plane from the lipid-protein interface over typically a few molecular diameters (the coherence length; see Figure I.5). The free energy change ($\Delta F$) accounts for changes in lipid chain order at the lipid-protein interface. Thus when the hydrophobic lengths of the membrane and protein are equal, $\Delta F > 0$ due to the loss of conformational entropy experienced by the lipid chains at the protein interface. In mismatch situations, when the protein is longer than the membrane, $\Delta F$ further increases due to the enhanced stretching of the lipid chains, and when the protein is shorter than the membrane, conformational
entropy increases due to compression, but $\Delta F$ increases due to an increase in interfacial free energy. Therefore, $\Delta F$ is at a minimum when the hydrophobic lengths of the protein and membrane are equal but is always positive.

It should be noted that theoretical models treat transmembrane proteins as smooth, rigid cylindrical impurities without vertical flexibility in the membrane phase (and are therefore considered to be symmetric in the membrane plane), characterized only by cross sectional area and hydrophobic thickness (Mouritsen and Bloom, 1993). At the lipid protein interface, the protein is taken to be a nearly planar, smooth hydrophobic wall. In addition, these models are only valid for proteins at the infinite dilution limit and therefore do not account for any possible protein-protein interactions (e.g., lateral aggregation). Importantly, theoretical models highlight membrane deformation as a vital consequence of mismatch. Membrane deformation is directly related to the material properties of the membrane (see Section I.2(iv)), and is therefore dependent on membrane composition, specifically cholesterol content (Needham, 1995).

(ii) Hydrophobic thickness of membranes and membrane proteins

The extent of mismatch between the hydrophobic thickness of the membrane and the protein would determine the extent of the mismatch response (Mouritsen and Bloom, 1993). A primary concern therefore is to experimentally determine the hydrophobic thickness of membrane proteins and the membrane bilayer. Determining membrane thickness is a non-trivial issue due to fluctuations of the membrane bilayer (as described earlier) in addition to the inherent variations in available bilayer structural data (Nagle and Tristram-Nagle, 2000). One method to obtain hydrophobic thickness is from continuous x-ray scattering which measures the Gaussian distribution of the phosphate groups, and therefore the phosphate-to-phosphate distance (Lewis and Engelman, 1983). The boundary of the hydrophobic thickness of the membrane is placed at the region where water ceases to be detected in the bilayer, at the sn-2 carbonyl carbon (Nagle and Tristram-Nagle, 2000).
Hydrophobic thickness can therefore be obtained from the phosphate-to-phosphate distance by subtracting the thickness of the polar head group region, known from neutron diffraction of specifically deuterated samples (Buîdt et al., 1979; Zaccai et al., 1979) to be 5.5 Å. Calculated this way, the hydrophobic thickness of pure fluid phase bilayers is found to vary linearly with acyl chain length (Lewis and Engelman, 1983). This fluid phase thickness can be used to calculate the thickness of the gel phase by accounting for lipid tilt (~30°) and increased thickness (~30%) due to the all-trans acyl chain conformation in the gel phase (Sperotto and Mouritsen, 1988). However, such a calculation would give only approximate values for gel phase bilayers since average lipid tilt is known to be dependent on chain length (Tristram-Nagle et al., 1993).

The hydrophobic thickness of membrane proteins is more difficult to assess due to difficulty in obtaining high-resolution structures of membrane proteins. Hydrophobic thickness may be determined directly from crystal structures when the structure contains resolved lipid molecules that would mark the membrane interface (Lee, 2003). In general, hydropathy profiles can provide an estimate of the number of residues in the transmembrane domain. The length of the hydrophobic (transmembrane) region can then be calculated assuming the transmembrane domain to be an α-helix, oriented parallel to the bilayer normal, with a vertical rise of 1.5 Å per residue. However, due to possible helical tilt, and contributions from the flanking residues, calculation of membrane protein thickness based on the length of the transmembrane domain may not always be straightforward. In addition, hydrophobic thickness of proteins has also been determined experimentally (Dumas et al., 1999; Powl et al., 2005).

(iii) Hydrophobic matching conditions modulate protein function and conformation

The function of several membrane proteins has been found to be dependent on the thickness of the host membrane (Lee, 2004). Protein activity is generally found to be maximal at a specific chain length and activity is reduced in longer or shorter membranes (Criado et al., 1984; Pilot et al., 2001; Cornelius, 2001; Dumas et al., 2000). The influence
of mismatch on activity is generally found to be specific to a particular aspect of protein function (such as ligand binding or rate of catalytic activity). In the case of Na⁺,K⁺-ATPase, the response to increased membrane thickness depends on how membrane thickness was increased. The effect of increased thickness due to the presence of high amounts of cholesterol (Nezil and Bloom, 1992) on protein activity was not equivalent to the effect of increased acyl chain length, indicating a specific role for cholesterol in the function of Na⁺,K⁺-ATPase (Cornelius, 2001; Cornelius et al., 2003). For melibiose permease, the protein is found to be maximally active in 'thickness matched' membranes, indicating that the loss of activity in shorter or longer membranes is a direct consequence of the thickness of the membrane (Dumas et al., 1999, 2000).

Modulation of protein activity by hydrophobic mismatch implies that the protein could undergo conformational changes in membranes of different hydrophobic thickness. Model transmembrane peptides are found to be preferentially associated with lipids of matching hydrophobic thickness (Webb et al., 1998), indicating that mismatched proteins could preferentially partition to 'thickness matched' regions of a mixed phase membrane (discussed in detail in (v)). In an early study, catalytic activity of Ca²⁺-ATPase was found to be dependent on fatty acyl chain length. However, the protein did not show preferential binding to potentially 'matched' lipids (Caffrey and Feigenson, 1981). Such lipid binding may reflect small conformational changes that change the hydrophobic thickness of the protein and reduce protein activity in mismatched bilayers. The β-barrel outer membrane protein F (OmpF) is found to preferentially bind lipids according to acyl chain length (O'Keefe et al., 2000). Theoretical binding constants of lipids of different acyl chain length to OmpF, calculated according to the mattress model (Mouritsen and Bloom, 1984; 1993) and Fattal and Ben-Shaul (1993), are found to be comparable to experimental values in a certain range of lipid lengths (O'Keefe et al., 2000). This would imply that OmpF behaves as a rigid structure (as discussed in Section (i)) that does not deform under conditions of hydrophobic mismatch. For very long lipids however, experimental binding constants are found to be much smaller than theoretical predictions, indicating that in such membranes OmpF undergoes some deformation to match the lipid bilayer thickness. In comparison to the relatively rigid β-barrel OmpF, binding constants to the α-helical
potassium channel KcsA from *S. lividans*, are found to be much smaller than theoretically predicted binding constants (Williamson *et al.*, 2002). This would imply that in all mismatch conditions both protein and lipid undergo deformation to achieve matching conditions. In addition, experiments utilizing depth dependent fluorescence quenching of the interfacially located tryptophans of KcsA (See Figure 1.1), suggested that matching is achieved by a change in tilt angle of the transmembrane α helices of KcsA (Williamson *et al.*, 2002). Interestingly, a recent report on the β-barrel OmpA protein, used ATR-FTIR to determine the orientation of the β-barrel under conditions of mismatch and showed that while the β-barrel structure itself is rigid (as in OmpF), the relative tilt with respect to the membrane is mismatch dependent (Ramakrishnan *et al.*, 2005). Thus, the relative tilt is reduced when the hydrophobic thickness of the membrane exceeds the thickness of the protein and vice versa. In addition, folding of OmpA in the membrane has been shown to be dependent on the thickness of the host membrane (Kleinschmidt and Tamm, 2002; Hong and Tamm, 2004). Since hydrophobic mismatch alters the lipid-protein interface, mechanosensitive proteins that respond to the mechanical deformation and curvature changes of the membrane bilayer would be particularly susceptible to mismatch. Gramicidin can function as a mechanosensitive protein since the formation of the functional channel is directly linked to membrane deformation (see Section 1.2(iii)). Interestingly, the response of gramicidin to mechanical deformation is dependent on membrane thickness (Martinac and Hamill, 2002). Thus, in very long membranes, membrane stretch activates gramicidin channels, while in shorter membranes the channel is inactivated. Hydrophobic mismatch can therefore inverse the response of a mechanosensitive protein to mechanical stimuli. Mismatch on the order of a few Å, is therefore capable of causing subtle conformational changes in membrane proteins that may lead to relevant functional changes. Such mismatch may therefore be an important source of membrane protein regulation in laterally heterogeneous cellular membranes (also see Section (v)).
Model systems provide a particularly convenient paradigm to understand hydrophobic mismatch due to the relative ease with which the important parameters (i.e., peptide and bilayer hydrophobic length) can be altered. One of the initial sequences designed to act as a model transmembrane peptide, P24, is a poly-Leu peptide with the sequence K2GL24K2A-amide (Davis et al., 1983). This peptide has a relatively rigid α-helical poly-Leu hydrophobic core designed to mimic the transmembrane region of membrane proteins. The poly-Leu core is flanked by two Lys residues that anchor the peptide at the membrane interface. Gly and Ala (flanking L24) were added for ease in peptide analysis after synthesis (Davis et al., 1983). This peptide has been shown to adopt a transmembrane orientation in membranes (Zhang et al., 1992a) and the hydrophobic thickness of the peptide can be varied by changing the number of Leu residues that form the hydrophobic core. Later, the addition of a single reporter tryptophan residue as a spectroscopic indicator (Bolen and Holloway, 1990) greatly increased the utility of these peptides (Ren et al., 1997, 1999).

While such poly-Leu peptides have been shown to span the bilayer (Zhang et al., 1992a), corresponding poly-Ala sequences are not sufficiently hydrophobic to adopt stable transmembrane orientations (Lewis et al., 2001). The WALP family of peptides (Killian et al., 1996), designed by Koepppe and Killian, contain a stretch of alternating Leu-Ala residues that form the hydrophobic core of the peptide. In addition, two Trp residues at both ends act as membrane interfacial anchors (as discussed in Section 1.1 (iii)). The WALP peptides were inspired by the ion channel gramicidin (see Section 1.2) and designed as α-helical mimics of gramicidin (de Planque and Killian, 2003). WALP peptides form rigid α-helices in the membrane environment (de Planque et al., 2001), and have proved very useful to understand basic aspects of mismatch adaptation (reviewed in de Planque and Killian, 2003).

An interesting aspect that has been illustrated by the use of such synthetic peptides is the role of anchoring residues in mismatch adaptation, due to the specific interactions of
amino acid side chains with the membrane interface. The effective hydrophobic thickness and therefore mismatch response of equivalent peptides (i.e., with the same number of residues in the hydrophobic core) have been shown to be dependent on the nature of the anchoring residues (Mall et al., 2000; de Planque et al., 2002; Strandberg et al., 2002). Trp-flanked (WALP) peptides induce a larger lipid response (i.e., acyl chain ordering) in shorter bilayers as compared to equivalent Lys-flanked (KALP) and Arg-flanked (RALP) peptides (de Planque et al., 1999, 2002), and are more effective in inducing mismatch relieving cubic and inverted hexagonal phases (de Planque et al., 2002). The effective hydrophobic length of WALP peptides is therefore reduced compared to equivalent KALP and RALP peptides. This is due to the ‘snorkeling’ of the lysine and arginine side chains (Segrest et al., 1990) such that the uncharged surface is buried in the hydrophobic interior of the membrane and the amphipathic side chain extends outward to the polar face of the membrane to insert the charged moiety into the aqueous milieu. Such ‘snorkeling’ has been found to be particularly effective in maintaining transmembrane orientations when charged Lys residues are placed in the hydrophobic core of a transmembrane peptide (Caputo and London, 2003a). Interestingly, the free energy change involved in snorkeling has been found to be relatively low (~0.07 to 0.7 kcal/mol) indicating that it may be a common mechanism adopted by membrane proteins to place charged moieties of amino acid side chains in a preferred environment (Strandberg and Killian, 2003). Glycosylation of in vitro translated sequences by the ER oligosaccharyl transferase provides a functional assay for transmembrane stability of specific sequences (Nilsson and von Heijne, 1993; Hessa et al., 2005). This ‘glycosylation mapping’ technique was utilized to determine the effect of peptide hydrophobic length on membrane incorporation (Monne and von Heijne, 1999). While peptide length in general is a strong determinant of glycosylation, the incorporation of Lys was found to strongly influence glycosylation of short hydrophobic peptides, possibly due to ‘snorkeling’.
Functional implications of hydrophobic mismatch in cellular membranes: thickness dependent sorting of membrane proteins

Mismatch driven modulation of protein conformation and function can have interesting consequences in the cellular milieu. As mentioned before, the importance of lateral heterogeneity in the distribution of proteins and lipids within the membrane is being increasingly recognized (Jacobson et al., 1995; Vereb et al., 2003). There is growing evidence for the organization of cellular membranes into dynamic domains of distinct lipid and protein composition with physiological significance (Edidin, 2003b; Mukherjee and Maxfield, 2004). An integral aspect of such membrane organization is the non-random distribution of cholesterol in biological and model membranes (Liscum and Underwood, 1995; Mukherjee and Chattopadhyay, 1996; Xu and London, 2000; Rukmini et al., 2001). In addition, such membrane domains are associated with a specific subset of proteins that would give the domain functional significance.

Lipid ‘rafts’ have been proposed as specialized membrane domains enriched in cholesterol and sphingolipids that could serve as platforms for the spatio-temporal clustering of signaling molecules (Simons and Ikonen, 1997). Significantly, cholesterol is known to increase the thickness of membranes (Simon et al., 1982; Nezil and Bloom, 1992) and make membranes more cohesive (Needham and Nunn, 1990). The partitioning of specific proteins to domains may therefore be driven by hydrophobic mismatch since membrane domains enriched in cholesterol and saturated lipids would have increased thickness (Sprong et al., 2001). A popular way to biochemically isolate and characterize such domains has been the resistance of these domains to solubilization by cold non-ionic detergents (Brown and Rose, 1992; Schroeder et al., 1998; Kalipatnapu and Chattopadhyay, 2004). Due to the higher content of cholesterol and sphingolipid, detergent resistant membranes would have distinct thickness and material properties as compared to the bulk membrane. Gandhavadi et al. (2002) used x-ray diffraction to measure the phosphate-to-phosphate distances for detergent resistant (DRM) and detergent soluble (DSM) membranes isolated from model ‘raft’ membranes (1:1:1 DOPC:cholesterol:sphingomyelin). The thickness of DRMs was found to be 5 Å more
than the bulk membrane and more importantly, 10 Å more than DSMs. Interestingly, it has been shown that mismatched peptides are excluded from thicker bilayers when mismatch (between the peptide and bilayer) exceeds 10 Å (Webb et al., 1998). When such lateral sorting between thick DRM and DSMs was tested using WALP peptides in model membranes, mismatched peptides failed to preferentially sort to thickness-matched DRM fractions (van Duyl et al., 2002). However, it is important to note that besides thickness, cholesterol/sphingomyelin enriched domains (isolated as DRMs) are known to be more ordered as compared to the relatively cholesterol-poor DSM domains.

It is known that transmembrane proteins in general are excluded from more ordered gel-phase and liquid-ordered domains (London and Feigenson, 1981; Fastenberg et al., 2003) due to the tighter packing of lipid acyl chains in such domains. In a theoretical analysis of the energetics of the partitioning of mismatched proteins between such lateral phase-separated membrane domains, Lundbæk et al., (2003) showed that hydrophobic mismatch alone is not sufficient to sort mismatched proteins out of thicker domains. However, when hydrophobic mismatch is coupled with decreased membrane compressibility due to higher cholesterol content, the energetic cost of membrane deformation is sufficiently high to sort mismatched proteins out of the domain. This effect of material properties on lateral sorting was tested by an analysis of the dependence of peptide partitioning to DRM and DSM fractions on the conditions used to isolate DRMs (McIntosh et al., 2003). Detergent resistance of ordered domains is based on the tight acyl-chain packing of these phase-separated regions that leads to reduced access to detergent molecules (London and Brown, 2000). In general, detergent treatment is carried out at low temperatures (4 °C) to enrich phase-separated cholesterol-rich domains. However, detergent treatment may also be carried out at elevated temperatures (Melkonian et al., 1995; Pucadyil and Chattopadhyay, 2004b). Detergent treatment was therefore carried out at elevated temperatures (37 °C) since at higher temperatures the material differences (compressibility) between DRM and DSM domains reduce. This is a result of the fact that higher temperatures decrease the compressibility of cholesterol containing bilayers (Evans and Needham, 1987). Thus at
higher temperatures, the increased compressibility of DRMs would allow partitioning of transmembrane peptides to these domains. Although partitioning to DRMs was found to be unfavorable for all peptides tested, the free energy of transfer (at 37 °C) was minimum for thickness-matched peptides. Therefore, while partitioning of transmembrane peptides to ordered domains is intrinsically unfavorable, hydrophobic mismatch can drive peptide partitioning towards thicker cholesterol-rich domains (DRMs).

In addition to lateral heterogeneity, cellular membranes are known to be heterogeneous due to the presence of a cholesterol gradient among intracellular membranes. Even though the endoplasmic reticulum is the site of cholesterol synthesis, it has the lowest cholesterol content in the membranes of the secretory pathway (Bretscher and Munro, 1993). Thus, cholesterol content increases progressively in the Golgi (along the cis-, medial-, and trans-Golgi stacks; Bretscher and Munro, 1993) and the plasma membrane has the highest concentration of cholesterol (~90% of total cellular cholesterol; Lange et al., 1989). This cholesterol gradient could set up a possible thickness gradient along the biosynthetic pathway of membrane proteins. In the biosynthetic pathway, expression at the cell surface is the default and specific signals are required for retention in the Golgi and ER (Weiland et al., 1987). However, so far no distinct protein motif has been identified as a retention signal for Golgi or ER proteins. Interestingly, several studies have pointed out the importance of the transmembrane domain (TMD) in retention of proteins in the Golgi and ER (Swift and Machamer, 1991; Munro, 1991; Tang et al., 1992; Masibay et al., 1993; Munro, 1995; Shi et al., 2004).

Significantly, the length of the TMD is found to be important rather than the specific sequence (Munro, 1991; Masibay et al., 1993). Therefore, replacement of the TMD of a Golgi protein (sialyltransferase) by a hydrophobic poly-Leu stretch of the same length resulted in targeting to Golgi. However, when the length of the sequence was increased, the protein was expressed at the cell surface (Munro, 1991). Similarly, a cell surface protein could be targeted to Golgi, by reducing the length of the TMD (Munro, 1995). Analysis of hydropathy plots of the sequences of a set of single TMD Golgi and plasma membrane proteins revealed that the average length of the transmembrane domain
in plasma membrane proteins is \( \sim 20 \) amino acids (Bretscher and Munro, 1993). However, for Golgi proteins the average length of the TMD is significantly reduced and is found to be \( \sim 15 \) amino acids (Bretscher and Munro, 1993; Masibay \textit{et al.}, 1993). As a result of hydrophobic mismatch, Golgi proteins may be sorted out from lateral cholesterol/sphingolipid rich domains in Golgi destined for the plasma membrane. This hypothesis is further supported by the prediction that short protein are efficiently sorted out of thicker cholesterol rich domains due to the high energetic penalty of deformation (Lundbaek \textit{et al.}, 2003). This ‘bilayer thickness model’ of Golgi protein retention is believed to work in conjunction with an ‘oligomerization/kin recognition’ model (Colley, 1997) to account for the enhancing effect of flanking residues on Golgi retention (Munro, 1991). The principal assumption behind the ‘bilayer thickness’ model viz., the increased thickness of the plasma membrane as compared to the intracellular membranes, due to increased cholesterol content, was recently tested using x-ray diffraction to measure phosphate-to-phosphate distances (Mitra \textit{et al.}, 2004). Surprisingly, this study did not report any significant gradient of thickness in Golgi, ER, and plasma membranes. In addition, depletion of a significant amount of cholesterol from these membranes did not lead to significant alterations in membrane thickness. However, depletion of protein from these membranes, and Golgi membrane in particular, was found to greatly influence membrane thickness (Mitra \textit{et al.}, 2004). Thus, cellular membranes as reported by this study, are not arranged in a thickness gradient as envisaged from the cholesterol gradient of these membranes. However, the thickness of cellular membranes, particularly organelle membranes (ER and Golgi) is modulated by the protein content.

This would imply that in natural systems, proteins may not always reside in ‘thickness matched’ membranes. On the other hand, a comparison of membrane thickness derived from capacitance measurements and x-ray diffraction shows that though phosphate-to-phosphate distances show a small increase with cholesterol content, the dielectric thickness (which would be a measure of the hydrophobic thickness) significantly increases (Simon \textit{et al.}, 1982). Therefore, while phosphate-to-phosphate distances do not reveal a thickness gradient, the hydrophobic thickness of these membranes may be graded
as a result of increasing cholesterol content in these membranes. Clearly, the ‘bilayer thickness’-dependent mechanism for Golgi retention is an evolving hypothesis. The sensitivity of physiologically relevant membrane proteins to small changes in membrane thickness in model systems (as discussed in Section (iii)), indicates that in cellular systems the location of proteins in lateral domains of variable thickness may have far-reaching functional consequences.