Chapter 1

Introduction
1.1. LIPID-PROTEIN INTERACTIONS IN MEMBRANES

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. Due to both lipid-protein as well as protein-protein interactions, the biological membrane constitutes the site of many important cellular functions (Shai, 2001). Several examples include energy metabolism, muscle contraction, nutrient absorption, signal transduction, ion transport, cell-cell contact and recognition, and variety of other processes. There has been a long-standing interest in how integral membrane proteins interact with the lipid molecules that surround them in the biological membrane. Clearly, the lipid and protein components of the biological membrane must have co-evolved to allow membrane proteins to function in the environment provided by the lipid bilayer and to allow membrane proteins to be inserted into the membrane without destroying it. The importance of understanding the organization and dynamics of membrane proteins lies in the fact that ~30% of the proteins coded by the human genome are membrane proteins (Smith et al., 2001). Interestingly, it is estimated that ~60% of drug targets in the pharmaceutical industry are membrane proteins (Yeagle and Lee, 2002). Knowledge of the structure and organization of membrane proteins therefore represents a major step toward understanding the function of membrane proteins. However, our understanding of the function of membranes, a complex organized molecular assembly, in terms of lipid-protein interactions at the molecular level is limited by the lack of high-resolution three-dimensional structures of membrane-bound proteins and peptides.

In spite of some recent successes (Doyle et al., 1998; Dutzler et al., 2002; Abramson et al., 2003), crystallization of membrane proteins and peptides for diffraction studies continues to be extremely difficult and challenging. Although the first complete x-ray crystallographic analysis of an integral membrane protein was successfully carried
out a number of years back (Deisenhofer et al., 1985), the number of membrane proteins whose structures have been solved to atomic resolution is still very small and represents ~50 unique structures* (Lee, 2003; Torres et al., 2003). This is in contrast to the number of soluble protein structures in the PDB structural database which exceeds the number of membrane protein structures by a ratio of ~1000:1 (Bowie, 2001; Preusch et al., 1998; Berman et al., 2000). This represents a huge deficit of information about membrane protein structure. Although detailed and precise structural information of proteins can be obtained from crystallographic diffraction data, such information is necessarily static. However, global and local dynamics exhibited by proteins and specific regions in them play important roles in their function. Further, a detailed crystallographic database is still not available in case of membrane proteins and peptides. The great disparity between our understanding of soluble proteins and membrane proteins is a consequence of many practical problems of working with membrane proteins. The main reason behind this extraordinary deficit of membrane protein structures is often associated with the extreme difficulty in crystallizing membrane-bound proteins and peptides for diffraction studies. Even high-resolution NMR methods have limited applications for membrane-bound proteins and peptides because of slow reorientation times in membranes (Opella, 1997) though solid-state NMR methods have been developed to solve the structures of simple membrane proteins in lipid bilayers which provide in some cases information that x-ray crystallography cannot (Torres et al., 2003; Yeagle and Lee, 2002).

Over the last few years, useful information about lipid-protein interactions has started to emerge from high-resolution structural studies of membrane proteins, which sometimes include resolved lipid molecules (Lee, 2003). These include bacteriorhodopsin, potassium channel KcsA, the photosynthetic reaction center, cytochrome c oxidase, cytochrome bc1 and formate dehydrogenase. Analysis of these

*Also see http://blanco.biomol.uci.edu/Membrane_Proteins_xtal.html
structures offers important clues on the location of aromatic residues (especially tryptophan) in membranes and this helps in identifying the ends of transmembrane α-helices of membrane proteins (see below).

**Role of Tryptophan Residues in Membrane Proteins and Peptides: Tryptophan and the Membrane Interface**

The presence of tryptophan residues as intrinsic fluorophores in most peptides and proteins makes them an obvious choice for fluorescence spectroscopic analyses of such systems. The role of tryptophan residues in the structure and function of membrane proteins has recently attracted a lot of attention (Reithmeier, 1995; Chattopadhyay et al., 1997; Clark et al., 2003; Raghuraman et al., 2003; Miller and Falke, 2004). The biological membrane provides a unique environment to membrane-spanning proteins and peptides thus influencing their structure and function. Membrane-spanning proteins have distinct stretches of hydrophobic amino acids that form the membrane-spanning domain and have been reported to have a significantly higher tryptophan content than soluble proteins (Schiffer et al., 1992). In addition, it is becoming increasingly evident that tryptophan residues in integral membrane proteins and peptides are not uniformly distributed and that they tend to be localized toward 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 1.1). For instance, 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. Statistical studies of sequence databases and available crystal structures of integral membrane proteins also show preferential clustering of tryptophan residues at the membrane interface (Reithmeier, 1995; Landolt-Marticorena et al., 1993; Ulmschneider and Sansom, 2001). Furthermore, for synthetic transmembrane
peptides, tryptophan has been found to be an efficient anchor at the membrane interface (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 function of membrane proteins is exemplified by the fact that substitution or deletion of tryptophans often results in reduction or loss of protein functionality (Becker et al., 1991; Fonseca et al., 1992; Miller and Falke, 2004).

Figure 1.1. A Schematic representation of the membrane bilayer showing the various regions of the bilayer displaying motional anisotropy. The membrane lipids shown have two hydrophobic tails with a PC headgroup. The preferred locations of various amino acids present in a transmembrane domain of a membrane protein are also shown. It is worth noting that the fluorescent tryptophan residues are localized in the membrane interface, a region characterized by unique organization, dynamics, hydration and functionality. See text for other details (from Raghuraman et al., 2003).

The exact location of the tryptophan residues at the membrane interface is, however, unclear. Some experiments suggest that tryptophan residues have a preference for the lipid headgroup side of the interface but others suggest that the preference is for
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the fatty acyl chain side (Yau et al., 1998; Persson et al., 1998; Jacobs and White, 1989; Brown and Heustis, 1993). Nevertheless, 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). The tryptophan rich aromatic belt at the membrane interface in transmembrane helices are thought to stabilize the helix with respect to the membrane environment (Landolt-Marticorena et al., 1993).

The tryptophan residue has a large indole side chain that consists of two fused aromatic rings. In fact, the tryptophan side chain has the largest volume of all the amino acid side chains, with a volume of 228 Å³ (Chothia, 1975) which is comparable to the volume of a PC headgroup, i.e., 319 Å³ (Petrache et al., 1997). In molecular terms, tryptophan is a unique amino acid since it is capable of both hydrophobic and polar interactions. In fact, the hydrophobicity of tryptophan, measured by partitioning into bulk solvents, has previously been shown to be dependent on the scale chosen (Fauchère, 1985). Tryptophan ranks as one of the most hydrophobic amino acids on the basis of its partitioning into polar solvents such as octanol (Fauchère and Pliska, 1983) while scales based on partitioning into nonpolar solvents like cyclohexane (Radzicka and Wolfenden, 1988) rank it as only intermediate in hydrophobicity. This ambiguity results from the fact that while tryptophan has the polar -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; Wimley and White, 1992). Wimley and White have recently shown from partitioning of model peptides to membrane interfaces that the experimentally determined interfacial hydrophobicity of tryptophan is the highest among the naturally occurring amino acid residues thus accounting for its specific interfacial localization in membrane-bound peptides and proteins (Wimley and White, 1996). Due to its aromaticity, the tryptophan residue is capable of π-π interactions and of weakly polar interactions (Burley and Petsko, 1988). The amphipathic character of tryptophan gives rise to its hydrogen bonding ability which could account for its orientation in
membrane proteins and its function through long-range electrostatic interaction (Fonseca et al., 1992). The amphipathic character of tryptophan also explains its interfacial localization in membranes due to its tendency to be solubilized in this region of the membrane, besides favorable electrostatic interactions and hydrogen bonding.

As mentioned earlier, lipid-protein interactions are crucial for many important cellular functions. Due to the inherent difficulty in crystallizing membrane proteins, most structural analyses of membrane-bound molecules have utilized other biophysical techniques with an emphasis on spectroscopic approaches. Spectroscopic techniques, which provide both structural and dynamic information, therefore become very useful for the analysis of membrane proteins. Fluorescence spectroscopy represents one such approach and is widely used in analysis of membrane protein structure and function. The advantages of using fluorescence techniques are intrinsic sensitivity, suitable time scale, non-invasive nature, and minimum perturbation (Lakowicz, 1980; Chattopadhyay, 1992; Demchenko, 1992; Mukherjee and Chattopadhyay, 1995). For the work reported in this thesis, I have utilized wavelength-selective fluorescence, a novel fluorescence approach, as a powerful tool to monitor the organization and dynamics of melittin in membranes and membrane-mimetic environments, in addition to other sensitive fluorescence-based approaches and CD spectroscopy. A brief introduction to the wavelength-selective fluorescence approach and its applications in monitoring the organization and dynamics of membrane-bound peptides and membrane proteins is provided below.
1.2. THE WAVELENGTH-SELECTIVE FLUORESCENCE APPROACH

Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system (Mukherjee and Chattopadhyay, 1995, Chattopadhyay, 2002; Chattopadhyay, 2003; Raghuraman et al., 2003). A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed the red edge excitation shift (REES) (Demchenko, 1988; Mukherjee and Chattopadhyay, 1995, Chattopadhyay, 2002; Chattopadhyay, 2003; Demchenko, 2002; Raghuraman et al., 2003). This effect is mostly observed with polar fluorophores in motionally restricted media such as very viscous solutions or condensed phases where the dipolar relaxation time for the solvent shell around a fluorophore is comparable to or longer than its fluorescence lifetime (Demchenko, 1988; Mukherjee and Chattopadhyay, 1995, Chattopadhyay, 2002; Chattopadhyay, 2003; Demchenko, 2002; Raghuraman et al., 2003; Galley and Purkey, 1970; Lakowicz and Keating-Nakamoto, 1984). REES arises due to slow rates of solvent relaxation (reorientation) around an excited state fluorophore which is a function of the motional restriction imposed on the solvent molecules in the immediate vicinity of the fluorophore. This approach therefore allows one to probe the mobility parameters of the environment itself (dynamics of solvation) represented by the relaxing solvent molecules using the fluorophore merely as a reporter group.

In addition to the dependence of fluorescence emission maxima on the excitation wavelength (REES), fluorescence polarization and lifetime are also known to depend on the excitation and emission wavelengths in viscous solutions and in otherwise motionally
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restricted media (Mukherjee and Chattopadhyay, 1995). Taken together, these approaches which are based on the red edge effect in fluorescence spectroscopy constitute the wavelength-selective fluorescence approach which can be used to directly monitor the environment and dynamics around a fluorophore in organized molecular assemblies such as membranes. Early applications of REES and wavelength-selective fluorescence to systems of biological relevance have been restricted mainly to indole, tryptophan, and other fluorescent probes in viscous solvents (Demchenko, 1988).

Membranes: An Appropriate System for the Wavelength-Selective Fluorescence Approach

A direct consequence of highly organized molecular assemblies such as membranes is the restriction imposed on the mobility of molecules incorporated in such assemblies. It is well known that interiors of biological membranes are viscous, with the effective viscosity comparable to that of light oil (Cone, 1972; Poo and Cone, 1974) or olive oil (Edidin, 2003). In addition, the membrane exhibits a considerable degree of anisotropy along the axis perpendicular to the membrane plane. Properties such as polarity, fluidity, segmental motion, ability to form hydrogen bonds and extent of water penetration vary in a depth-dependent manner in the membrane. The interfacial region in membranes is characterized by unique motional and dielectric characteristics distinct from both the bulk aqueous phase and the hydrocarbon-like interior of the membrane (Seelig, 1977; Ashcroft et al., 1981; Stubbs et al., 1985; Perochon et al., 1992; White and Wimley, 1994; Slater et al., 1993; Venable et al., 1993; Gawrisch et al., 1995) as shown in Figure 1.1. It is a chemically heterogeneous region composed of lipid headgroups, water and portions of the acyl chain (Wiener and White, 1992). Overall, the interfacial region of the membrane accounts for 50% of the thermal thickness of the bilayer (White and Wimley, 1994). This specific region of the membrane exhibits slow rates of solvent
relaxation and is also known to participate in intermolecular charge interactions (Yeagle, 1987) and hydrogen bonding through the polar headgroup (Gennis, 1989; Shin et al., 1991; Boggs, 1987). These structural features which slow down the rate of solvent reorientation have previously been recognized as typical features of solvents giving rise to significant red edge effects (Itoh and Azumi, 1975). It is therefore the membrane interface which is most likely to display red edge effects and is sensitive to wavelength-selective fluorescence measurements (Chattopadhyay and Mukherjee, 1993). This makes membrane peptides and proteins ideally suited for studies using the wavelength-selective fluorescence approach since as discussed above, the fluorescent tryptophan residues are preferentially localized in this region of the membrane. The use of this approach becomes all the more relevant in view of the fact that no detailed crystallographic database for membrane-bound proteins and peptides exists to date due to the inherent difficulty in crystallizing such molecules. Moreover, any information obtained has a dynamic component generally absent in x-ray crystallographic data.

Our group has previously shown that the wavelength-selective fluorescence approach in general and REES in particular, serve as a powerful tool to monitor the organization and dynamics of probes and peptides bound to membranes (Chattopadhyay and Rukmini, 1993; Chattopadhyay and Mukherjee, 1993; Ghosh et al., 1997; Chattopadhyay and Mukherjee, 1999a; 1999b; Kelkar et al., 2003; Mukherjee et al., 2004; Mukherjee and Chattopadhyay, 1994; Chattopadhyay et al., 1997) and membrane-mimetic media such as micelles (Rawat et al., 1997; Rawat and Chattopadhyay, 1999; Raghuraman and Chattopadhyay, 2004; Raghuraman et al., 2004) and reverse micelles (Chattopadhyay et al., 2002; Raghuraman and Chattopadhyay, 2003). In addition, we have previously used the wavelength-selective approach to analyze the organization and dynamics of tryptophans in the soluble hemolytic protein α-toxin (Raja et al., 1999) and cytoskeletal proteins tubulin (Guha et al., 1996) and spectrin (Chattopadhyay et al., 2003) which are components of the cytoskeletal network in eukaryotes. The application of the
wavelength-selective fluorescence approach to membranes, membrane-mimetic systems and membrane protein structure and dynamics has been recently reviewed (Chattopadhyay, 2002; Demchenko, 2002; Chattopadhyay, 2003; Raghuraman et al., 2003).

In summary, knowledge of dynamics of hydration at the molecular level is of considerable importance in understanding cellular structure and function (Crowe and Crowe, 1984; Rand and Parsegian, 1989; Ho and Stubbs, 1992; Fischer et al., 1994; Sankararamakrishnan and Sansom, 1995; Kandori et al., 1995; Häussinger, 1996; Mentré, 2001; Kouyama et al., 2004). As mentioned earlier, REES is based on the change in fluorophore-solvent interactions in the ground and excited states brought about by a change in the dipole moment of the fluorophore upon excitation, and the rate at which solvent molecules reorient around the excited state fluorophore. Since for biological systems, the ubiquitous solvent is water, the information obtained in such cases will come from the otherwise 'optically silent' water molecules. The unique feature about REES is that while all other fluorescence techniques such as fluorescence quenching, energy transfer and polarization measurements yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics which is not possible to obtain by other fluorescence techniques. This makes the use of REES and the wavelength-selective fluorescence approach powerful in membrane biology since hydration plays a crucial modulatory role in a large number of cellular events involving the membrane such as lipid-protein interactions (Ho and Stubbs, 1992) and ion transport (Crowe and Crowe, 1984; Rand and Parsegian, 1989; Ho and Stubbs, 1992). REES and the wavelength-selective fluorescence approach, in combination with novel molecular biology approaches for site-specific incorporation of unnatural fluorophores at specific sites (Cohen et al., 2002) could prove to be an extremely powerful tool to probe organization and dynamics of membrane proteins and peptides.
In order to understand the lipid-protein interactions at the molecular level, both natural and synthetic membrane peptides have been used. These include melittin (Dempsey, 1990), gramicidin A (Andersen et al., 1999), alamethicin (Nagaraj and Balaram, 1981), magainin (Matsuzaki, 1998), cecropin (Bechinger, 1997), and KALP and WALP peptides (de Planque et al., 1999). The small size and easy availability of these peptides makes it possible to study lipid-protein interactions in depth by a variety of biophysical techniques. These peptides offer a convenient tool to study the specific role of membranes on the orientation, incorporation, stability and function of such peptides (Chung, 1992; Lundbaek et al., 1997; Webb et al., 1998; Ren et al., 1999). This thesis is focused on exploring lipid-protein interactions using the hemolytic peptide melittin and a variety of membranes and membrane-mimetic systems.

1.3. MELITTIN: A PEPTIDE WITH DIVERSE FUNCTIONS

Melittin, the principal toxic component in the venom of the European honey bee *Apis mellifera*, is a cationic, hemolytic peptide (Habermann, 1972; Habermann and Jentsch, 1967). It constitutes 50% of the dry weight of the bee venom. It is a small linear peptide composed of 26 amino acid residues (NH₂-GIGAVLKVLTTGLPALISWIKRKQRQQ-CONH₂) in which the amino-terminal region (residues 1-20) is predominantly hydrophobic whereas the carboxy-terminal region (residues 21-26) is hydrophilic due to the presence of a stretch of positively charged amino acids. The amphiphilic property of this peptide makes it water-soluble and yet it spontaneously associates with natural and artificial membranes (Dufourcq et al., 1984; Bernheimer and Ruby, 1986; Dempsey, 1990; Sansom, 1991; Saberwal and Nagaraj, 1994). Such a sequence of amino acids, coupled with its amphiphilic nature, is characteristic of many membrane-bound peptides and putative transmembrane helices of
membrane proteins (Dempsey, 1990; Shai, 1995). These include apolipoproteins and peptide hormones (DeGrado et al., 1982; Kaiser and Kezdy, 1983; Morii et al., 1994), signal peptides (Bello et al., 1982; Garnier et al., 1980; Golding and O’Shea, 1995), the envelope glycoprotein gp41 from the human immunodeficiency virus (Eisenberg and Wesson, 1990; Rabenstein and Shin, 1995), the pore-forming peptide of pathogenic Entamoeba histolytica (Leippe et al., 1991; 1992) and the 25-residue presequence (p25) of subunit IV of yeast cytochrome oxidase (Clague and Cherry, 1988). Interestingly, melittin exhibits sequence and structural similarities to a region of the TMV CP known to be critical for protein-protein and protein-RNA interactions (Marcos et al., 1995). Further, understanding of melittin-membrane interaction assumes significance due to the recent finding that melittin mimics the structure of N-terminal of HIV-1 virulence factor Nef1-25 (Barnham, 1997). This has resulted in melittin being used as a convenient model for monitoring lipid-protein interactions in membranes.

Melittin is intrinsically fluorescent due to the presence of a single tryptophan residue, Trp-19, which makes it a sensitive probe to study the interaction of melittin with membranes and membrane-mimetic systems (Chandani and Balasubramanian, 1986; Chattopadhyay and Rukmini, 1993; Bradrick et al., 1995; Ghosh et al., 1997; Cajal and Jain, 1997; Oren and Shai, 1997). This is particularly advantageous since there are no other aromatic amino acids in melittin and this makes interpretation of fluorescence data less complicated due to lack of interference and heterogeneity. Importantly, it has been shown that the sole tryptophan residue of melittin is crucial for its powerful hemolytic activity (see section 1.3.2.c). The organization and dynamics of the tryptophan residue therefore become important for the function of the peptide. Numerous studies have been undertaken to determine the nature of interaction of melittin with membranes, both with the aim of understanding the molecular mechanisms of melittin-induced hemolysis, and as a model for studying the general features of the structures of membrane proteins and their interactions with membrane lipids. The consideration of the properties of melittin in
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water is relevant to its effects on membranes since melittin reaches target membranes through the aqueous phase. Hence, a comprehensive account of the solution properties of melittin is presented first followed by a discussion on the actions of melittin on membranes. In the process, the structural requirements for the action of melittin, its orientation, aggregation state, and current view of pore formation as well as its various cellular actions are discussed.

1.3.1. Structure and Conformation of Melittin in Aqueous Solution

Melittin, like other membrane-binding peptides and proteins, is predominantly hydrophobic. Yet, the peptide has a net charge of +6, four of which are at a stretch in the highly basic C-terminal region (Lys-Arg-Lys-Arg) and the remaining two in the N-terminal amino group and Lys-7 in the N-terminal region. There is an asymmetric distribution of polar and non-polar amino acids which makes melittin amphipathic when the peptide is aligned in an α-helical configuration as shown in Figure 1.2 as a helical wheel diagram (Dathe and Wieprecht, 1999). Though melittin has a high proportion of

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\text{Figure 1.2. Helical wheel projection and schematic drawing of the amphipathic helix of melittin illustrating different structural features. The one letter code for amino acids is used. Hydrophobic residues are shown in white, polar residues in gray and cationic residues in black circles. N is the number of residues, Q is the net charge, H is the mean residue hydrophobicity, } \mu \text{ is the hydrophobic moment calculated as the vector sum of the hydrophobicities of all residues for } \alpha \text{-helical region only (residues 1-21) (from Dathe and Wieprecht, 1999).}
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non-polar amino acids, it is highly soluble in water (>250 mg/ml) and moderately soluble in methanol (up to 20 mg/ml). At low concentration, melittin is monomeric and adopts essentially a random coil conformation in aqueous solution. Importantly, it adopts an \( \alpha \)-helical conformation and aggregates into tetramers depending on several factors as described below (see Section 1.3.1.b).

(a) High-Resolution Structures of Melittin

The three-dimensional structure of the aqueous melittin tetramer is known to atomic resolution as determined by the x-ray crystallographic analysis at 2 Å resolution from melittin crystals grown from solutions of high ionic strength (Terwilliger and Eisenberg, 1982a,b; Terwilliger et al., 1982). The four melittin monomers in the tetramer are nearly identical in conformation. In the crystalline tetramer (see Figure 1.3) each melittin chain is composed of two \( \alpha \)-helical segments and the overall shape is that of a 'bent rod'. The peptide helix is bent due to the presence of proline at position 14 so that lines drawn through the helix axes of residues 1-10 and 16-26 intersect with an angle of about 120°. The large helix bend allows optimal packing of hydrophobic side chains within the melittin tetramer. Despite a large bend around Pro-14, the crystalline melittin monomer has an essentially helical conformation throughout. The degree of helicity of crystalline tetrameric melittin is greater than expected from the value determined by CD for tetrameric melittin in solution which is around 50-60% (Knöppel et al., 1979; Bello et al., 1982). Overall, the melittin chain can be classified into three regions based on the asymmetric distribution of polar and apolar side chains: (i) a hydrophobic amino terminal region, (ii) a central region with hydrophobic and hydrophilic faces, and (iii) an entirely hydrophilic C-terminal region.
In addition to the x-ray structure of tetrameric melittin in aqueous solution, high-resolution structures of monomeric melittin in methanol, and in DPC micelles have also been determined using high-resolution proton NMR and amide exchange analysis (Bazzo et al., 1988; Dempsey, 1988; Inagaki et al., 1989). Melittin is monomeric and α-helical in methanol (Bazzo et al., 1988; Dempsey, 1988) and the structure has been found to be similar to that found in melittin crystals. Surprisingly, the structure of the hinge region in the melittin chain is shown to be significantly different from that of the crystal structure, leading to a considerably smaller angle between the two helical segments. This indicates that the Pro-14 residue need not induce a large bend in the melittin structure and this
should be considered in models of the membrane-bound conformation in the absence of
direct determination of perturbation induced by the proline residue. Interestingly, the
NMR structure of melittin bound to micelles (Brown and Wüthrich, 1981; Brown et al.,
1982; Inagaki et al., 1989; Yuan et al., 1996) shares most of the features of the NMR
structure obtained in methanol and aqueous solutions (Brown et al., 1980). These high-
resolution structures of melittin in the crystalline state, in solution of low dielectric
constant and in micelles have been considered as good models for the membrane-bound
conformation. Interestingly, the secondary structure of membrane-bound melittin has
also been shown to be predominantly α-helical (see Section 1.3.2).

(b) Aggregation/Self-Association of Melittin in Solution

In aqueous solution, aggregation of monomeric melittin to a tetramer is promoted
by high salt, high melittin concentration, and high pH. These factors strongly suppress
the charge of melittin and promote the self-association of melittin monomers into
tetramers since melittin has a high charge density and aggregation would be prevented by
electrostatic repulsions. For instance, Talbot et al. (1979) have shown by optical rotatory
dispersion (ORD), gel filtration and fluorescence measurements that melittin is
monomeric and adopts a random coil conformation when the peptide concentration and
ionic strength are low at physiological pH. This is in agreement with fluorescence
experiments which show that melittin is completely monomeric at very low salt
concentration (Quay and Condie, 1983). In contrast, the aggregation of melittin is
promoted by high concentration of the peptide and/or high ionic strength at neutral pH
(Talbot et al., 1979; Faucon et al., 1979). The effect of increasing melittin concentration
on tetramer formation has been supported by an increase in the helical content of the
peptide (Lauterwein et al., 1980). In addition, Knöppel et al. (1979) reported a change in
the conformation of melittin to tetramer at high pH although the concentration of the
peptide and the ionic strength are low. Further, Brown et al. (1980) studied the $^1$H NMR spectrum and ultracentrifugation of melittin and found aggregation to tetramer at (i) elevated concentration at neutral pH, (ii) at low pH in 1.5 M NaCl, (iii) at high concentration in low pH, salt-free solution, and (iv) at pH 9 in salt-free solution. It has been shown that deprotonation of the α-amino group enhances the tendency to aggregate which is in agreement with an extensive study on the effect of pH and concentration on the conformation and aggregation of melittin (Bello et al., 1982). These studies point out that the conformation of melittin in aqueous solution is a complex function of peptide concentration, ionic strength and pH. Further, the dependence of aggregation of melittin on temperature has also been reported (Wilcox and Eisenberg, 1992). Interestingly, the tetramer formation has been shown to occur when the net charge of melittin is modified by acetylation (net charge becomes +2) or succinylation (net charge becomes −2) and this conversion is promoted by an increase in peptide concentration (Bello et al., 1982).

The complexity of the aggregation behavior of melittin in solution with reference to electrostatic repulsion has been demonstrated by Ramalingam et al. (1992) using anionic melittin analogues. They changed the net charge of melittin to −6 by acetylation and showed that this anionic derivative forms a tetrameric helix at neutral pH, without salt, and at relatively low concentration, conditions under which the native melittin is monomeric and adopts random coil conformation. This observation suggests that a high net charge is not sufficient to prevent the association and helix formation of melittin. Surprisingly, the anionic melittin analogues in which all five lysine and arginine residues are replaced with glutamate (net charge −4), and the acetyl and succinyl derivatives of this anionic melittin (net charges −5 and −6, respectively) were resistant to helix formation and required much higher salt concentration for helix formation than does cationic melittin. Even divalent cations are found to be less effective in promoting helix formation in these derivatives when compared with melittin (Ramalingam et al., 1992).
Overall, the aggregation/self-association of melittin in aqueous solution is a complex phenomenon and strictly depends on the interplay between the peptide concentration and solution properties (ionic strength and pH), which in turn affect the hydrophobic, electrostatic, and helix-dipole interactions at the N-terminus of melittin.

1.3.2. Melittin-Membrane Interactions

Melittin targets the membrane from the aqueous phase and partitions into PC membranes with a partition coefficient ($K_p$) of $\sim10^4 \text{ M}^{-1}$ (Beschiaschvili and Baeuerle, 1991; Beschiaschvili and Seelig, 1990; Kuchink and Seelig, 1989; Schwarz and Beschiaschvili, 1989). The association of melittin with PC membranes takes place in the order of milliseconds (Sekharam et al., 1991; Bradrick et al., 1995; Wolfe et al., 1998). The kinetics of the association of melittin with membranes is controversial since both monophasic (Sekharam et al., 1991; Bradrick et al., 1995) as well as biphasic (Wolfe et al., 1998) kinetics of this phenomenon have been reported. Melittin adopts an $\alpha$-helical conformation in membranes which is supported by a number of studies carried out in membrane-mimetic systems such as micelles and reverse micelles, and in liposomes using a variety of techniques (Brown et al., 1981; Chandani and Balasubramanian, 1986; Vogel, 1987; Inagaki et al., 1989; Weaver et al., 1992; Bismuto et al., 1993; Ghosh et al., 1997; Yang et al., 2001; Ladokhin and White, 2001; Raghuraman and Chattopadhyay, 2003; Raghuraman and Chattopadhyay, 2004). Studies using CD and Raman spectroscopy indicate that the helix contains about 20 amino acids in DTPC membranes (Vogel and Jähnig, 1986; Vogel, 1987), suggesting that the C-terminal region consisting of a stretch of charged residues may adopt a non-helical conformation. Recently, NMR studies of membrane-bound melittin have also shown it to be $\alpha$-helical with a kink in the middle (Naito et al., 2000; Lam et al., 2001). Further, it has been shown that the angle between the N- and C- terminal helical segments of melittin bound to DMPC bilayer is
~140° or ~160°, which is larger than the value of 120° determined by x-ray diffraction studies in aqueous solution (Naito et al., 2000). However, despite the availability of high-resolution crystal structure of tetrameric melittin in aqueous solution (Terwilliger and Eisenberg, 1982a,b), the structure of the membrane-bound form is not yet resolved by x-ray crystallography.

(a) **Phospholipase A₂ Contamination: A Major Concern in Melittin-Membrane Interactions**

Apart from melittin, bee venom also contains an endogenous phospholipase A₂ (Shipolini et al., 1971) and this presents a major concern for artifacts in experiments with melittin involving phospholipid membranes unless the peptide is purified free of the enzyme. Although baseline resolution of melittin and phospholipase A₂ may be achieved in several chromatographic systems, melittin frequently retains phospholipase activity, probably as a result of binding of the peptide to the enzyme (Banks et al., 1981). Moreover, the phospholipase A₂ activity is not inhibited by low concentrations of EDTA, indicating very tight complexing of Ca²⁺ with the enzyme (Shipolini et al., 1971; Dasseux et al., 1984; Dempsey and Watts, 1987). A number of reports on melittin-induced increased thermal transition temperature in melittin-DMPC complexes have been shown to result from contaminating phospholipase A₂ activity (Dasseux et al., 1984; Dempsey and Watts, 1987). Apparently, trace amounts of phospholipase A₂ are shown to be present in melittin obtained from Sigma Chemical Co. (St. Louis, MO). However, the effect of phospholipase A₂ is detectable only if a significant amount of Ca²⁺ is added to the sample. In fact, results from both Sigma melittin and pure synthetic melittin are shown to be indistinguishable (Yang et al., 2001). Nevertheless, as a precautionary measure, it is advisable to use buffers with EDTA at concentrations of 5 mM in experiments involving melittin-membrane interactions to ensure the suppression of residual phospholipase A₂ activity (Dempsey, 1990). In addition, it has been shown that
the phospholipase activity, assayed using radiolabeled phospholipids, could not be detected in lipid samples containing 5 mM EDTA in buffer even after keeping for a few days at room temperature (Ghosh et al., 1997).

(b) Actions of Melittin on Membranes

(i) Hemolytic Activity: The characteristic action of melittin is its hemolytic activity since the target for the action of melittin is erythrocyte membrane (Habermann, 1972; Sessa et al., 1969). At sub micromolar concentrations and higher, melittin binds rapidly to erythrocytes (within seconds) and induces the release of hemoglobin into the extracellular medium (DeGrado et al., 1982). The apparent dissociation constant has been found to be \(10^{-7}\) (DeGrado et al., 1982) to \(3 \times 10^{-8}\) M (Tosteson et al., 1985) depending on the conditions used for the assay. Interestingly, SPR experiments have shown that the apparent dissociation constant for melittin varies from \(10^{-5}\) to \(10^{-7}\) M depending on the surface charge density of membranes (Lee et al., 2001). There are about \(1.8 \times 10^7\) binding sites for melittin per erythrocyte (DeGrado et al., 1982; Tosteson et al., 1985), indicating that the primary site of interaction is the membrane lipid rather than specific 'receptors'. It has been shown that melittin-induced hemolysis at room temperature occurs by a colloid osmotic mechanism (Tosteson et al., 1985; Kubota and Yang, 1986). This conclusion is based on the observation that the permeability of ions is rapid during the first few minutes of exposure to melittin and the release of hemoglobin is secondary to the formation of ion-permeable (hemoglobin-impermeable) lesions or 'pores' (Tosteson et al., 1985). This colloid osmotic mechanism is supported by the hemolytic action of truncated melittin analogues and the size of the lesions or 'pores' is estimated to be \(\sim 20\) Å (Subbalakshmi et al., 1999).

Melittin-induced hemolysis follows reproducible, temperature-dependent biphasic kinetics (Hider et al., 1983; DeGrado et al., 1982) with characteristic fast and slow
phases which dominate lysis at 4 °C and 37 °C, respectively (DeGrado et al., 1982). This biphasic kinetics of melittin-induced permeabilization has been supported by experiments carried out using model membranes (Ghosh et al., 1997; El Jastimi and Lafleur, 1999; Gómara et al., 2003; Allende and McIntosh, 2003) as well as in nucleated mammalian cells (Su et al., 2001). Interestingly, the rates of the fast and slow kinetic phases are concentration-dependent and these rates have been interpreted in terms of the molecularity of the melittin species involved in each phase (DeGrado et al., 1982). At 4 °C in iso-osmotic sucrose (10 mM phosphate), the rate of the fast phase increases linearly with increasing peptide concentration while the rate of the slow phase increases as the square of the melittin concentration. The fast phase is interpreted as resulting from the perturbation of membrane structure and organization due to the rapid accumulation of melittin in the outer leaflet of the erythrocyte membrane and its decay into a slow phase is a result of the reorganization of peptide and membrane lipid to recover favorable packing geometry. It has been proposed that the internalization of melittin dimer underlies the slow phase of hemoglobin release because of the second-order dependence of the rate on peptide concentration (DeGrado et al., 1982). However, interpretation of the nature of molecularity of melittin from kinetic experiments produced variable and contrasting results depending on the concentration of peptide and temperature used. These variations could be due to the complex aggregation behavior of melittin in solution and possibly in membranes.

Importantly, it has been shown that the binding of melittin to erythrocytes as a monomer is necessary for its hemolytic activity (Hider et al., 1983). This is based on the observation that phosphate suppresses hemolysis to an extent which correlates with its effect on inducing aggregation of melittin to tetramers in solution. This is in agreement with the finding that the linear aggregates of melittin, obtained by cross-linking with dimethylsuberimidate, retain the lytic activity at high phosphate concentrations indicating that cross-linking suppresses phosphate-induced self-association of melittin in solution.
(DeGrado et al., 1982). Whether the hemolysis induced by melittin requires reassociation of monomeric membrane-bound melittin to specific aggregates remains a challenging yet an interesting issue.

(ii) Voltage-gated channel formation: In the presence of a trans-negative membrane potential, melittin has been reported to induce increased permeability of ions in planar lipid membranes (Tosteson and Tosteson, 1981). This observed change in conductance, under high ionic strength conditions, exhibits discrete multilevel conductances (Hanke et al., 1983; Tosteson et al., 1987). However, these are erratic and less well-defined currents. The voltage-dependent increase in conductance is consistent with the formation of channels in response to a voltage-dependent change in orientation of melittin in the membrane. The voltage-gated pores show selectivity of anions over cations, probably due to the accumulation of positive charges on the C-terminal region of melittin (Pawlak et al., 1991; Tosteson and Tosteson, 1981). Interestingly, amino-acetylated melittin has also been shown to induce voltage-dependent conductance, indicating that the change in the orientation of melittin under the influence of transmembrane potential is not driven by the interaction of N-terminal or Lys-7 amino groups with the membrane potential (Hanke et al., 1983). This is supported by the observation that synthetic melittins having a blocked N-terminal amino group or a Lys-7 to Asn-7 substitution show voltage-dependent ion permeability (Tosteson et al., 1988).

The melittin-induced increase in ion permeability is characterized by a fourth power dependence on melittin concentration indicating that a tetrameric structure of melittin may be responsible for channel formation (Tosteson and Tosteson, 1981, 1984; Tosteson et al., 1990; Fattal et al., 1994; Stankowski et al., 1991). However, the observation that melittin exhibits multiple conductance levels in POPC (Hanke et al., 1983) and asolectin (Tosteson et al., 1987) indicates heterogeneity in the 'pore' structure. In addition, structurally modified analogues of melittin show different concentration
dependencies in their ion permeability (Tosteson et al., 1988). These studies therefore indicate that strictly tetrameric models for the voltage-dependent melittin channel formation are probably unjustified. In fact, it has been shown that the number of melittin monomers required to form molecular aggregates vary depending on the lipid composition and the physical conditions of the bilayer (see 1.3.2.g). Interestingly, the direct interaction of tricyclic tranquilizers with melittin has been proposed for the activation of melittin channels in PC membranes (Tanaka et al., 1992). Taken together, these observations provide a plausible explanation for the colloid osmotic mechanism of melittin-induced hemolysis (DeGrado et al., 1982; Hider et al., 1983; Tosteson et al., 1985; Subbarao and MacDonald, 1994).

(iii) Micellization and Fusion of bilayers: Melittin-induced permeabilization of membranes is known to cause the breakdown of membranes into micelles at high concentration. This is similar to the solubilization of membranes by detergents. Interestingly, micellization of melittin specifically occurs in membranes composed of pure saturated PC which has an interesting dependence on the lipid phase transition temperature (liquid crystalline-to-gel state phase transition of the pure lipid, T_m) (Monette et al., 1993). Though the morphology of liquid crystalline PC bilayer remains unaltered in presence of low concentration of melittin (~5 mol%), the presence of intermediate amounts of melittin induces the liposome to fragment into small particles (micelles) upon lowering of temperature (below T_m) to form gel phase. This temperature dependence of bilayer to micelle formation by melittin and [Ala-14] melittin analogue in DMPC vesicles has been shown to be reversible using NMR spectroscopy and freeze-fracture electron microscopy (Dempsey and Watts, 1987; Dempsey and Sternberg, 1991; Dufourcq et al., 1986). Freeze-fracture electron microscopy, light scattering and gel filtration experiments indicate that disk-shaped micelles with an approximate diameter of 235 Å are formed (Dufourcq et al., 1986; Faucon et al., 1995). Interestingly, these discs are
thermodynamically unstable and their formation shows lipid acyl chain length
dependence. The resistance of saturated phosphatidylcholine bilayers to melittin-induced
gel phase micellization increases with increasing acyl chain length supporting the idea
that bilayer disruption is maximized when cohesion between lipid acyl chains is
minimized in gel phase membranes (Lafleur et al., 1987). Similarly, the stability of gel
phase micellar discs is inversely related to the acyl chain length, with long chain
phosphatidylcholine-melittin micelles reforming bilayer sheets or large vesicles over
extended periods by fusion without warming above the phase transition temperature
(Lafleur et al., 1987). These studies conclude that melittin induces a reorganization of
lipid assemblies which include vesicularization of multibilayers, fusion of small lipid
vesicles, fragmentation into discs and micelles depending on the experimental conditions.
Interestingly, the presence of cholesterol in membranes inhibits melittin-induced fusion
and formation of small discs (Monette et al., 1993; Pott and Dufourc, 1995). In contrast,
melittin exhibits bilayer stabilizing effects when mixed with phosphatidylethanolamine
membranes under conditions where the pure lipid arranges in a hexagonal phase (H_{II})
(Batenburg et al., 1988).

In common with a number of basic amphipathic peptides (Svenaga et al., 1989),
melittin exhibits fusogenic activity. Even at very low concentration, melittin fuses a
variety of phosphatidylcholine membranes when the temperature is cycled through the
lipid phase transition temperature (Morgan et al., 1983; Pott and Dufourc, 1995). In
negatively charged lipids, fusion occurs under isothermal conditions (Morgan et al.,
1983). The mechanism of melittin-induced fusion of vesicles is probably different from
the fusion of bilayer disc micelles but may require a local disruption of bilayer structure
and the apposition of vesicular membranes (Morgan et al., 1983; Murata et al., 1987).
Interestingly, the kinetics of fusion of DPPC vesicles depends on the association state of
melittin in solution (Bradrick and Georghiou, 1987; Bradrick et al., 1989).
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(c) Structural and Charge Requirements for the Activity of Melittin

It was initially believed that the action of melittin in membranes is mediated through channel formation. Structure-function studies of melittin indicate that replacement of Pro-14 with alanine (P14A melittin) removes the bend in the helical structure of melittin. Interestingly, it has been shown that P14A melittin is 2.5 fold more hemolytic than native peptide, but it forms less stable voltage-dependent channels, thereby indicating that channel formation may not dictate the hemolytic activity of melittin (Dempsey, 1990). In another study, it has been shown that P14A melittin affects the self-association, membrane binding and pore-formation kinetics of melittin due to changes in structural and electrostatic properties (Rex, 2000). The amphiphilic helical segment of melittin between residues 1-20 appears to play only a structural role in its activity. Though membrane-active, the segment 1-20 of melittin does not possess any lytic activity. Interestingly, the cationic segment 20-26 has also been shown to be inactive (Schroder et al., 1971; Dawson et al., 1978). However, Lys-23 and Arg-24 residues are important in binding of melittin to membranes (Otoda et al., 1992).

In an excellent study, Blondelle and Houghten (1991a) analyzed the antibacterial and hemolytic activities of 24 individual omission analogues of melittin. The results indicate that deletion of Leu-6, Leu-9, Leu-13, Leu-16, Iso-17 and Trp-19 results in considerable decrease in hemolytic activity whereas apart from these residues, the deletion of Ala-4 and Lys-7 results in considerable reduction in antibacterial activity relative to native melittin. Analysis of these residues suggests that the residues making up the two separate helical regions (i.e., residues 1-9 and 13-20), except Pro-14 are important for the hemolytic action of melittin whereas the residues making up the C-terminal regions of melittin had no effect as mentioned earlier. However, using surface plasmon resonance and hybrid bilayer membrane systems, Mozsolits et al. (2001) demonstrated that the C-terminal region of melittin is essential for binding to both
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zwitterionic and anionic membranes. These observations suggest that the structural requirements for the binding and the functional activity of melittin are entirely different. Based on the analysis of retention times during reverse-phase high-performance liquid chromatography, it has been suggested that deletion of amino acids that caused lower hemolytic activity had lower amphiphilicity (decreased amphiphilicity was correlated with early retention times). The study, therefore, clearly indicated that amphiphilic structure is crucial for the hemolytic activity of melittin. This is supported by experiments which utilizes individual substitution analogs in the C-terminal region of melittin (Yan et al., 2003). Interestingly, the requirement of amphiphilic structure does not seem to be important for the antibacterial activity (Blondelle and Houghten, 1991a). In contrast, hydrophobicity has been shown to be required for the antibacterial activity of melittin (Yan et al., 2003).

The requirement of amphiphilic helical structure for hemolytic activity but not for antimicrobial activity is also borne out by studies on diastereomeric, retro and retro-enantiomers of melittin (Juvvadi et al., 1996; Oren and Shai, 1997). Diastereomeric melittin, where Val-5, Val-8, Iso-17 and Lys-21 residues were replaced by D-isomers, showed a very low propensity for secondary structure and did not have any appreciable hemolytic activity but had significant antimicrobial activity (Oren and Shai, 1997). This specificity is achieved due to the fact that melittin diastereomers bind to and destabilize only negatively charged membranes in contrast to native melittin, which binds strongly to both zwitterionic and anionic membranes. However, the partition coefficient, the depth of penetration into the membrane, and the membrane-permeabilizing activity of the diastereomers with negatively charged phospholipids are similar to that of native melittin. On the other hand, retro and retro-enantio analogues of melittin, which have high propensity for β-structure in aqueous medium but have high helical content in the presence of hexafluoroisopropanol, possess antimicrobial activity comparable to melittin but considerably lower hemolytic activity. Thus, the hemolytic activity appears to be
sensitive to the direction of amide bonds and helix dipole moment (Juvvadi et al., 1996). Interestingly, using the retro and retro-enantio analogues of cecropin-melittin hybrids, it was concluded that chirality of the peptide was not a critical feature, and full antibacterial activity could be achieved with peptides containing either all L- or all D-amino acids in their respective right-handed or left-handed helical conformations (Merrifield et al., 1995). These studies emphasized the importance of amphipathicity and α-helicity not only to the cytolytic activity of melittin but for the activity of other cytolytic peptides. An additional attribute, common among native amphipathic helices including melittin, is peptide linearity. Experiments using the synthetic cyclic melittin analogue reveal that cyclization causes 4-30 fold decrease in melittin binding depending on the charge of the host membranes. Interestingly, the cyclic melittin analogue has increased antibacterial activity but decreased hemolytic activity (Unger et al., 2001). These results indicate that the linearity of the peptide is not essential for the disruption of the target phospholipid membrane, but rather provides the means to reach it.

Studies involving single amino acid omission analogues of melittin revealed the importance of the sole tryptophan of melittin (Trp-19) in its hemolytic activity (Blondelle and Houghten, 1991a). This is further demonstrated by the remarkable decrease in activity observed upon photooxidation of the tryptophan (Habermann and Kowallek, 1970), upon substitution of Trp-19 by leucine (Blondelle and Houghten, 1991b) and the introduction of a second tryptophan residue in the melittin sequence (Blondelle et al., 1993). However, the position of Trp in the sequence does not seem to be very critical for activity, as analogues of melittin containing a single Trp residue at positions 9, 11 or 17 also exhibited hemolytic activities with potencies in the order Trp-17 > Trp-19 = Trp-11 > Trp-9 (Weaver et al., 1989). Importantly, analogues of melittin containing a single Trp residue at positions 9, 11 or 17 do not form tetramers in solution at high peptide or salt concentrations unlike native melittin. Hence, the ability to form soluble tetrameric structures appears to be unrelated to the hemolytic activity of melittin. This is in contrast
to the previous observation in which it was shown that single amino acid substitution of Lys-7 prevented the self-association of melittin and those substitutions, which prevented the inducible amphipathic folding ability, were found to result in a loss in hemolytic and antimicrobial activity (Pérez-Payá et al., 1995).

In addition to amphipathicity, \( \alpha \)-helicity and amino acid specificity, certain charge constraints have also been shown to have an important role in the specificity of action of melittin, either hemolytic or antimicrobial. As mentioned earlier, the net charge of melittin is +6 in solution. It has been shown that the presence of at least two positive charges is essential for the activity of melittin. Interestingly, melittin with extra positive charges introduced on the hydrophilic face of the helix possesses hemolytic activity greater than that of native melittin (Werkmeister et al., 2002). Further, a 15-residue synthetic peptide, corresponding to the C-terminal region of melittin, exhibits 5-7 fold less antimicrobial activity than melittin. The hemolytic activity, on the other hand, is 300 times less than that of melittin. An analogue of this peptide in which two cationic residues have been transposed to the N-terminal region from the C-terminal region, has antibacterial activity comparable to that of melittin but has considerably lower hemolytic activity (Subbalakshmi et al., 1999). The biological activities of these peptides have been rationalized on the basis of their structure and aggregation properties.

(d) Energetics of Folding of Melittin in Membranes

The membrane interface has a potent ability to induce secondary structure in melittin and this is also true for a wide range of other membrane-active peptides such as hormones, toxins and antimicrobial peptides (Kaiser and Kezdy, 1983, 1984; Schwyzer, 1992; Maloy and Kari, 1995; White and Wimley, 1998). In addition, it has been shown that membrane interfacial properties such as bilayer area compressibility modulus modulate the binding of amphipathic peptides (Allende et al., 2003). Wimley and White
(1996) have shown that the partitioning of peptides in membranes is dominated by the
extremely unfavorable free energy cost of partitioning the peptide bonds (+1.2 kcal mol\(^{-1}\)
per peptide bond for phosphocholine bilayers). They proposed that hydrogen bonding of
peptide bonds reduces this high free energy cost and thereby promotes the formation of
secondary structures of peptides/proteins in membranes.

In general, the folding of peptides is tightly coupled to their partitioning in
membranes. Determination of the energetics of helix folding on membranes is therefore
extremely difficult due to the experimental inaccessibility of the unfolded membrane-
bound form of peptides. Since melittin adopts unfolded (random coil conformation) and
folded forms (α-helical) in solution and membranes, respectively, it has been used to test
the hypothesis that hydrogen bonding reduces the high cost of partitioning the peptide
bonds along with its diastereomeric analogue which has four D-amino acids (D\(_4\),L-
melittin). Since D\(_4\),L-melittin has little secondary structure either in its free or bound
form due to the presence of D-amino acids (Oren and Shai, 1997), it serves as a model for
the experimentally inaccessible unfolded bound form of native melittin. This is based on
the assumption that membrane-bound D\(_4\),L-melittin has only six residues for helix
formation when compared to 18 residues in native melittin (Dempsey and Butler, 1992).
Thus, native melittin has 12 more residues in an α-helical conformation than
D\(_4\),L-melittin when membrane-bound. The free energy of partitioning of unfolded
melittin from the aqueous phase to the bilayer interface of POPC vesicles as the folded
form has been estimated to be −7.6 kcal mol\(^{-1}\) using mole fraction partition coefficients
(Ladokhin and White, 1999). The partitioning of native melittin into POPC membranes
is 5.0 kcal mol\(^{-1}\) (−3 fold) more favorable than the partitioning of D\(_4\),L-melittin which is
2.6 kcal mol\(^{-1}\). These findings show that the free energy reduction per residue
accompanying the folding of melittin in membrane interfaces is about 0.4 kcal mol\(^{-1}\),
consistent with the hypothesis that hydrogen bonding reduces the high cost of partitioning
of peptide bonds. This value has potential implications in estimating the energetic
consequences of membrane-induced secondary structure formation of peptides/proteins. Further, the cumulative effect of these relatively small per residue free energy reductions can be very large and assumes significance when tens or hundreds of residues are involved as in the assembly of the β-barrel transmembrane domain of α-hemolysin (Song et al., 1996) that buries ~100 residues in the membrane.

(e) Orientation of Membrane-Bound Melittin

The transmembrane potential has been conjectured to affect the orientation of melittin in membranes (Tosteson and Tosteson, 1981; Tosteson et al., 1985). In an excellent study, Kempf et al. (1982) show a voltage-dependent change in the orientation of melittin helix from a bilayer surface orientation to a transmembrane one under the influence of transmembrane negative potential. This process has been found to be reversible when the voltage is trans-positive. A recent study confirms the change in the orientation of membrane-bound melittin under the influence of transmembrane potential using a combination of HPLC and liquid secondary ion mass spectrometry (LSIMS) (Niu et al., 2000). All other studies concerning the orientation of membrane-bound melittin have been done in model systems in the absence of a transbilayer potential. In addition, utilizing the effective energy function for proteins in membranes, it has been reported that the lowest-energy orientation of melittin varies depending on the hydrocarbon thickness of the bilayer (Lazaridis, 2003). Determination of the orientation of α-helical melittin bound to membranes using conventional CD spectroscopy is rather impossible since this technique cannot discriminate between the parallel and perpendicular orientations of the membrane-bound peptide. Hence, the direct determination of helix orientation has been carried out using oriented bilayers utilizing IR spectroscopy and oriented circular dichroism (OCD) spectroscopy (see below).
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The orientation of melittin in membranes is dependent on the lipid composition and the physical condition of the membrane. One of the important controlling parameters that determine the orientation of membrane-bound peptides is their concentration. At low concentrations, melittin adopts parallel orientation with respect to the membrane surface whereas when the concentration of peptide increases above a certain threshold value, an increasing fraction of peptide molecules change to the perpendicular orientation as explained by the two-state model for the action of antimicrobial peptides (Huang, 2000). The orientation of α-helical segments of melittin in membranes is also found to be dependent on other important variables such as hydration, temperature and the phase state of the lipid (Frey and Tamm, 1991; Vogel, 1987; Naito et al., 2000). The sensitivity of these variables changes with the peptide and lipid. For example, in gel phase DTPC or DMPC membranes at low hydration levels (6% w/w corresponding to 2-3 water molecules per lipid), the α-helical segments of melittin are oriented roughly perpendicular to the plane of the membrane (Vogel and Jähnig, 1986). The perpendicular orientation of melittin is also observed in DLPC, DTPC and DMPC bilayers when the temperature is well above the phase transition temperature (Yang et al., 2001; Naito et al., 2000). In contrast, it has been shown that melittin is always oriented parallel to the DPhPC bilayers with little dependence on temperature and hydration (Yang et al., 2001).

This is also true in case of melittin bound to DOPC membranes (Hristova et al., 2001). The parallel orientation in the membrane-water interface is such that the apolar residues face the hydrophobic core of the membrane and the polar residues face the bulk aqueous phase. This corresponds to the "wedge" model that has been described in the literature (Dawson et al., 1978; Terwilliger et al., 1982; Bernèche et al., 1998) and is consistent with the experimental observations of the orientation of melittin in bilayers (Bradshaw et al., 1994; Citra and Axelsen, 1996; Dempsey and Butler, 1992). Interestingly, in POPC bilayers, melittin can orient either parallel or perpendicular to the plane of the membrane depending on the peptide concentration (Ladokhin et al., 1997; Yang et al., 2001).
The orientation of melittin in membranes is further complicated by the observation that melittin does not adopt a fully transmembrane configuration (Wall et al., 1995). Using fluoresceinphosphatidylethanolamine (FPE) as a real-time probe for peptide-membrane interaction and stopped-flow fluorescence measurements, it has been shown that melittin binds and partially inserts into PC membranes. This is supported by the molecular dynamics simulation study on the interaction of melittin with fully hydrated DMPC bilayers (Bachar and Becker, 2000). These studies, therefore, point out the role of lipid composition and the physical factors that affect the properties of membranes in the orientation of melittin in membranes.

(f) Aggregation State of Melittin in Membranes

The aggregation state of melittin in membranes is an important issue since this property is presumed to be associated with the function of melittin. This can be appreciated by the fact that melittin forms voltage-gated channels (Tosteson and Tosteson, 1981, Tosteson et al., 1985) which may require self-association of melittin monomers to form pores in membranes. It is not known whether pore-forming melittin aggregates pre-exist in the membrane in the absence of an applied membrane potential. Further, extrapolating knowledge of the aggregation properties of melittin in solution to membranes is extremely debatable since the membrane is a two-dimensional anisotropic fluid. This lack of one dimension in membranes may increase the apparent local concentrations of melittin monomers and therefore favor self-association in membranes. In addition, it is well documented that lipids play a crucial role in the insertion and the pore formation of melittin (see Sections 1.3.2.e and 1.3.2.g). Several studies have attempted to determine the aggregation state of membrane-bound melittin using modified (Vogel and Jähnig, 1986), spin-labeled (Altenbach and Hubbell, 1988) and N-methyl anthraniloyl-labeled melittins (Hermetter and Lakowicz, 1986) utilizing various sensitive
techniques such as fluorescence quenching, electron paramagnetic resonance (EPR) and fluorescence resonance energy transfer (FRET). The results show that there is no consensus regarding the aggregation properties of membrane-bound melittin and lead to contradictory conclusions which are summarized as follows:

From the fluorescence energy transfer from Trp-19 to a melittin with a modified tryptophan, Vogel and coworkers proposed a detailed model of tetrameric aggregation (Vogel and Jähnig, 1986) in membranes (at lipid/melittin ratio above 1000:1), whereas utilizing FRET between Trp-19 and N-methyl anthraniloyl residue attached to Lys-21, Hermetter and Lakowicz (1986) concluded that the membrane-bound melittin is monomeric (at lipid/melittin ratio above 50:1). Fluorescence studies with melittin dansylated at the N-terminus confirm this result which is further supported by energy transfer measurements involving chemically modified tryptophan that show a lack of association of melittin bound to membranes at low salt concentration (Schwarz and Beschiaschvili, 1989). However, a related study in which the N-bromosuccinimide oxidized Trp-19 analogue of melittin has been used indicates the aggregation of melittin bound to fluid phase DMPC, PG or egg PC in the presence of NaCl (Talbot et al., 1987).

The dependence of the state of aggregation of membrane-bound melittin on the salt concentration has been reported earlier (Bradrick et al., 1989). In addition, it has been speculated that melittin bound to DMPC membranes exists in both monomeric as well as aggregated forms in presence of 2 M NaCl (Kaszycki and Wasylewski, 1990). Using spin-labeled melittin analogues in DOPC and DOPC:DOPA (9:1) at lipid/protein ratios of about 200:1, Altenbach and Hubbell (1988) not only confirmed that membrane-bound melittin is monomeric but also showed that melittin is bound parallel to the membrane surface. Interestingly, these results are consistent even in the presence of high salt concentration. Several studies support the monomeric model of membrane-bound melittin for more indirect reasons (Lavialle et al., 1982; Podo et al., 1982; Talbot et al., 1982; Terwilliger et al., 1982).
The marked variation in these experimental results is most readily explained by differences in the aggregation properties of the differently labeled melittins and by the different conditions of ionic strength and membrane composition employed. Overall, the results of experiments attempted to determine the aggregation state of membrane-bound melittin indicate that melittin is monomeric at peptide/lipid molar ratios below about 1:100 in fluid phase membranes at low ionic strength (Dempsey, 1990).

Kinetic studies suggest that the aggregation of melittin helices on the membrane surface act as a precursor of pore formation, with dimerization being the rate-limiting step in many (DeGrado et al., 1992; Schwarz and Beschiaschvili, 1989; Schwarz et al., 1992) but not all (Rex and Schwarz, 1998) cases. Recently, Takei et al. (1999) provided direct evidence for the kinetic importance of melittin association in pore formation through studies of cysteine-substituted melittin analogues [(melittin K23C)2 and (melittin K23Q, Q25C)2] linked by disulfide bridges under low ionic strength conditions. The initial rate of melittin-induced pore formation increased with the square of the peptide concentration, whereas both disulfide-dimerized melittin analogues showed a first-order dependence of pore formation rates on peptide concentration indicating that peptide dimerization is the rate-limiting step for melittin-induced pore formation. Further, it has been shown that a dimeric form of melittin (Q25C) analogue causes larger structural perturbations in membranes when compared to native melittin at low concentrations under identical conditions (Hristova et al., 2001). Recently, the self-association and membrane-binding behavior of mono- and tetra- 5,5,5-trifluoroleucine-labeled melittin (which have enhanced hydrophobicity) has also been examined (Niemz and Tirrel, 2001).

(g) Melittin and Pore Formation

It is commonly believed that multimeric pore formation is the mode of action of many naturally produced peptides such as antimicrobial peptides and toxins (Boman et
Under certain conditions, melittin molecules insert into the lipid bilayer and form multiple aggregated forms that are controlled by temperature, pH, ionic strength, lipid composition and lipid-to-peptide ratio. Neutron diffraction studies show that melittin-induced transmembrane pores are present only when the peptide is primarily oriented perpendicular to the membrane bilayer. No pores are detected when the peptide orients parallel to the bilayers. Hence, the perpendicular orientation of melittin with respect to the plane of a bilayer represents an important criterion for the pore formation of melittin. Several studies have been attempted to monitor the structure and function of such pores and their results show that melittin forms pores that have a rather wide distribution of sizes. For example, the sizes of the melittin pores which are characterized by the inner pore diameter, have been reported to be in the range of 10-60 Å (Rex, 1996), 13-24 Å (Matsuzaki et al., 1997), and 25-30 Å (Ladokhin et al., 1997) from vesicle leakage experiments. The diameter of these pores is expected to increase when the peptide concentration is increased. On the other hand, utilizing neutron diffraction, which detects and measures the size of transmembrane pores, it has been shown that the inner pore diameter of melittin-induced pores is 44 Å (Yang et al., 2001). Interestingly, the pore size is shown to be independent of peptide concentration (peptide/lipid ratio ≥ 1/30) in this case. These results are consistent with the studies on osmotic protection of erythrocytes which show an estimated pore size of ~20-30 Å at high melittin concentrations (Katsu et al., 1988).

The first model of peptide-induced pores (barrel-stave model) was proposed by Baumann and Mueller (1974) to account for the single channel conductance induced by alamethicin in black lipid membranes. In this model, alamethicin helices associate to form a bundle with a central lumen, like a barrel made of helical peptides as staves. Ever since its introduction, the barrel-stave model has been viewed as the prototype of peptide-
induced transmembrane pores, which include the pore induced by melittin (Vogel and Jähnig, 1986; Sansom, 1991; Naito et al., 2000). Utilizing the peptide orientation-sensitive technique oriented circular dichroism (OCD) and neutron diffraction, melittin pores have been shown to be consistent with the toroidal model (Yang et al., 2001) that was proposed to describe magainin-induced pores (Matsuzaki et al., 1996; Ludtke et al., 1996). The toroidal model differs from the barrel-stave model in that the peptides are always associated with the lipid headgroups even when they are perpendicularly inserted in the lipid bilayer. In forming such a pore, the lipid monolayer bends continuously from the top to the bottom in the fashion of a toroidal hole, so that the pore is lined by both the peptides and the lipid headgroups (see Figure 1.4). In addition, molecular dynamics simulation study supports the toroidal pore model for melittin-induced pores (Lin and Baumgaertner, 2000). Importantly, membrane-thinning effect caused by the partitioning of peptides in the parallel orientation in membranes has been shown to be responsible for the insertion and pore formation of melittin (Chen et al., 2003; Lee et al., 2004).

Lipid composition and phase separation appears to play a critical role in melittin-induced pore formation. For instance, it has been shown that SM, one of the main lipids of erythrocyte plasma membrane, affects the ability of melittin to permeabilize lipid vesicles and stimulates melittin pore formation in electrically neutral bilayers (Gómara et al., 2003). Analysis of the leakage data according to a kinetic model of pore formation (Nir and Nieva, 2000) shows a good fit for hexameric/octameric pores in SM-containing neutral vesicles at low melittin concentration. This is attributed to the coexistence of gel and fluid phases induced by the presence of SM in POPC bilayers. Interestingly, addition of cholesterol to this binary mixture reduces the efficiency of melittin pore formation since cholesterol induces the coexistence of fluid and liquid ordered (l_0) phases in SM/POPC membranes. In another study, melittin-induced release of encapsulated fluorescent dextran markers from POPC vesicles is shown to be mediated by transmembrane pores (Ladokhin et al., 1997; Ladokhin and White, 2001) whereas release
from anionic POPG vesicles is found to be non-selective, i.e., ‘detergent-like’ (Ladokhin

Figure 1.4. Schematic representation of the barrel-stave model (top) and the toroidal model (bottom). The dark layers represent the headgroup regions of bilayers. Peptide monomers are represented by the cylinders (from Yang et al., 2001). See text for more detailed description.

and White, 2001). This is supported by SPR studies that melittin forms pores only in zwitterionic membranes and not in negatively charged membranes (Papo and Shai, 2003a). These studies clearly point out the importance of lipid composition and phase separation on the aggregation behavior and pore formation of melittin.

1.3.3. Cellular Activities of Melittin

(a) Action of Melittin on Membrane Proteins

Apart from its ability to disrupt lipid bilayers, melittin affects the dynamics of membrane proteins. For instance, it has been shown that lytic concentrations of melittin dramatically reduce the rotational mobility of band 3 protein in human erythrocyte
membranes (Dufton et al., 1984; Clague and Cherry, 1988; Hui et al., 1990) and of bacteriorhodopsin in lipid vesicles (Hu et al., 1985). Further, melittin causes aggregation of membrane proteins including band 3 protein (Clague and Cherry, 1988), bacteriorhodopsin (Hu et al., 1985) and the Ca\(^{2+}\)-ATPase (Voss et al., 1991; Mahaney and Thomas, 1991; Mahaney et al., 1992). In addition, melittin is a potent inhibitor of the Ca\(^{2+}\)-ATPase (Voss et al., 1991; Mahaney and Thomas, 1991), the H\(^{+}\)K\(^{+}\)-ATPase (Cuppoletti et al., 1989; Cuppoletti, 1990) and the Na\(^{+}\)K\(^{+}\)-ATPase (Cuppoletti and Abbott, 1990). The experiments with bacteriorhodopsin suggest that immobilization due to aggregation of membrane proteins is a result of direct melittin-protein interactions rather than an indirect consequence of melittin-lipid interactions (Hu et al., 1985). This is supported by the observation that melittin effectively immobilizes membranes proteins in the plane of the lipid bilayer in erythrocyte membrane ghosts (Watala and Gwozdzinski, 1992). Interestingly, the role of melittin-lipid interactions has also been shown to be responsible, along with more specific binding of melittin with membrane proteins, for the inhibition of the Ca\(^{2+}\)-ATPase (Baker et al., 1995) and protein kinase C (Raynor et al., 1991).

(b) Transfection activity of melittin and nonviral gene delivery

Entry of exogenous DNA into the cytoplasm and subsequent transport into the nucleus are major cellular barriers for nonviral gene delivery vectors. Melittin, covalently attached to PEI, has been successfully used to enhance the transfection activity of PEI-DNA complexes in a broad range of cell lines including different tumor cell lines and also primary hepatocytes and human umbilical vein endothelial cells (Ogris et al., 2001). It has been shown that melittin not only enables efficient release of non-viral gene transfer particles into the cytoplasm due to its membrane lytic activity as monitored by fluorescence microscopy and flow cytometry, but also enhances their transport into the
nucleus. The latter effect of melittin has been attributed to the presence of the cationic cluster KRKR at the C-terminus of the peptide whose characteristics are similar to classical nuclear localization sequence (NLS). These results suggest that naturally occurring melittin possesses a dual endosomolytic and nuclear-homing functionality that can form the basis of a powerful transfection agent and makes it an interesting candidate for the further development of systemic gene delivery in vivo.

In addition, melittin has also been used in peptide-mediated RNA delivery, a novel approach for the enhanced transfection of primary and post-mitotic cells (Bettinger et al., 2001). Endosomolytic activity was incorporated by conjugating PEI of 2 kDa with melittin and resulting PEI 2 kDa-melittin/RNA polyplexes mediated high transfection levels in certain cell lines. This suggests that melittin-modified low molecular weight polycations possess endosomolysis which enables efficient non-viral mRNA transfection of quiescent and post-mitotic cells.

(c) Melittin and cell transformation

Oncogenes play an important role in the initiation and progression of the neoplastic phenotype. The ras oncogene is especially important with respect to human cancer (Bos, 1989) since at least one-third of all human colorectal tumors analyzed express an activated ras oncogene (Bos et al., 1987; Forrester et al., 1987). It has been demonstrated that melittin specifically selects against cells in culture that express high levels of the ras oncogene (Sharma, 1992). Hence, melittin exerts its anti-transformation effect(s) by specifically eliminating cells that express the oncoprotein. Further, acquisition of resistance to melittin is accompanied by a decrease in the number of copies of the ras genes, decrease in expression of the ras oncoprotein and a concomitant reversion of transformed cells to a normal morphology in a strict dose-dependent manner (Sharma, 1992). Interestingly, it has been shown that the biochemical basis for melittin-
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mediated counterselection of ras-transformed cells is due to the ability of melittin to hyperactivate cellular phospholipase A2 (PLA2) in ras-transformed cells by the mediation of enhanced influx of calcium ions (Sharma, 1993).

(d) Melittin and Signal Transduction

It is known that cationic amphiphilic peptides such as mastoparan and melittin directly stimulate nucleotide exchange by heterotrimeric GTP-binding proteins (G-proteins) in a manner similar to that of G protein-coupled receptors (Higashijima et al., 1990; Higashijima et al., 1988; Ross and Higashijima, 1994). Further, it has been shown that melittin inhibits the activity of adenylyl cyclase in synaptic membranes. Both mastoparan and melittin are known to stimulate Gi or Go activities in various cells, which may possibly be due to their common amphiphilic structure (Higashijima et al., 1990). In addition, melittin has also been shown to inhibit Gs activity by reducing the affinity of both GTP (or GTPγS) and GDP to Gs (Fukushima et al., 1998). Hence, it has been proposed that Gi stimulation and Gs inhibition are involved in melittin-induced inhibition of adenylyl cyclase. Interestingly, melittin represents the first metabostatic peptide that inhibits the intrinsic activity of G protein (Gs) activity.

(e) Leishmanicidal Activity of Melittin

Melittin induces membrane permeabilization and lyse prokaryotic as well as eukaryotic cells in a non cell-selective manner (Papo and Shai, 2003b). This mode of action is responsible for its hemolytic, anti-microbial (Blondelle and Houghten, 1991a; Bechinger, 1997), anti-fungal (Lazarev et al., 2002), anti-tumor (Winder et al., 1998) and leishmanicidal (Diaz-Achirica et al., 1998) activities of melittin. The protozoan mammalian parasite Leishmania is the causative agent of leishmaniasis, which afflicts 12 to 14 million people worldwide (Herwaldt, 1999). Though melittin has leishmanicidal
activity, the cytolytic activity of melittin is considered an obstacle for its potential therapeutic use. Interestingly, it has been shown that cecropin A-melittin hybrid peptides such as CA(1-18)M(1-18) and CA(1-7)M(2-9) show remarkable leishmanicidal activity with minimal cytolytic activity (Diaz-Achirica et al., 1998; Luque-Ortega et al., 2003; Chicharro et al., 2001). It has been shown that the action of CA(1-18)M(1-18) involves targeting of the plasma membrane of *Leishmania donavani* promastigotes, whereas the amastigote form is far more resistant to this peptide hybrid (Diaz-Achirica et al., 1998). Further, N-terminal fatty acylation has been shown to increase the leishmanicidal activity of CA(1-7)M(2-9) (Chicharro et al., 2001). These hybrid peptides, therefore, show promise for the development of specific anti-*Leishmania* peptides.

**Anti-Viral Activity of Melittin**

It has been shown that melittin reduces HIV-1 production in a dose-dependent manner (Wachinger et al., 1992). The reduction in viral infectivity is proposed to be due to the affinity of melittin for the gag/pol precursor, thereby preventing the processing of gag/pol by the HIV protease. This intracellular action of melittin is supported by a recent study which shows that melittin decreases the levels of Gag antigen and HIV-1 mRNAs (Wachinger et al., 1998). Further, it has been indicated that melittin has a direct suppressive effect on the activity of the HIV long terminal repeat. Taken together, these studies point out that antimicrobial peptides such as melittin and cecropin are capable of inhibiting replication of HIV-1 by suppressing viral gene expression. In yet another study, melittin has also been shown to inhibit plant virus infection (Marcos et al., 1995). Synthetic analogues of melittin that have sequence and structural similarities to an essential domain of TMV coat protein have been found to possess highly specific antiviral activity.