Chapter 3

Effect of Micellar Charge on the Conformation and Dynamics of Melittin
3.1. INTRODUCTION

The net electrical charge of the biological membrane represents an important parameter in the organization, dynamics and function of the membrane. The electrostatic interfacial potential generated at the membrane-solution interface due to the membrane charge plays a crucial role in a variety of membrane associated-phenomena including activity of membrane proteins and receptors, ion binding and transport, ligand recognition, and interaction with other membranes (McLaughlin, 1989). It is well established that electrostatic interaction plays a crucial role in stabilizing lipid-protein interactions in membranes and in the process of membrane binding of proteins and peptides (McLaughlin, 1989; McLaughlin and Aderem, 1995). In addition, electrostatic forces are important in determining the transmembrane orientation and topology of membrane-spanning α-helices of integral membrane proteins (von Heijne, 1992). Several naturally occurring peptides have been used to characterize lipid-protein interactions in model membranes (Sitaram and Nagaraj, 1999). A particularly interesting peptide which has been widely used is the hemolytic bee venom peptide, melittin (Dempsey, 1990).

Melittin is known to interact selectively with negatively charged lipids (Dempsey, 1990; Ghosh et al., 1997; Beschiaschvili and Seelig, 1990; Kleinschmidt et al., 1997). The affinity of melittin for membranes composed of negatively charged lipids has been shown to be 100-fold greater than for zwitterionic lipids (Batenburg et al., 1988; Lee et al., 2001). Interestingly, the presence of negatively charged lipids in the membrane has been shown to inhibit membrane lysis by melittin and this inhibition is enhanced with increasing surface charge density (Ghosh et al., 1997; Benachir and Lafleur, 1995; Monette and Lafleur, 1995; Hincha and Crowe, 1996). We have previously monitored the microenvironment experienced by the sole tryptophan of melittin when bound to membranes utilizing the wavelength-selective fluorescence approach. Our results show
that the tryptophan residue is located in a motionally restricted region in the membrane (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997) and the tryptophan environment is modulated by surface charge of the membrane which could be related to the difference in the lytic activity of the peptide observed in membranes of varying charge (Ghosh et al., 1997). In this study, we have utilized micelles of various charge types to monitor the effect of varying surface charge on the organization and dynamics of micelle-bound melittin.

Detergents are soluble amphiphiles and above a critical concentration (strictly speaking, a narrow concentration range), known as the critical micelle concentration (CMC), self-associate to form thermodynamically stable, noncovalent aggregates called micelles (Tanford, 1978). The studies on micellar organization and dynamics assume special significance in light of the fact that the general principles underlying the formation of micelles are common to other related assemblies such as reverse micelles, bilayers, liposomes, and biological membranes (Tanford, 1978; Israelachvili et al., 1980; Tanford, 1980; Tanford, 1987). Micelles have also been applied as membrane mimetics to characterize membrane proteins and peptides (Franklin et al., 1994; Improta et al., 1994; Lenz et al., 1995; Sham et al., 2003). Further, micelles have been used as a model for the anesthetic action of certain pharmacological compounds (Desai et al., 1994) and for creating nanostructures (Laval et al., 1995).

Micelles are highly cooperative, organized molecular assemblies of amphiphiles and are dynamic in nature (Menger, 1979; Lindman and Wennerstrom, 1982). A direct consequence of such organized systems is the restriction imposed on the dynamics and mobility of their constituent structural units. Micelles offer certain inherent advantages in fluorescence studies over membranes since micelles are smaller and optically transparent, have well-defined sizes, and are relatively scatter-free. Further, micelles can be of any desired charge type and can adopt different shapes and internal packing, depending on the chemical structures of the constituent monomers and the ionic strength.
of the medium (Missel et al., 1982; Ikeda, 1984; Porte and Appel, 1984). Furthermore, structural transition can be induced in charged micelles at a given temperature by increasing the ionic strength of the medium or amphiphile concentration (Missel et al., 1982; Ikeda, 1984; Porte and Appel, 1984). The organization and dynamics of micellar environments, namely, the core, the interface, and the immediate layers of water on the interface, have been investigated using experimental (Rawat et al., 1997; Rawat and Chattopadhyay, 1999; Maiti et al., 1997; Saroja and Samanta, 1995; Sarkar et al., 1996) and theoretical (MacKerell, 1995) approaches. It is fairly well established now that practically all types of molecules have a surface-seeking tendency in micelles (due to very large surface area to volume ratio) and that the interfacial region is the preferred site for solubilization, even for hydrophobic molecules (Shobha et al., 1989).

We have shown earlier that the wavelength-selective fluorescence approach serves as a powerful tool to monitor organization and dynamics of a variety of micelles differing in charge and shape (Rawat et al., 1997; Rawat and Chattopadhyay, 1999; Raghuraman et al., 2004). In this study, we have monitored the change in the organization and dynamics of melittin bound to micelles of various charge types (see Figure 3.1) utilizing fluorescence and circular dichroism spectroscopy. Our study assumes significance in the overall context of the role of surface charge of membranes and membrane-mimetic media such as micelles in the organization and dynamics of membrane-active, amphiphilic peptides in general and melittin in particular.

3.2. MATERIALS AND METHODS

Materials

CTAB, SDS, CHAPS, and melittin were obtained from Sigma Chemical Co. (St. Louis, MO). To check for any residual phospholipase A₂ contamination in melittin,
phospholipase activity was assayed using $^{14}$C-labeled dioleoyl-sn-glycero-3-phosphocholine as described earlier (Argiolas and Pisano, 1983). No appreciable activity could be detected this way. Brij 35 was from Pierce (Rockford, IL) and DPH was purchased from Molecular Probes (Eugene, OR). Ultra pure grade acrylamide was from Gibco BRL (Rockville, MD). The purity of acrylamide was checked from its absorbance using its molar extinction coefficient ($\varepsilon$) of 0.23 M$^{-1}$cm$^{-1}$ at 295 nm and optical transparency beyond 310 nm (Eftink, 1991). Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. Solvents used were of spectroscopic grade. The concentration of melittin in aqueous solution was calculated from its molar absorption coefficient ($\varepsilon$) of 5,570 M$^{-1}$cm$^{-1}$ at 280 nm (Ghosh et al., 1997). The purity of the detergents were checked by measuring their CMC values and comparing with literature CMC. The CMC of detergents were determined fluorimetrically using a widely used method previously developed by one of us (Chattopadhyay and London, 1984) which utilizes enhancement of DPH fluorescence upon micellization.

Sample Preparation

Concentrations of detergents used were above the CMC of the respective detergents to ensure that they are in the micellar state. Melittin was added to micellar systems from an aqueous stock solution to give the peptide to detergent molar ratio of 1:500 (mol/mol) for CTAB and Brij 35, 1:1000 (mol/mol) for SDS, and 1:4000 (mol/mol) for CHAPS. The molar ratio of fluorophore to detergent was carefully chosen to give optimum signal to noise ratio with minimal perturbation to the micellar organization and negligible interprobe interactions. At such a low peptide to detergent molar ratio, not more than one peptide molecule would be present per micelle on an average which rules out any peptide aggregation effects, especially keeping in mind the aggregation number of ~10-160 for the detergents used (Bhairi, 2001). Background
samples were prepared the same way except that melittin was not added to them. Samples were equilibrated for 12 h in dark before measuring fluorescence. All experiments were done at room temperature (23 °C).

**Steady State Fluorescence Measurements**

Steady state fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements. All spectra were recorded using the correct spectrum mode. Background intensities of samples in which melittin was omitted were subtracted from each sample spectrum to cancel out any contribution due to the solvent Raman peak and other scattering artifacts. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ± 1 nm of the ones reported. Fluorescence polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated using Eq. 2.1 (Chapter 2). All experiments were done with multiple sets of samples and average values of polarization are shown in the figure.

**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 Eq. 2.2 (Chapter 2). The decay parameters were recovered as discussed in Chapter 2, Section 2.2. Mean (average) lifetimes for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using Eq. 2.3 (Chapter 2).
Acrylamide quenching experiments of melittin fluorescence in micelles and in aqueous solution were carried out by measurement of fluorescence intensity of melittin in various micellar systems in separate samples containing increasing concentrations of acrylamide taken from a freshly prepared stock solution (8 M) in water. Samples were incubated 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 micellar system. Corrections for inner filter effect were made using the following equation (Lakowicz, 1999):

$$F = F_{\text{obs}} \text{antilog} \left[ \frac{(A_{\text{ex}} + A_{\text{em}})}{2} \right]$$  \hspace{1cm} (3.1)

where $F$ is the corrected fluorescence intensity and $F_{\text{obs}}$ is the background subtracted fluorescence intensity of the sample. $A_{\text{ex}}$ and $A_{\text{em}}$ are the measured absorbance at the excitation and emission wavelengths. The absorbance of the samples was measured using a Hitachi U-2000 UV-visible absorption spectrophotometer. Quenching data were analyzed by fitting to the Stern-Volmer equation (Lakowicz, 1999):

$$\frac{F_\text{o}}{F} = 1 + K_{SV} [Q] = 1 + k_q \tau_o [Q]$$  \hspace{1cm} (3.2)

where $F_\text{o}$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, respectively, $[Q]$ is the molar quencher concentration and $K_{SV}$ is the Stern-Volmer quenching constant. The Stern-Volmer quenching constant $K_{SV}$ is equal to $k_q \tau_o$ where $k_q$ is the bimolecular quenching constant and $\tau_o$ is the lifetime of the fluorophore in the absence of quencher.
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.

3.3. RESULTS

Figure 3.1 shows the chemical structures of the detergents used. These detergents show diversity in chemical structure, charge, shape and size of the micelles they form when present in aqueous solutions at concentrations comparable to or higher than CMC (Bhairi, 2001). Among the charged detergents, CTAB is cationic while SDS is anionic. Both form large spherical micelles with aggregation numbers of 170 and 62, respectively. Brij 35 is a nonionic detergent with a very low CMC and a typical aggregation number of 40. CHAPS is a derivative of the naturally occurring bile salts. It is one of the most commonly used detergent in membrane biochemistry and is zwitterionic in nature (Hjelmeland, 1980; Chattopadhyay and Harikumar, 1996). It is mild and denaturing, and combines useful features of both the bile salt hydrophobic group and the N-alkyl sulfobetaine type polar group. It forms micelles with lateral association of monomers with a low aggregation number (~10).

For experiments involving melittin incorporated into micelles, we wanted to work in conditions in which melittin was bound to the micelles which would avoid problems in spectroscopic measurements due to ground state heterogeneity. Table 3.1 shows the fluorescence emission maxima and polarization of melittin incorporated into various types of micelles under a wide range of detergent to melittin ratio (mol/mol). These two fluorescence parameters are sensitive to binding of melittin to micelles. The table shows that these two fluorescence parameters are invariant over a large range of detergent to melittin ratio indicating that melittin is bound to micelles under these conditions. We
therefore chose to work in conditions where the peptide is bound to micelles. Thus, the peptide to detergent molar ratio used was 1:500 (mol/mol) for CTAB and Brij 35, 1:1000 (mol/mol) for SDS, and 1:4000 (mol/mol) for CHAPS.

![Chemical structures of the detergents used.](image)

**Figure 3.1.** Chemical structures of the detergents used.
Table 3.1. Binding of Melittin in Micelles of Different Charge Types

<table>
<thead>
<tr>
<th>Medium</th>
<th>Detergent to Melittin Ratio (mol/mol)</th>
<th>Emission Maximum (nm)</th>
<th>Fluorescence Polarizationa</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTAB</td>
<td>100-2500</td>
<td>349</td>
<td>0.035</td>
</tr>
<tr>
<td>CHAPS</td>
<td>500-5000</td>
<td>339</td>
<td>0.083</td>
</tr>
<tr>
<td>Brij 35</td>
<td>400-1000</td>
<td>338</td>
<td>0.097</td>
</tr>
<tr>
<td>SDS</td>
<td>100-2000</td>
<td>334</td>
<td>0.103</td>
</tr>
</tbody>
</table>

*Calculated using Eq. 2.1. The values shown are means of three independent experiments.

REES of Melittin in Micelles of Various Charge Types

The shifts in the maximum of fluorescence emission of the tryptophan residue of melittin when bound to micelles of SDS, Brij 35, and CHAPS as a function of excitation wavelength are shown in Figure 3.2. We have previously reported that the fluorescence emission maximum of melittin incorporated in model membranes of dioleoyl-sn-glycero-3-phosphocholine (DOPC), in which the localization of melittin has been shown to be interfacial is ~335 nm (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997). Figure 3.2 shows that upon excitation at 280 nm, the fluorescence emission maxima of melittin is found to be at 334, 338, 339, and 349 nm in micelles of SDS, Brij 35, CHAPS, and CTAB, respectively. The magnitude of these emission maxima suggests that the tryptophan residue of melittin is localized at the interfacial region of the micelles.
Figure 3.2. Effect of changing excitation wavelength on the wavelength of maximum emission of melittin in micelles of SDS (■), Brij 35 (∆), CHAPS (●) and CTAB (○). The ratio of melittin to detergent was 1:1000 (mol/mol) for micelles of SDS, 1:4000 (mol/mol) for micelles of CHAPS, and 1:500 (mol/mol) for Brij 35 and CTAB micelles. The concentration of melittin ranged from 1.6-16 μM. See Materials and Methods for other details.

As the excitation wavelength is changed from 280 to 307 nm, the emission maxima of micelle-bound melittin are shifted from 334 to 337 nm (in case of SDS micelles), 338 to 343 nm (Brij 35), and 339 to 345 nm (CHAPS), which correspond to a REES of 3-6 nm in each of these cases. Such dependence of the emission spectra on the excitation wavelength is characteristic of the red edge effect. Observation of this effect in micelles implies that melittin, when incorporated in these micelles, is in an environment where its mobility is considerably reduced. Since the tryptophan residue of melittin is localized in the interfacial region (see above), such a result would directly imply that this region of the micelle offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state fluorophore. Interestingly, the magnitude of REES is less for melittin in anionic SDS micelles (3 nm) compared to
REES obtained in zwitterionic CHAPS micelles (6 nm) indicating that REES is sensitive to micellar charge. This is in agreement with our previous observation that the presence of negatively charged lipids in membranes decreases the magnitude of REES in case of membrane-bound melittin (Ghosh et al., 1997).

Interestingly, the fluorescence emission maximum of melittin in positively charged CTAB micelles is found to be 349 nm which is similar to the fluorescence emission maximum of monomeric melittin in aqueous solution (Chattopadhyay and Rukmini, 1993; Ghosh et al., 1997). More importantly, as the excitation wavelength is changed from 280 to 307 nm, the emission maximum of melittin in CTAB micelles remain invariant at 349 nm irrespective of the excitation wavelength. Melittin therefore does not exhibit REES in CTAB micelles. Taken together, these observations suggest that melittin does not bind to cationic CTAB micelles due to electrostatic repulsion between the positively charged headgroups of CTAB micelles and melittin which is also positively charged (Dempsey, 1990; Bechinger, 1997). This is further supported by low fluorescence polarization values of melittin in presence of CTAB micelles (see below). The fluorescence emission maximum of melittin displays a 7 nm blue shift (i.e., the maximum is at 342 nm) when CTAB micelles are used in presence of 100 mM NaCl which helps screen the charge and therefore enhances binding (data not shown). However, even in this condition, melittin does not display REES implying lesser degree of immobilization probably due to inefficient binding.

Fluorescence Polarization of Micelle-bound Melittin

The fluorescence polarization of melittin in micelles of various charge types and in aqueous solutions is shown in Figure 3.3. The polarization value of melittin is low in aqueous solution since melittin is in a monomeric unordered state under this condition (Bello et al., 1982). Figure 3.3 shows that the fluorescence polarization of melittin in aqueous solution does not show any appreciable change in presence of 100 mM NaCl
ruling out any aggregation at this condition. On the other hand, the fluorescence polarization of melittin in micelles of SDS, Brij 35, and CHAPS is high indicating restriction to rotational mobility of melittin when bound to micelles. Interestingly and in accordance with our earlier observation (see above), the fluorescence polarization of melittin in CTAB micelles does not show much increase when compared to values obtained in aqueous solutions. This reinforces our earlier conclusion that cationic melittin does not bind to CTAB micelles which are positively charged due to unfavorable electrostatic interaction. This is further confirmed by an increase in polarization value of melittin in CTAB micelles in presence of NaCl which effectively screens the charge as shown in Figure 3.3.

![Figure 3.3](image)

**Figure. 3.3.** Fluorescence polarization of melittin in various aqueous and micellar systems. The excitation wavelength was 280 nm in all cases. Emission was monitored at the fluorescence emission maximum of melittin in each case (349 nm in aqueous and CTAB micelles; 339, 338 and 334 nm for micelles of CHAPS, Brij 35 and SDS, respectively). All other conditions are as in Figure 3.2. See Materials and Methods for other details.
Time-Resolved Fluorescence Measurements of Micelle-bound Melittin

A typical decay profile of tryptophan residue of melittin incorporated in CHAPS micelles with its biexponential fitting and the various statistical parameters used to check the goodness of the fit is shown in Figure 3.4.

Figure 3.4. Time-resolved fluorescence intensity decay of melittin incorporated in CHAPS micelles. Excitation wavelength was at 297 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 339 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. All other conditions are as in Figure 3.2. See Materials and Methods for other details.

The fluorescence lifetimes of melittin in water and micelles of various charge types are shown in Table 3.2. As seen from the table, all fluorescence decays could be fitted well with a biexponential function. As shown in the table, the mean fluorescence lifetime of monomeric melittin in aqueous solution is 3.48 ns in agreement with previous
literature (McDowell et al., 1985; Pandit et al., 2003). The mean fluorescence lifetimes of melittin in micelles of various surface charges are different indicating that the local environment experienced by the tryptophan residue is different in these cases. Interestingly, melittin in CTAB micelles shows a mean lifetime of 3.40 ns which is similar to the mean lifetime value obtained in water. This further shows that melittin does not bind to CTAB micelles due to electrostatic repulsion. The most drastic reduction in mean fluorescence lifetime is observed in the case of melittin in SDS micelles with a mean lifetime of 2.16 ns. The shortening of fluorescence lifetime could be due to steric interaction between Trp-19 and Lys-23 which will be in close proximity in a helical arrangement (Weaver et al., 1992) induced by the micellar surface upon melittin binding. The binding itself is enhanced by the favorable electrostatic interaction between cationic melittin and anionic SDS micelles. In fact, CD measurements show that SDS micelles induce maximum helicity in melittin (see later, Figure 3.6). Since Lys-23 in melittin has a pKₐ of 8.6 (Quay and Tronson, 1983), it will be positively charged in aqueous medium. Interestingly, only the protonated form of the amino group of Lys-23 is believed to be an efficient quencher of tryptophan fluorescence which is observed as a reduction in fluorescence lifetime (Weaver et al., 1992). This represents a noteworthy example of cation-π interaction which has an important role in biological interactions (Dougherty, 1996). Such preferential shortening of fluorescence lifetime of melittin has previously been reported for melittin bound to negatively charged membranes (Ghosh et al., 1997). Further, an increase in the polarity of the environment is known to reduce the lifetime of tryptophans due to fast deactivating processes in polar environments (Kirby and Steiner, 1970). An additional factor for the shortening of fluorescence lifetime of melittin when bound to SDS micelles therefore could be the increased polarity encountered by the melittin tryptophan in the negatively charged micelles.
### Table 3.2. Lifetimes of Melittin in Water and in Micelles of Different Charge Types

<table>
<thead>
<tr>
<th>Medium&lt;sup&gt;a&lt;/sup&gt;</th>
<th>$\alpha_1$</th>
<th>$\tau_1$ (ns)</th>
<th>$\alpha_2$</th>
<th>$\tau_2$ (ns)</th>
<th>$&lt;\tau&gt;$&lt;sup&gt;b&lt;/sup&gt; (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>0.62</td>
<td>2.18</td>
<td>0.38</td>
<td>4.50</td>
<td>3.5</td>
</tr>
<tr>
<td>CTAB</td>
<td>0.87</td>
<td>2.25</td>
<td>0.13</td>
<td>6.20</td>
<td>3.4</td>
</tr>
<tr>
<td>CHAPS</td>
<td>0.88</td>
<td>1.65</td>
<td>0.12</td>
<td>6.30</td>
<td>3.2</td>
</tr>
<tr>
<td>Brij 35</td>
<td>0.85</td>
<td>2.33</td>
<td>0.15</td>
<td>5.47</td>
<td>3.3</td>
</tr>
<tr>
<td>SDS</td>
<td>0.93</td>
<td>0.95</td>
<td>0.07</td>
<td>5.13</td>
<td>2.2</td>
</tr>
</tbody>
</table>

<sup>a</sup>The ratio of melittin to detergent was 1:500 (mol/mol) for CTAB and Brij 35 micelles, 1:4000 (mol/mol) for micelles of CHAPS, and 1:1000 (mol/mol) for SDS micelles. The concentration of melittin ranged from 1.6-16 μM. The excitation wavelength was 297 nm; Emission was monitored at the fluorescence emission maximum of melittin in each case. See Materials and Methods for other details.

<sup>b</sup>Calculated using Eq. 2.3.

In order to ensure that the observed change in steady state polarization is not due to any change in lifetime, the apparent rotational correlation times for the tryptophan residue of melittin in micelles were calculated using Eq. 2.5 (Chapter 2). The values of the apparent rotational correlation times, calculated this way using a value of $r_o$ of 0.09 (Weber, 1960), are shown in Table 3.3. The apparent rotational correlation time of melittin in CTAB micelles is the least which confirms our earlier conclusion that melittin does not bind to cationic CTAB micelles. These values of apparent rotational correlation times show that the observed change in polarization values (Figure 3.3) were not due to any lifetime-induced artifacts.
Table 3.3. Apparent Rotational Correlation Times of Melittin in Water and in Micelles of Different Charge Types

<table>
<thead>
<tr>
<th>Medium</th>
<th>$\tau_c^a$ (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.0</td>
</tr>
<tr>
<td>CTAB</td>
<td>1.2</td>
</tr>
<tr>
<td>CHAPS</td>
<td>5.0</td>
</tr>
<tr>
<td>Brij 35</td>
<td>9.6</td>
</tr>
<tr>
<td>SDS</td>
<td>8.2</td>
</tr>
</tbody>
</table>

$^a$Calculated using mean fluorescence lifetimes from Table 3.2 and using Eq. 2.5. All other conditions are as in Table 3.2.

Acrylamide Quenching of Melittin Tryptophan Fluorescence

To examine the accessibility and relative location of melittin in various types of micelles, fluorescence quenching experiments were performed with acrylamide which is a widely used neutral aqueous quencher of tryptophan fluorescence (Eftink, 1991). The results of these experiments for quenching of the tryptophan residue of melittin by acrylamide in various micellar systems are plotted in Figure 3.5 as Stern-Volmer plots. The slope of such a plot ($K_{SV}$) is related to the degree of exposure (accessibility) of the melittin tryptophan to the aqueous phase. In general, the higher the slope, the greater the degree of exposure, assuming that there is not a large difference in fluorescence lifetime. The bimolecular quenching constant ($k_q$) is a more accurate measure of the degree of exposure since it takes into account the differences in fluorescence lifetime (see Eq. 3.2).
Figure 3.5. Representative data for Stern-Volmer analysis of acrylamide quenching of melittin fluorescence in micelles of different charge types. \( F_0 \) is the fluorescence in the absence of quencher, \( F \) is the corrected fluorescence in the presence of quencher. The excitation wavelength was 295 nm and emission was monitored as in Figure 3.3. The ratio of melittin to detergent was 1:500 (mol/mol) for micelles of CTAB (O), Brij 35 (△) and SDS (■), and 1:2000 (mol/mol) for micelles of CHAPS (●). The concentration of melittin ranged from 1.6-24 μM. See Materials and Methods for other details.

The quenching parameters obtained by analyzing the Stern-Volmer plots are shown in Table 3.4. It is apparent from the table that the tryptophan in melittin is the most exposed in case of melittin in water. This is expected since melittin is monomeric and unordered in this case. In agreement with our earlier observations, the melittin tryptophan in CTAB micelles appears to be the most exposed among all the micelles studied. This shows once again that melittin does not bind CTAB micelles under these conditions. Quenching parameters obtained with CHAPS, Brij 35 and SDS micelles show that the melittin tryptophan is much less accessible in these micelles indicating partitioning of melittin into deeper regions of these micelles.
Table 3.4. Acrylamide Quenching of Tryptophan Fluorescence of Melittin in Aqueous and Micellar Systems

<table>
<thead>
<tr>
<th>Medium</th>
<th>$K_{sv}$</th>
<th>$k_q \times 10^{-9}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>18.33 ± 1.2</td>
<td>5.24</td>
</tr>
<tr>
<td>CTAB</td>
<td>15.76 ± 1.1</td>
<td>4.64</td>
</tr>
<tr>
<td>CHAPS</td>
<td>7.15 ± 0.1</td>
<td>2.23</td>
</tr>
<tr>
<td>Brij 35</td>
<td>5.43 ± 0.3</td>
<td>1.65</td>
</tr>
<tr>
<td>SDS</td>
<td>4.05 ± 0.5</td>
<td>1.84</td>
</tr>
</tbody>
</table>

*The ratio of melittin to detergent was 1:500 (mol/mol) for CTAB, Brij 35 and SDS micelles, and 1:2000 (mol/mol) for micelles of CHAPS. The concentration of melittin ranged from 1.6-24 μM.

*Calculated using Eq. 3.2. The quenching parameter shown represents mean ± standard error of at least three independent measurements while quenching data shown in Figure 3.5 are from representative experiments. See Materials and Methods for other details.

*Calculated using mean fluorescence lifetimes from Table 3.2 and using Eq. 3.2. See Materials and Methods for other details.

Secondary Structure of Micelle-bound Melittin

To investigate the effect of micellar surface charge on the secondary structure of melittin, we carried out far-UV CD spectroscopy in micellar systems of various charge types. The CD spectra of melittin in micelles of different charge types are shown in Figure 3.6. It has been reported that monomeric melittin in aqueous solution shows essentially random coil conformation (Ghosh *et al.*, 1997; Bello *et al.*, 1982). Our results show that melittin in CTAB micelles shows secondary structure similar to that in aqueous solution which is in agreement with the previous literature (Chandani and Balasubramanian, 1986). This further confirms that melittin does not partition into CTAB micelles under these conditions. The effect of micellar charge on the secondary
structure of micelle-bound melittin is apparent from the secondary structure of melittin in zwitterionic (CHAPS), nonionic (Brij 35) and anionic (SDS) host micelles. Figure 3.6 shows that these micelles induce significant helicity to melittin. The differences in CD spectra among different micelles could be due to the fact that micelles with varying shape and charge would present a different surface to melittin for binding. This is most apparent in the case of CHAPS (see Figure 3.6) since the micellar organization and association in CHAPS is distinctly different than the other micelles due to lateral association of monomers and a low aggregation number. Thus the penetration of melittin and the resulting packing in the host micelles could contribute to the differences in the CD spectra.

Figure 3.6. Far-UV CD spectra of melittin in micelles of CTAB (---), CHAPS (-- - - -), Brij 35 (— — —) and SDS (———). The ratio of melittin to detergent was 1:500 (mol/mol) for micelles of CTAB and Brij 35, 1:1000 (mol/mol) for micelles of CHAPS and SDS. The concentration of melittin ranged from 13.3-16 μM. See Materials and Methods for other details.
3.4. DISCUSSION

Lipid-protein and protein-protein interactions in biological membranes are of vital importance in understanding various cellular processes (Shai, 1995; Lee, 2003). Cellular plasma membranes are characterized by an asymmetric distribution of zwitterionic and negatively charged phospholipids in such a way that the latter is localized predominantly in the inner leaflet of the bilayer, imparting a net negative charge to the cytoplasmic surface of the cell membrane (Gennis, 1989). Membrane proteins such as MARCKS, protein kinase C, and the Src family of tyrosine protein kinases have been shown to have clusters of basic amino acids (a feature which is shared by melittin) that interact electrostatically with negatively charged phospholipids (Resh, 1994). These proteins are cytosolic in nature, and reversibly associate with the cytoplasmic face of the plasma membrane, where the charge is negative due to the presence of anionic phospholipids (Gennis, 1989), in order to initiate cellular signaling events (Resh, 1994). The electrostatic interaction between their basic patch and the negatively charged phospholipids contributes considerable membrane binding energy to stabilize their interaction with membrane (Kim et al., 1991). Such charge-dependent membrane binding often leads to domain formation at the membrane interface trigged by lipid-protein interaction (Lentz, 1995).

The interaction of melittin with membranes of different charge types has important functional consequence. For example, it has been shown that the presence of negatively charged lipids inhibits membrane lysis induced by melittin and this inhibition increases with increasing charge density in the membrane (Ghosh et al., 1997; Benachir and Lafleur, 1995; Monette and Lafleur, 1995; Hincha and Crowe, 1996). The modulation of lytic activity is believed to be related to the electrostatic interaction between the peptide and the membrane surface (Ghosh et al., 1997). In this study, we have monitored the change in the organization and dynamics of melittin bound to
micelles of various charge types by utilizing fluorescence approaches and circular dichroism spectroscopy. Our results show that melittin does not partition into CTAB micelles due to electrostatic repulsion between melittin and CTAB micelles both of which carry positive charge. This charge repulsion appears to be attenuated by the presence of sodium chloride as evidenced by the blue shifted emission maximum and higher polarization value of melittin in CTAB micelles in presence of sodium chloride.

In case of the interaction of melittin with zwitterionic or neutral detergents, the predominant driving force for the peptide-micelle interaction will be the interaction of the hydrophobic region of the detergent with the apolar face of the melittin amphiphilic helix. The lack of specific and strong electrostatic interaction between the peptide and the zwitterionic or neutral detergent headgroup leads to penetration of the interfacial water in the micelle interior thereby allowing such water molecules in the proximity of the tryptophan residue. Such water molecules will be motionally restricted giving rise to a REES of 5-6 nm as observed in case of melittin bound to Brij 35 and CHAPS micelles (Figure 3.2). In case of interaction of melittin with SDS micelles, the major driving force for peptide-micelle interaction will be the favorable electrostatic interaction between the negatively charged detergent and the positively charged residues of the peptide. The close interaction of the charged peptide with the oppositely charged detergent headgroup results in poor water penetration in the micelle interior, especially around the interfacial tryptophan residue. This results in a relatively nonpolar environment around the tryptophan in SDS micelles leading to a blue shift of the fluorescence emission maximum (334 nm) compared to electrically neutral/zwitterionic micelles (338/339 nm) and a reduction of REES to 3 nm (as opposed to 5-6 nm for neutral/zwitterionic micelles). Alternatively, the location of the tryptophan could be different for melittin bound to various micelles. Tryptophan residues in membrane peptides and proteins are known to be preferentially localized at the membrane interfacial region (Killian and von Heijne, 2000). In a micellar environment, however, the thermal thickness of the interfacial
region is less. The location (and hence the immediate environment) of the melittin tryptophan could be different in various micelles which could account for differences in the extent of REES.

Taken together, we observe a REES of 3-6 nm for the tryptophan of melittin when bound to anionic, nonionic and zwitterionic micelles. This suggests that melittin is localized in a restricted environment probably in the interfacial region of the micelles. Further, the rotational mobility of melittin is considerably reduced in these micelles and is found to be dependent on the surface charge of micelles. The highest rotational correlation time is observed with the electrically neutral Brij 35 and anionic SDS micelles (see Table 3.3). In addition, the fluorescence lifetime of melittin is modulated in micelles of different charge types. The lowest mean fluorescence lifetime (2.16 ns) is observed in the case of melittin in anionic SDS micelles. We attribute this short lifetime to steric interaction between the closely spaced Trp-19 and Lys-23 in a helical arrangement (Quay and Tronson, 1983) induced by the micellar surface. This is reinforced by results from circular dichroism spectroscopy which show that SDS micelles induce maximum helicity in melittin.

In summary, our results clearly show that micellar surface charge can modulate the organization and dynamics of melittin. These results are relevant to understand the role of surface charge of membranes in the interaction of membrane-active, amphiphilic pepides with membranes.