Chapter 5

Interaction of Melittin with Membrane Cholesterol
5.1. INTRODUCTION

Cholesterol is an essential component of eukaryotic membranes and plays a crucial role in membrane organization, dynamics, function and sorting (Liscum and Underwood, 1995; Simons and Ikonen, 2000; Yeagle, 1985). It is often found distributed nonrandomly in domains or pools in biological and model membranes (Liscum and Underwood, 1995; Simons and Ikonen, 2000; Yeagle, 1985; Schroeder et al., 1995; Mukherjee and Chattopadhyay, 1996; Simons and Ikonen, 1997; Xu and London, 2000; Rukmini et al., 2001). Many of these domains are believed to be important for the maintenance of membrane structure and function. Recent observations suggest that cholesterol exerts many of its actions by maintaining a specialized type of membrane domain, termed “lipid raft”, in a functional state (Simons and Ikonen, 1997; Edidin, 2003). Although the existence of lipid rafts in membranes has not been unequivocally shown (Munro, 2003), they are thought of as lateral organizations on the plane of the membrane that are enriched in cholesterol and sphingolipids and specific proteins that are implicated in cell signaling and traffic. The integrity of the raft regions of the membrane is thought to be crucial to regulate signal transduction events (Simons and Toomre, 2000) and entry of pathogens into the cell (van der Goot and Harder, 2001; Pucadyil et al., 2004).

Lipid-protein interactions play a crucial role in maintaining the structure and function of biological membranes (Lee, 2003). Effects on membrane function are presumed to be mediated by membrane proteins and for this reason, monitoring lipid-protein interactions assumes significance. A significant portion of any given membrane peptide or protein remains in contact with the membrane lipid environment. This raises the obvious possibility that the membrane could be an important modulator of membrane protein structure and function. In view of the importance of cholesterol in relation to
membrane domains (see above), the interaction of cholesterol with membrane proteins (Yeagle, 1985) represents an important determinant in functional studies of such proteins.

It has been proposed that the rigid ring system and perpendicular orientation in relation to the plane of the membrane make cholesterol an attractive target for many bacterial toxins and fungal antibiotics (de Kruijff, 1990). In addition, it has been suggested that the tryptophans in the toxins could potentially form a stable complex with the rigid ring system of the cholesterol molecule. In this work, we have monitored the effect of cholesterol on the organization and dynamics of melittin. The functional significance of the interaction of melittin with membrane cholesterol is brought out by the fact that the presence of cholesterol in the membrane inhibits the lytic activity of melittin (Benachir et al., 1997). Interestingly, the natural target for melittin is the erythrocyte membrane which contains high amounts of cholesterol (Yeagle, 1985). The organization and dynamics of the sole tryptophan residue of melittin, which has been earlier shown to be crucial for its hemolytic activity (Habermann and Kowallek, 1970; Blondelle and Houghten, 1991a; 1991b), therefore becomes an important issue in cholesterol-containing membranes.

5.2. MATERIALS AND METHODS

Materials

Melittin, calcein, DHE, L-tryptophan and NATA were obtained from Sigma Chemical Co. (St. Louis, MO). DOPC and spin-labeled phospholipids (5- and 12 PC) were purchased from Avanti Polar Lipids (Alabaster, AL). Anthroyloxy-labeled fatty acids (2- and 12-AS) were from Molecular Probes (Eugene, OR). To check for any residual phospholipase A2 contamination in melittin, phospholipase activity was assayed using 14C-labeled DOPC (Amersham International) as described earlier (Ghosh et al.,
Lipids were checked for purity by thin layer chromatography on silica gel precoated plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and were found to give only one spot in all cases with a phosphate-sensitive spray and on subsequent charring (Dittmer and Lester, 1964). The concentration of DOPC was determined by phosphate assay subsequent to total digestion by perchloric acid (McClare, 1971). DMPC was used as an internal standard to assess lipid digestion. The concentration of melittin in aqueous solution was calculated from its molar extinction coefficient (ε) of 5570 M⁻¹cm⁻¹ at 280 nm (Ghosh et al., 1997). Ultra pure grade acrylamide was from Gibco BRL (Rockville, MD). The purity of acrylamide was checked from its absorbance using its molar extinction coefficient (ε) of 0.23 M⁻¹cm⁻¹ at 295 nm and optical transparency beyond 310 nm (Eftink, 1991a). All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout.

Sample Preparation

SUVs of DOPC containing increasing concentrations (0-40 mol%) of cholesterol and 0.5 mol% melittin (1 mol% for energy transfer and CD measurements) were prepared. The vesicles were prepared by drying 2560 nmol (1280 nmol for energy transfer experiments) of total lipid (DOPC or DOPC/cholesterol) under a stream of nitrogen while being warmed gently (35 °C) and then under a high vacuum for at least 3 h. The lipids were swollen by adding 1.5 ml of 10 mM MOPS, 150 mM NaCl, pH 7.2 buffer containing 5 mM EDTA (to suppress any residual phospholipase A₂ activity), and vortexed for 3 min to disperse the lipid. The lipid dispersions were then sonicated until they were clear using a bath sonicator (Laboratory Supplies, Hicksville, NY) under argon. To incorporate melittin into membranes, a small aliquot containing 12.8 nmol (25.6 nmol for CD measurements) of melittin was added from a stock solution in water to the pre-formed vesicles and mixed well. For acrylamide quenching experiments, the total
lipid used was less to avoid scattering artifacts. For these experiments, 320 nmol of total lipid (DOPC or DOPC/cholesterol) was used to prepare vesicles as mentioned above and melittin was incorporated into membranes by adding a small aliquot containing 1.6 nmol of melittin from a stock solution in water to the pre-formed vesicles and mixed well to give membranes containing 0.5 mol% melittin. Samples were kept in the dark for 12 h before measuring fluorescence. Background samples were prepared the same way except that melittin was not added to them. All experiments were done at room temperature (23 °C).

Depth Measurements using the Parallax Method

The actual spin (nitroxide) content of the spin-labeled phospholipids (5- and 12-PC) was assayed using fluorescence quenching of anthroyloxy-labeled fatty acids (2- and 12-AS) as described earlier (Abrams and London, 1993). For depth measurements, liposomes were made by the ethanol injection method (Kremer et al., 1977). These samples were made by drying 640 nmol of total lipid (DOPC or DOPC/cholesterol) containing 15 mol% spin-labeled phospholipid (5- or 12-PC) under a stream of nitrogen while being warmed gently (35 °C) followed by further drying under a high vacuum for at least 3 h. The dried lipid film was dissolved in ethanol to give a final concentration of 40 mM. The ethanolic lipid solution was then injected into 10 mM MOPS, 150 mM NaCl, pH 7.2 buffer containing 5 mM EDTA, while vortexing to give a final concentration of 0.43 mM total lipid in the buffer. Melittin was incorporated into membranes by adding a small aliquot containing 3.2 nmol of melittin from a stock solution in water to the pre-formed vesicles and mixed well to give membranes containing 0.5% melittin. The lipid composition of these samples were as follows: (i) DOPC (85%) and 5- (or 12)-PC (15%), (ii) DOPC (65%), 5- (or 12)-PC (15%) and cholesterol (20%) and (iii) DOPC (45%), 5- (or 12)-PC (15%) and cholesterol (40%). Duplicate samples were prepared in each case except for samples lacking the quencher.
(5- or 12-PC) where triplicates were prepared. Background samples lacking the fluorophore (melittin) were prepared in all experiments, and their fluorescence intensity was subtracted from the respective sample fluorescence intensity. Samples were kept in the dark for 12 h before measuring fluorescence.

**Steady State Fluorescence Measurements**

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes as described earlier (see Chapter 2, Section 2.2). Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated from the Eq. 2.1 (Chapter 2). All experiments were done with multiple sets of samples and average values of polarization are shown in Table 5.1.

**Time-Resolved Fluorescence Measurements**

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode as described in Chapter 2 (Section 2.2). All experiments were performed using excitation and emission slits with a nominal bandpass of 4 nm or less. Intensity decay curves so obtained were fitted as a sum of exponential terms using the Eq. 2.2 (Chapter 2). The decay parameters were recovered as discussed in Chapter 2 (Section 2.2). Mean (average) lifetimes $<\tau>$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the Eq. 2.3 (Chapter 2).
Fluorescence Quenching Measurements

Acrylamide quenching experiments of melittin tryptophan fluorescence in membranes of varying degrees of unsaturation were carried out as described in Chapter 3 (Section 3.2). Samples were kept in dark for 1 h before measuring fluorescence. The excitation wavelength used was 295 nm and emission was monitored at the fluorescence emission maximum of melittin in the given membrane system. Corrections for inner filter effect were made using the Eq. 3.1 (Chapter 3). Quenching data were analyzed by using the Eq. 3.2 (Chapter 3).

Energy Transfer Measurements

All experiments were done using SUVs containing 1280 nmol of DOPC with varying amounts of DHE (0-4 mol%) and 1 mol% of melittin. The tryptophan residue of melittin served as the donor while DHE was used as the acceptor. The energy transfer efficiencies were calculated using the equation (Lakowicz, 1999):

\[ E = (1 - F/F_0) \] (5.1)

where \( E \) is the efficiency of energy transfer and \( F \) and \( F_0 \) are fluorescence intensities of the donor (tryptophan of melittin) in the presence and absence of the acceptor (DHE), respectively. The quantum yield \( (Q_D) \) of membrane-bound melittin was determined using the equation (Parker and Rees, 1960; Chen, 1965):

\[ Q_D = Q_S (F_D/F_S) (A_S/A_D) (n_D^2/n_S^2) \] (5.2)

where the subscripts ‘S’ and ‘D’ refer to the reference standard and the donor, respectively. \( F \) is the wavenumber-integrated area of the corrected emission at constant slit openings, \( A \) is the absorbance at the excitation wavelength (less than 0.1 to avoid inner filter effect); and \( n \) is the refractive index of the medium in which the standard or the sample is placed. Both L-tryptophan \([Q_S = 0.13 \text{ in water (Eftink, 1991c)}]\) and NATA \([Q_S = 0.14 \text{ in water (Szabo and Rayner, 1980)}]\) were used as reference standards and the internal consistency of the results was checked by measuring the quantum yield of one
standard with respect to the other. Solutions were freshly prepared and degassed by bubbling high purity nitrogen before use. Refractive index was measured using DUR refractometer (Schmidt-Haensch, Germany).

**CD Measurements**

CD measurements were carried out at room temperature (23 °C) on a JASCO J-715 spectropolarimeter as discussed in Chapter 2 (section 2.2).

**Assay for the Permeabilization of Lipid Vesicles**

The assay for the permeabilization of lipid vesicles was carried out as described in Chapter 4 (section 4.2) using SUVs of DOPC containing different amounts of cholesterol.

### 5.3. RESULTS

**Cholesterol Inhibits Melittin-induced Lysis**

The effect of cholesterol on the lytic activity of melittin is shown in Figure 5.1. The figure shows the release of calcein entrapped in vesicles composed of DOPC with increasing concentration of cholesterol induced by melittin. Initially, a low background fluorescence ($I_b$) is observed in all cases since the high concentration (70 mM) of calcein resulted in self quenching of fluorescence. Upon addition of melittin, the entrapped calcein is released into the buffer due to lysis induced by melittin. This leads to the dilution of the dye which gives rise to increased fluorescence ($I_f$). The extent of increase in fluorescence intensity is a measure of the lytic power of melittin in a given membrane environment. Relative lytic efficiencies are determined by complete disruption of the vesicle membranes with Triton X-100 which corresponds to the total fluorescence ($I_T$). Since cholesterol modifies the physical properties of the bilayer (Yeagle, 1985), we examined whether these changes influence the lytic ability of melittin.
Figure 5.1. Release of entrapped calcein induced by melittin in DOPC vesicles containing (a) 0, (b) 10, and (c) 30 mol% cholesterol. $I_B$ is the background (self-quenched) intensity of calcein encapsulated in vesicles, while $I_F$ represents the enhanced fluorescence intensity resulting from dilution of the dye in the medium caused by melittin-induced release of entrapped calcein. $I_T$ is the total fluorescence intensity after complete permeabilization is achieved upon addition of Triton X-100. Lipid concentration was 41.9 μM and melittin to lipid ratio was 1:50 (——), 1:75 (−−−−−−), 1:100 (⋯⋯), and 1:400 (−−−−) (mol/mol). See Materials and Methods for other details.

The percentage of calcein release was calculated according to Eq. 4.1 and is shown in Figure 5.2. As is apparent from Figures 5.1 and 5.2, the lytic efficiency of melittin increases with increasing peptide concentration, i.e., with decreasing lipid/peptide ratio (mol/mol) in all cases, irrespective of the presence of cholesterol in the membrane. More importantly, the lytic efficiencies of melittin appear to be dependent on the composition of the membrane. Our results show that the presence of cholesterol in the membrane inhibits the lytic power of melittin and the inhibition is enhanced with increasing cholesterol concentration. This is in agreement with earlier results in which it was shown that the presence of cholesterol inhibits melittin-induced lysis (Benachir et al., 1997).
Figure 5.2. Calcein release as a function of melittin/lipid (mol/mol) ratio for DOPC vesicles containing 0 (O), 10 (●), 20 (□), 30 (■) and 40 (△) mol% cholesterol. All other conditions are as in Figure 5.1. See Materials and Methods for other details.

**Binding of Melittin to Membranes Containing Cholesterol**

Since the presence of cholesterol in the membrane could affect the binding (and hence lytic activity) of melittin to membranes, we monitored the binding of melittin to DOPC membranes containing varying amounts of cholesterol utilizing the intrinsic fluorescence of the sole tryptophan residue in melittin. The binding of melittin to membranes can be quantitated by the blue shift of emission maximum (336 nm) upon binding to the lipid vesicles which is due to the change in the polarity of the surrounding medium since melittin in buffer exhibits an emission maximum at 352 nm. The increase in fluorescence intensity ratio of $F_{336}/F_{352}$ therefore represents the fraction of membrane-bound melittin. Figure 5.3a shows the binding curve for melittin binding to DOPC vesicles monitored this way. The binding of melittin to DOPC vesicles is efficient and reaches a plateau at a lipid/peptide ratio of ~50 (mol/mol). The binding becomes less efficient with incorporation of increasing amounts of cholesterol in the membrane. Thus,
with DOPC vesicles containing 40 mol% cholesterol, complete binding of melittin is only achieved at a lipid/peptide ratio of ~200 (mol/mol). This suggests that the presence of cholesterol reduces the binding of melittin to the DOPC vesicles. This is further supported by measurements of fluorescence polarization as a function of increasing lipid/melittin ratio (mol/mol) (see Figure 5.3b). We therefore chose conditions for our experiments in such a way so as to ensure that melittin is completely membrane-bound and there is no ground state heterogeneity.

Figure 5.3. Binding of melittin to DOPC vesicles containing different amounts of cholesterol measured by changes in melittin fluorescence: (a) the ratio of fluorescence intensity monitored at 336 and 352 nm; and (b) fluorescence polarization plotted as a function of total lipid/melittin ratio (mol/mol) for vesicles containing 0 (●), 10 (O), and 40 (■) mol% cholesterol. The excitation wavelength used was 280 nm and fluorescence polarization was monitored at 336 nm. The concentration of melittin was 4.26 μM in all cases. See Materials and Methods for other details.
Fluorescence of Membrane-Bound Melittin

The fluorescence emission spectra of melittin in DOPC vesicles containing different amounts of cholesterol are shown in Figure 5.4a. The maximum of fluorescence emission of melittin in DOPC vesicles is 336 nm, as has been reported previously (Ghosh et al., 1997). The fluorescence emission maximum is slightly shifted toward longer wavelength (338 nm) in the presence of high amounts (40 mol%) of cholesterol (see Table 5.1). This is accompanied by a marked reduction (36%) in peak fluorescence intensity (see Figure 5.4b). The reduction in fluorescence intensity could possibly be due to both specific and general bilayer effects induced by cholesterol. The specific effect could be due to the close proximity of cholesterol to melittin tryptophan which could imply specific interaction between melittin and cholesterol. On the other hand,
tryptophan fluorescence intensity is known to be sensitive to the presence of water in its immediate environment (Ladokhin, 2000). The decrease in the fluorescence intensity as well as the red shift of the emission maximum could therefore be due to increased water penetration in the interfacial region (where the tryptophan residue in membrane-bound melittin is localized (Ghosh et al., 1997)) of the membrane induced by cholesterol (Subczynski et al., 1994). Control experiments with TOE, a model tryptophan analogue for membrane-bound tryptophans characterized by an interfacially localized tryptophan (Chattopadhyay et al., 1997), showed that reduction in fluorescence intensity observed with membrane-bound melittin is not solely due to general bilayer effects.

Table 5.1. Fluorescence Emission Characteristics of Melittin in DOPC Membranes Containing Cholesterol

<table>
<thead>
<tr>
<th>Cholesterol (mol%)</th>
<th>Fluorescence Emission Maximum (nm)</th>
<th>REES (nm)</th>
<th>Fluorescence Polarization$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>336</td>
<td>6</td>
<td>0.132 ± 0.001</td>
</tr>
<tr>
<td>10</td>
<td>336</td>
<td>6</td>
<td>0.133 ± 0.002</td>
</tr>
<tr>
<td>20</td>
<td>337</td>
<td>6</td>
<td>0.132 ± 0.001</td>
</tr>
<tr>
<td>30</td>
<td>337</td>
<td>5</td>
<td>0.133 ± 0.002</td>
</tr>
<tr>
<td>40</td>
<td>338</td>
<td>5</td>
<td>0.127 ± 0.001</td>
</tr>
</tbody>
</table>

$^a$Calculated using Eq. 2.1. The polarization value shown represents mean ± standard error of at least three independent measurements. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 8.53 μM. See Materials and Methods for other details.
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REES and Fluorescence Polarization of Membrane-Bound Melittin

The magnitude of REES of the tryptophan residue of membrane-bound melittin with increasing amounts of cholesterol is shown in Table 5.1. As the excitation wavelength is changed from 280 to 307 nm, the maximum of emission wavelength the melittin tryptophan is shifted from 336 to 342 nm in case of vesicles containing only DOPC and DOPC/10 mol% cholesterol, and from 337 to 343 nm for DOPC vesicles containing 20 mol% cholesterol. In all these cases, the magnitude of REES corresponds to 6 nm. It is possible that there could be further red shift if excitation is carried out beyond 307 nm. We found it difficult to work in this wavelength range due to low signal to noise ratio and artifacts due to the solvent Raman peak that sometimes remained even after background subtraction. Such dependence of the emission maximum on excitation wavelength is characteristic of REES. This implies that the tryptophan residue in melittin is localized in a motionally restricted region of the membrane in these cases. This is consistent with the interfacial localization of the melittin tryptophan when bound to membranes (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997). Interestingly, the magnitude of REES does not appear to be critically dependent on the cholesterol content of the membrane (see Table 5.1). Melittin in membranes containing higher amounts (>20 mol%) of cholesterol exhibits a REES of 5 nm possibly due to the fact that the effect of cholesterol is more pronounced in the hydrophobic region of the membrane (Yeagle, 1985) than in the interfacial region where the tryptophan residue of melittin is localized.

The steady state fluorescence polarization of melittin in DOPC vesicles containing increasing amounts of cholesterol is shown in Table 5.1. These values are representative of motionally restricted tryptophan environments (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997) and do not change with increasing cholesterol concentrations up to 30 mol%. A small (~4%) reduction in polarization is observed in
DOPC vesicles containing 40 mol% cholesterol, probably due to change in headgroup packing induced by cholesterol (Yeagle, 1985) which could increase the rotational mobility of the tryptophan residue. In addition, fluorescence polarization is also known to be dependent on excitation and emission wavelengths in motionally restricted media (Mukherjee and Chattopadhyay, 1995). We measured polarization changes of melittin bound to DOPC and DOPC/cholesterol vesicles as a function of excitation and emission wavelengths. Our results show that membrane-bound melittin exhibits wavelength-dependent changes in polarization for both excitation and emission. This reinforces that melittin is localized in a motionally restricted interfacial region of the membrane in these cases (data not shown).

**Time-Resolved Fluorescence Measurements of Membrane-Bound Melittin**

A typical decay profile of tryptophan residue of membrane-bound melittin with its biexponential fitting and the statistical parameters used to check the goodness of the fit is shown in Figure 5.5.

![Figure 5.5](image.png)

**Figure 5.5.** Time-resolved fluorescence intensity decay of melittin in DOPC vesicles. Excitation wavelength was at 297 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 336 nm. The sharp peak on the left is the lamp profile. The relatively broad peak on the right is the decay profile, fitted to a biexponential function. The two lower plots show the weighted residuals and the autocorrelated function of the weighted residuals. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 8.53 μM in all cases. See Materials and Methods for other details.
The fluorescence lifetimes of melittin bound to membranes containing varying amounts of cholesterol are shown in Table 5.2.

Table 5.2. Fluorescence Lifetimes of Melittin in DOPC Membranes Containing Cholesterol

<table>
<thead>
<tr>
<th>Cholesterol (mol%)</th>
<th>( \alpha_1 )</th>
<th>( \tau_1 ) (ns)</th>
<th>( \alpha_2 )</th>
<th>( \tau_2 ) (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.89</td>
<td>0.64</td>
<td>0.11</td>
<td>3.24</td>
</tr>
<tr>
<td>10</td>
<td>0.92</td>
<td>0.67</td>
<td>0.08</td>
<td>3.48</td>
</tr>
<tr>
<td>20</td>
<td>0.92</td>
<td>0.83</td>
<td>0.08</td>
<td>3.54</td>
</tr>
<tr>
<td>30</td>
<td>0.90</td>
<td>0.64</td>
<td>0.10</td>
<td>3.08</td>
</tr>
<tr>
<td>40</td>
<td>0.92</td>
<td>0.60</td>
<td>0.08</td>
<td>2.99</td>
</tr>
</tbody>
</table>

*a*Calculated using Eq. 2.3. The excitation wavelength was 297 nm; emission was monitored at 336 nm. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 8.53 \( \mu \)M. See Materials and Methods for other details.

As seen from the table, all fluorescence decays could be fitted well with a biexponential function. The mean fluorescence lifetimes were calculated using Eq. 2.3 (Chapter 2) and are shown in Figure 5.6. As shown in the figure, there is a progressive decrease (amounting to ~20% in case of DOPC vesicles containing 40 mol% cholesterol) in the mean fluorescence lifetime of the tryptophan residue of membrane-bound melittin with increasing cholesterol concentration. Since an increase in polarity of the tryptophan environment is known to reduce the lifetime of tryptophans (Kirby and Steiner, 1970), the decrease in the fluorescence lifetime could therefore possibly be due to increased water penetration in the interfacial region of the membrane induced by cholesterol (Subczynski *et al.*, 1994; Stubbs *et al.*, 1995). This is consistent with our earlier
observation of reduction in fluorescence intensity of membrane-bound melittin with increasing amounts of cholesterol in the membrane (see Figure 5.4).

Figure 5.6. Mean fluorescence lifetime of melittin in DOPC vesicles containing increasing concentration of cholesterol. The excitation wavelength used was 297 nm, and emission was monitored at 336 nm. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 8.53 μM in all cases. Mean lifetimes were calculated from Table 5.2 using Eq. 2.3. See Materials and Methods for other details.

Acrylamide Quenching of Melittin Tryptophan Fluorescence

Figure 5.7 shows the Stern-Volmer plots of acrylamide quenching of the tryptophan residue in membrane-bound melittin in DOPC vesicles of varying cholesterol content. The slope of such a plot, Stern-Volmer constant ($K_{SV}$), is related to the degree of exposure (accessibility) of the melittin tryptophan to acrylamide, which is a water soluble quencher. In general, the higher the slope, the greater the degree of exposure, assuming that there is not a large difference in fluorescence lifetime. The quenching parameter ($K_{SV}$) obtained by analyzing the Stern-Volmer plots is shown in Table 5.3. The $K_{SV}$ value of melittin in DOPC vesicles was found to be 2.1 M$^{-1}$. Incorporation of cholesterol to DOPC vesicles leads to an apparent increase in the accessibility of the melittin...
tryptophan as evident from an increase in $K_{SV}$ (see Table 5.3). This suggests that the tryptophan residue in membrane-bound melittin is more exposed (relatively shallow) in membranes containing cholesterol.

![Graph showing Stern-Volmer analysis of acrylamide quenching of melittin fluorescence in DOPC vesicles containing 0 (●), 20 (○), and 40 (■) mol% cholesterol. Fo is the fluorescence in the absence of quencher, and F is the corrected fluorescence in the presence of quencher. The excitation wavelength was 295 nm and emission was monitored at 336 nm in all cases. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 1.07 μM in all cases. See Experimental Procedures for other details.]

The interpretation of $K_{SV}$ values is, however, complicated due to its intrinsic dependence on fluorescence lifetime (see Eq. 3.2 in Chapter 3). The bimolecular quenching constant ($k_q$) is therefore a more accurate measure of the degree of exposure since it takes into account the differences in fluorescence lifetime. The $k_q$ values, calculated using mean fluorescence lifetimes from Figure 5.6 and Eq. 3.2, are shown in Table 5.3. The $k_q$ values are in overall agreement with $K_{SV}$ values implying that the conclusions derived by the analysis of Stern-Volmer constants are not influenced by changes in lifetime. The reason for higher accessibility of the tryptophan in membranes containing cholesterol could be due to increased permeability to small molecules by
cholesterol (as shown above by increased water penetration). In addition, increased accessibility could also be due to any change in the tryptophan location (depth) in membranes containing cholesterol. Analysis of membrane penetration depth confirms the latter possibility (see below).

Table 5.3. Acrylamide Quenching of Tryptophan Fluorescence of Membrane-bound Melittin

<table>
<thead>
<tr>
<th>Cholesterol&lt;sup&gt;a&lt;/sup&gt; (mol%)</th>
<th>$K_{SV}$&lt;sup&gt;b&lt;/sup&gt; $(M^{-1})$</th>
<th>$k_q (x 10^{-9})$&lt;sup&gt;c&lt;/sup&gt; $(M^{-1}s^{-1})$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2.1 ± 0.15</td>
<td>1.28</td>
</tr>
<tr>
<td>20</td>
<td>2.4 ± 0.27</td>
<td>1.54</td>
</tr>
<tr>
<td>40</td>
<td>3.3 ± 0.10</td>
<td>2.48</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 1.07 μM in all cases.

<sup>b</sup>Calculated using Eq. 3.2. The quenching parameter shown represents mean ± standard error of at least three independent measurements. See Materials and Methods for other details.

<sup>c</sup>Calculated using mean fluorescence lifetimes from Table 5.2 and using Eq. 3.2. See Materials and Methods for other details.

Cholesterol Influences Tryptophan Depth in Membrane-Bound Melittin

Membrane penetration depth represents an important parameter in the study of membrane structure and organization (Chattopadhyay, 1992; London and Ladokhin, 2002). Knowledge of the precise depth of a membrane embedded group or molecule often helps define the conformation and topology of membrane probes and proteins. In addition, properties such as polarity, fluidity, segmental motion, ability to form hydrogen
bonds and the extent of solvent penetration are known to vary in a depth dependent manner. In order to gain a better understanding of the organization and conformation of membrane-bound melittin in presence of cholesterol, penetration depths of the sole tryptophan residue of melittin in membranes were determined. Depth of the tryptophan residue was calculated by the parallax method (Chattopadhyay and London, 1987) using the equation:

\[
z_{cF} = L_{c1} + \left\{ \left( \frac{-1}{\pi C} \right) \ln \left( \frac{F_1}{F_2} \right) - \frac{L_{21}^2}{2} \right\}^{1/2}
\]

where \(z_{cF} = \) the depth of the fluorophore from the center of the bilayer, \(L_{c1} = \) the distance of the center of the bilayer from the shallow quencher (5-PC in this case), \(L_{21} = \) the difference in depth between the two quenchers (i.e., the transverse distance between the shallow and the deep quencher), and \(C = \) the two-dimensional quencher concentration in the plane of the membrane (molecules/Å²). Here \(F_1/F_2\) is the ratio of \(F_1/F_0\) and \(F_2/F_0\) in which \(F_1\) and \(F_2\) are fluorescence intensities in the presence of the shallow (5-PC) and deep quencher (12-PC), respectively, both at the same quencher concentration \(C; F_0\) is the fluorescence intensity in the absence of any quencher. All the bilayer parameters used were the same as described previously (Chattopadhyay and London, 1987). The depths of penetration of the tryptophan residue for melittin bound to DOPC vesicles containing varying amounts of cholesterol are shown in Table 5.4. The depth of penetration of the tryptophan residue for melittin bound to DOPC vesicles are found to be 10.6 Å from the center of the bilayer, in agreement with our previous results (Ghosh et al., 1997), and with the value determined by fluorescence quenching using brominated phospholipids and the distribution analysis method (Ladokhin and Holloway, 1995). Interestingly, the depth of penetration of the tryptophan residue of membrane-bound melittin changes to 17.4 and 18.2 Å in DOPC vesicles containing 20 and 40 mol% cholesterol, respectively (see Table 5.4). This indicates that the tryptophan is localized at a relatively shallow depth in membranes containing cholesterol. This observation is consistent with the relatively red shifted fluorescence emission maximum of membrane-bound melittin in
presence of cholesterol (see Table 5.1) and the acrylamide quenching results (Table 5.3) in which it was shown that the tryptophan is more exposed to the aqueous phase in DOPC vesicles containing cholesterol.

Table 5.4. Penetration Depth of the Tryptophan in Membrane-Bound Melittin by the Parallax Method

<table>
<thead>
<tr>
<th>Cholesterol(^a) (mol%)</th>
<th>Distance from the Center of the Bilayer, (z_{cF}) (Å)(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>10.6</td>
</tr>
<tr>
<td>20</td>
<td>17.4</td>
</tr>
<tr>
<td>40</td>
<td>18.2</td>
</tr>
</tbody>
</table>

\(^a\)Corrections were made for the altered concentrations of spin-labeled lipids (for lateral distribution) and the depths of the quenchers used in membranes containing cholesterol (Kaiser and London, 1998).

\(^b\)Depths were calculated from fluorescence quenchings obtained with samples containing 15 mol\% of 5-PC and 12-PC and using Eq. 5.3. Samples were excited at 280 nm, and emission was collected at 336 nm. The ratio of melittin to total lipid was 1:200 (mol/mol) and the concentration of melittin was 2.13 μM in all cases. See Materials and Methods for other details.

Secondary Structure of Membrane-bound Melittin

To investigate the effect of cholesterol on the secondary structure of melittin, far-UV CD spectroscopy of melittin was carried out in membranes containing various amounts of cholesterol. The CD spectra of melittin in buffer and when bound to membranes composed of varying concentrations of cholesterol are shown in Figure 5.8. Our results show that the secondary structure of membrane-bound melittin does not appear to be sensitive to the presence of membrane cholesterol.
Interaction of Melittin with Membrane Cholesterol: Resonance Energy Transfer with Dehydroergosterol

To explore whether there is any specific interaction of melittin with membrane cholesterol, energy transfer measurements were carried out using melittin tryptophan as the donor and dehydroergosterol (DHE) as the acceptor. DHE is a naturally occurring fluorescent analogue of cholesterol which is found in yeast and differs from cholesterol in having three additional double bonds and a methyl group. A number of reports have shown that DHE faithfully mimics natural cholesterol in biophysical, biochemical, and cell biological studies (Schroeder et al., 1995; Schroeder et al., 1991; Mukherjee et al., 1998). For example, up to 85% of the endogeneous sterol in cultured fibroblast cells could be replaced with DHE with no significant effect on growth property, membrane composition or activities of membrane enzymes (Schroeder et al., 1995; Schroeder et al., 1991).
Figure 5.9. Spectral overlap (shown as shaded area) between the corrected fluorescence emission spectrum of melittin (—) and the absorption spectrum of DHE (-- -- --) in DOPC vesicles. The excitation wavelength used for melittin was 280 nm. See Materials and Methods for other details.

Figure 5.9 shows that there is substantial spectral overlap between the emission spectrum of membrane-bound melittin tryptophan with the absorption spectrum of membrane-bound DHE which is an essential criterion for efficient energy transfer. This makes melittin and DHE a good donor and acceptor pair for energy transfer experiments. The distance (in Å) between the donor and acceptor ($R_o$) that results in 50% energy transfer efficiency can be calculated using the equation (Lakowicz, 1999):

$$R_o = 9.79 \times 10^3 \left(\frac{J \kappa^2 n^4}{Q_D n^4}\right)^{1/6}$$

(5.4)

where $J$ is the spectral overlap integral (in M$^{-1}$cm$^3$) between the emission spectrum of the donor and the absorption spectrum of the acceptor, $\kappa^2$ is the dipole-dipole orientation factor for the donor and acceptor, $Q_D$ is the quantum yield of the donor in the absence of acceptor, and $n$ is the refractive index of the medium. The fluorescence quantum yield ($Q_D$) of membrane-bound melittin was determined to be 0.06. $J$ was determined from the fluorescence emission of melittin and the absorption of DHE (Figure 5.9) by using the equation (Lakowicz, 1999):
where $F_D(\lambda)$ is the fractional fluorescence intensity of the donor at wavelength $\lambda$, $e_A(\lambda)$ is the molar extinction coefficient of the acceptor at wavelength $\lambda$. The value of $J$ was calculated using a previously written program (Kumar and Chatterji, 1990) and was found to be $2.3 \times 10^{-15} \text{ M}^{-1} \text{cm}^3$. The value of $\kappa^2$, which is a function of the relative orientation of donor emission and acceptor absorption moments, was assumed to be $2/3$, the dynamic average of random orientation of the donor and acceptor. This value of $\kappa^2$ implies that there is rapid, isotropic reorientation of the donor and acceptor moments during the donor emission lifetime. This assumption could be rationalized for membrane-bound donors and acceptors (Estep and Thompson, 1979). $R_o$ for melittin-DHE pair was calculated using Eq. 5.4 and was found to be 16.0 Å.

The extent of energy transfer can be quantitated by determining the extent of tryptophan quenching. The dependence of efficiency of energy transfer on surface densities of the acceptor (DHE) for various $R_o$ is shown in Figure 5.10. The experimentally obtained energy transfer efficiencies were then compared with calculated efficiencies of energy transfer for a random distribution of donors and acceptors in a two-dimensional plane using the formalism developed earlier (Fung and Stryer, 1978). The series of theoretical plots of energy transfer efficiency vs. acceptor surface density in the membrane, for pairs of donors and acceptors with varying $R_o$ and randomly distributed in the plane of the bilayer, were generated by numerical integration of the equation (Fung and Stryer, 1978):

$$J = \int_0^\infty F_D(\lambda) e_A(\lambda) \lambda^4 d\lambda$$

(5.5)
Figure 5.10. Efficiency of energy transfer from melittin to DHE as a function of DHE/phospholipid ratio. The figure shows a series of theoretical plots (solid lines) of energy transfer at various acceptor densities in the membrane bilayer for pairs of donors and acceptors with varying $R_0$ ($16 \text{ Å} > R_0 > 30 \text{ Å}$) assuming random distribution of donors and acceptors in the plane of the bilayer. These plots were generated according to the formalism developed earlier (Fung and Stryer, 1978). The experimentally determined energy transfer efficiencies for melittin/DHE pair (●), calculated from tryptophan (donor) quenching (by DHE) using eq 6, are superimposed on the theoretical plots. $R_0$ for the melittin/DHE pair was calculated to be 16 Å (see Results). A $R_0$ value of 24 Å gave the best fit to the experimental data. The measured efficiency of energy transfer thus substantially exceeded the calculated value of $R_0$ (16 Å). The donor (melittin) concentration was fixed at 1 mol% of the total amount of lipid used which corresponds to a ratio of 1:100 (mol/mol) melittin to total lipid (DOPC and DHE). Acceptor density was varied from 0 to 4 mol% of the total lipid used. Excitation wavelength used was 280 nm and emission was collected at 336 nm. See Materials and Methods for other details.
Chapter 5

\[
E = 1 - (1/\tau_0) \int_0^\infty [F(t)/F(0)] dt
\]  

(5.6)

where \( \tau_0 \) is the excited state lifetime of the donor in the absence of acceptor, and \( F(t) \) is the fluorescence intensity of the donor in an infinite plane at time \( t \) and is given by

\[
F(t) = F(0) e^{-t/\tau_0} e^{-\sigma S(t)}
\]  

(5.7)

where \( e^{-\sigma S(t)} \) is the energy transfer term, \( F(0) \) is the initial fluorescence intensity, \( \sigma \) is the surface density of the acceptor (acceptor per phospholipid), and \( S(t) \) is given by

\[
S(t) = \int_0^\infty \left[ 1 - e^{-\left(t/\tau_0\right)(Ro/r)^{6}} \right] 2\pi r dr
\]  

(5.8)

where \( a \) is the distance of closest approach of donor and acceptor (9.9 Å for melittin-DHE pair), and the expression \( 2\pi r dr \) represents the probability of finding an acceptor within a distance \( r \) from the donor in two dimensions and \( R_o \) is the distance (in Å) between the donor and acceptor at which 50% energy transfer takes place. The dependence of energy transfer efficiency on the surface density of the acceptor for values of \( R_o \) ranging from 16 to 30 Å, calculated by numerical integration of equations 14 and 15 and assuming random distribution of donors and acceptors in the plane of the bilayer (Fung and Stryer, 1978), is shown in Figure 5.10. The experimental data points were superimposed on the theoretical curves and the experimental data fitted best to a \( R_o \) of 24 Å. The measured efficiency of energy transfer thus substantially exceeded the calculated value of \( R_o \) (16.0 Å). This would be expected if there is a preferential association of the donor and acceptor in the membrane (Lakowicz, 1999). These results therefore suggest that there is a close molecular interaction between melittin and dehydroergosterol at low sterol concentrations.
5.4. DISCUSSION

Cholesterol is a ubiquitous membrane component of eukaryotic organisms (Bloch, 1983) and has been reported to be necessary for the functional activity of many membrane proteins and receptors (Yeagle, 1985; Gimpl et al., 1997). Cholesterol affects the physical properties of model and biological membranes (Yeagle, 1985). In addition, it increases the order of the acyl chains in fluid membranes and as a consequence, leads to tighter acyl chain packing, a thicker bilayer and a reduced lipid surface area (Yeagle, 1985; Nezil and Bloom, 1992).

The distribution of cholesterol in the membranes of the cellular organelles is not uniform and the cholesterol content in eukaryotic plasma membranes is usually rather high (e.g., ~45 mol% in human erythrocytes) whereas the internal organelle membranes have much less amounts of cholesterol (Yeagle, 1985). Cholesterol is often laterally associated with sphingolipids in plasma membranes to form lateral membrane heterogeneities called membrane microdomains or rafts which have been implicated in membrane traffic and cell signaling in mammalian cells (Simons and Ikonen, 2000; Simons and Ikonen, 1997; Simons and Toomre, 2000). In view of the importance of the cholesterol in relation to membrane domains, the interaction of cholesterol with membrane proteins and peptides assumes significance with respect to their organization and function. Interestingly, these microdomains act as concentration platforms to facilitate entry of pore-forming toxins (Abrami and van der Goot, 1999). In addition, it has been proposed that cholesterol could act as a target for many pore-forming toxins (de Kruijff, 1990).

We have monitored the organization and dynamics of the hemolytic peptide melittin in membranes containing cholesterol by utilizing the intrinsic fluorescence properties of its functionally important sole tryptophan residue and CD spectroscopy. The significance of this study lies on the fact that the natural target for melittin is
erythrocyte membrane which contains high amounts of cholesterol. Our results show that the presence of cholesterol inhibits melittin-induced leakage of lipid vesicles and the extent of inhibition appears to be dependent on the concentration of membrane cholesterol. A similar inhibition by cholesterol has been reported for melittin-induced leakage from POPC vesicles (Benachir et al., 1997). In addition, cholesterol has been shown to inhibit the lytic activity of mastoparan, a wasp venom toxin with a strong structural resemblance to melittin (Katsu et al., 1990), and Gardnerella vaginalis cytolysin (Cauci et al., 1993). Tight lipid packing and increased deformation energy (see later) induced by cholesterol could account for such effects. Further, our results show that the presence of increasing amounts of cholesterol strongly reduces the binding of melittin to the lipid vesicles i.e., three fold excess lipid is needed in order for melittin to be completely bound to DOPC membranes in the presence of cholesterol (Figure 5.3). The reduced lytic effect of melittin in the presence of cholesterol could also be due to reduced binding of the peptide to the bilayer.

The fluorescence parameters such as intensity, emission maximum, and lifetime of membrane-bound melittin imply a change in polarity in the immediate vicinity of the tryptophan probably due to increased water penetration in presence of cholesterol. This is supported by results from fluorescence quenching experiments using acrylamide as the quencher. Tryptophan penetration depths for membrane-bound melittin were analyzed by the parallax method. Since melittin has a single tryptophan, the interpretation of depth values obtained is devoid of complications that often arise for depth analysis of multi tryptophan proteins (Chattopadhyay and McNamee, 1991). Our results show that the depth of penetration of the tryptophan residue for melittin bound to DOPC vesicles to be 10.6 Å from the center of the bilayer. The depth of penetration of the tryptophan residue of membrane-bound melittin changes to 17.4 and 18.2 Å in DOPC vesicles containing 20 and 40 mol% cholesterol suggesting that the tryptophan is localized at a relatively shallow depth in membranes containing cholesterol (see Figure 5.11). Similar reduction
in the depth of penetration has been reported for peptides such as temporin L (Zhao and Kinnunen, 2002) and amphipathic class A peptide (Gorbenko et al., 2003). This is probably due to the increase in elastic modulus (and hence increase in bilayer deformation energy) by several folds in the presence of high amounts of cholesterol (Needham, 1995).

Figure 5.11. Schematic representation of the membrane bilayer showing the orientation and location of membrane-bound melittin in the absence (upper panel) and presence (lower panel) of cholesterol. The small v-shaped structures represent membrane associated water molecules. The sole tryptophan residue (W) of melittin is shown to be localized in the interfacial region of membranes in both cases. However, melittin tryptophan is localized at a relatively shallow depth in membranes containing cholesterol (Table 5.4). The specific interaction of melittin with membrane cholesterol is shown as cluster of cholesterol around melittin tryptophan (Figure 5.10). In addition, the global bilayer effects of cholesterol is indicated by a change in headgroup packing and an increase in water penetration in the immediate vicinity of the tryptophan residue. The dotted line indicates the center of the bilayer. See text for other details.
Resonance energy transfer experiments were carried out using melittin/DHE as the donor/acceptor pair to investigate the possibility of specific interaction of melittin with cholesterol. Analysis of energy transfer results indicates that DHE is not randomly distributed in the membrane and is rather preferentially localized around the tryptophan residue of membrane-bound melittin, even at the low concentrations used (1-4 mol%). Since DHE is a naturally occurring fluorescent cholesterol analogue which has been shown to faithfully mimic cholesterol, we interpret this result as specific interaction of melittin with membrane cholesterol (see Figure 5.11). Interestingly, it has been shown earlier by us (Mukherjee and Chattopadhyay, 1996; Rukmini et al., 2001) and others (Loura and Prieto, 1997) that both cholesterol and DHE can exhibit local order and organization even at such low concentrations.

The existence of membrane domains in human erythrocytes has previously been reported (Rodgers and Glaser, 1991; Samuel et al., 2001). While the exact nature of these domains is not still resolved, high amounts of cholesterol in the erythrocyte membrane could induce raft-like membrane microdomains (Samuel et al., 2001), which may have sphingolipids as the other lipid component. The distribution of lipids in the erythrocyte membrane therefore is not homogenous. The fact that cholesterol inhibits the lytic activity of melittin, whose target is the erythrocyte membrane with high amounts of cholesterol, raises an interesting scenario. It is interesting to note that the interaction of another hemolytic protein, the earthworm hemolysin eiseniapore, with lipid membranes has been shown to require spingolipids (Lange et al., 1997). Interestingly, the presence of cholesterol enhances the hemolytic activity of eiseniapore toward sphingomyelin-containing vesicles probably due to its specific interaction with sphingomyelin. In addition, it has been recently shown that cholesterol, along with cone shaped lipids, increases membrane permeabilization by the cholesterol-specific cytolysins (Zitzer et al., 2001). However, the membrane permeabilization is inefficient if only sterol is present. Although our study shows that cholesterol inhibits the lytic activity of melittin, the
possibility of preferential localization of melittin in cholesterol-sphingolipid-rich domains cannot be ruled out since cholesterol-sphingolipid-rich plasma membrane microdomains (rafts) has been reported to act as concentration platforms for pore-forming toxins (Abrami and van der Goot, 1999). It should be noted that the Nef protein of HIV-1, whose N-terminal region (Nef1-25) resembles melittin (Barnham et al., 1997), has been shown to associate with membrane rafts for its activity (Wang et al., 2000). Whether melittin requires a similar association with raft-like domains in erythrocytes represents an exciting possibility. In summary, our results are relevant in understanding the interaction of melittin with membranes in general, and interaction with cholesterol-containing membranes in particular.