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H. Raghuraman · Amitabha Chattopadhyay

Effect of micellar charge on the conformation and dynamics of melittin

Abstract Electrostatic interactions play a crucial role in modulating and stabilizing molecular interactions in membranes and membrane-mimetic systems such as micelles. We have monitored the change in the conformation and dynamics of the cationic hemolytic peptide melittin bound to micelles of various charge types, utilizing fluorescence and circular dichroism (CD) spectroscopy. The sole tryptophan of melittin displays a red-edge excitation shift (REES) of 3–6 nm 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, and this region offers considerable restriction to the reorientational motion of the solvent dipoles around the excited state tryptophan in melittin. Further, the rotational mobility of melittin is considerably reduced in these micelles and is found to be dependent on the surface charge of micelles. Interestingly, our results show that melittin does not partition into cetyltrimethylammonium bromide (CTAB) micelles owing to electrostatic repulsion between melittin and CTAB micelles, both of which carry a positive charge. In addition, the fluorescence lifetime of melittin is modulated in micelles of different charge types. The lowest mean fluorescence lifetime is observed in the case of melittin bound to anionic sodium dodecyl sulfate (SDS) micelles. CD spectroscopy shows that micelles induce significant helicity to melittin, with maximum helicity being induced in the case of melittin bound to SDS micelles. Fluorescence quenching measurements using the neutral aqueous quencher acrylamide show differential accessibility of melittin in various types of micelles. Taken together, our results show that micellar surface charge can modulate the conformation and dynamics of melittin. These results could be relevant to understanding the role of the surface charge of membranes in the interaction of membrane-active, amphiphilic peptides with membranes.

H. Raghuraman · A. Chattopadhyay (✉)
Centre for Cellular and Molecular Biology,
Uppal Road, 500 007 Hyderabad, India
E-mail: amit@ccmb.res.in
Tel.: +91-40-27192578
Fax: +91-40-27160311

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 the 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, the principal toxic component in the venom of the European honey bee, *Apis mellifera*, is a cationic hemolytic peptide (Habermann 1972). It is a small linear peptide composed of 26 amino acids (NH_2-GIGA-VLKVLTTGLPALISWIKRKRQQ-$CONH_2$) 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. This amphiphilic property of melittin makes it water soluble and yet it spontaneously associates with natural and artificial membranes (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). This has resulted in melittin being used as a convenient model for monitoring lipid–protein interactions in membranes. Apart from its powerful hemolytic activity, melittin causes bilayer micellization and membrane fusion and has also been reported to form voltage-dependent ion channels across planar lipid bilayers (Dempsey 1990; Bechinger, 1997).

Melittin adopts a predominantly random coil conformation as a monomer in aqueous solution (Bello et al. 1982). However, at high ionic strength, pH, or peptide concentration, it self-associates to form an α-helical tetrameric structure, driven by the formation of a hydrophobic core (Bello et al. 1982; Dempsey 1990). Interestingly, melittin adopts an α-helical conformation when bound to membranes or micelles (De Jongh et al. 1994). NMR studies of membrane-bound melittin have shown it to be α-helical with a kink in the middle (Naito et al. 2000; Lam et al. 2001). However, despite the availability of a high-resolution crystal structure of tetrameric melittin in aqueous solution (Terwilliger and Eisenberg 1982), the structure of the membrane-bound form is not yet resolved by X-ray crystallography. Yet the importance of the membrane-bound form stems from the observation that the amphiphilic α-helical conformation of this hemolytic toxin in membranes resembles those of apolipoproteins and peptide hormones (Kaiser and Kezdy 1983; Morii et al. 1994), signal peptides (Golding and O'Shea 1995), and the envelope glycoprotein gp41 from the human immunodeficiency virus (HIV) (Rabenstein and Shin 1995). Furthermore, understanding of melittin–membrane interactions assumes greater significance due to the recent finding that melittin mimics the N-terminal of HIV-1 virulence factor Nef1-25 (Barnham et al. 1997).

Melittin is intrinsically fluorescent due to the presence of a single tryptophan residue, Trp19, which makes it a sensitive probe to study the interaction of melittin with membranes and membrane-mimetic systems (Bradrick et al. 1995; Cajal and Jain 1997; Ghosh et al. 1997; Oren and Shai 1997). This is particularly advantageous, since there are no other aromatic amino acids in melittin and this makes the interpretation of fluorescence data less complicated owing to the lack of interference and heterogeneity. More importantly, it has been shown that the sole tryptophan residue of melittin is crucial for its powerful hemolytic activity, since a dramatic reduction in activity is observed upon photooxidation (Habermann and Kowallek 1970) or substitution by leucine (Blondelle and Houghten 1991a). This is further reinforced by studies with single amino acid omission analogues of melittin (Blondelle and Houghten 1991b). These reports point out the crucial role played by the uniquely positioned tryptophan in maintaining the structure and hemolytic activity of melittin. The organization and dynamics of the tryptophan therefore become important for the function of the peptide.

Melittin is known to interact selectively with negatively charged lipids (Beschiaschvili and Seelig 1990; Ghosh et al. 1997; 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 (Benachir and Lafleur 1995; Monette and Lafleur 1995; Hincha and Crowe 1996; Ghosh et al. 1997). 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 the 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 paper, we have utilized micelles of various charge types to monitor the effect of varying surface charge on the conformation 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). Micelles have also been applied as membrane mimetics to characterize membrane proteins and peptides (Franklin et al. 1994; Lenz et al. 1995; Sham et al. 2003).

Micelles are highly cooperative, organized molecular assemblies of amphiphiles and are dynamic in nature (Menger 1979). A direct consequence of such organized

systems is the restriction imposed on the dynamics and mobility of their constituent structural units. We have previously shown that the microenvironment of molecules bound to micelles and other organized molecular assemblies can be conveniently monitored using the wavelength-selective fluorescence approach (see Chattopadhyay et al. 2002; Chattopadhyay 2003 and references therein; Kelkar et al. 2003; Raghuraman and Chattopadhyay 2003; Mukherjee et al. 2004; Raghuraman et al. 2004). 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 (Ikeda 1984; Porte and Appel 1984). Furthermore, structural transitions can be induced in charged micelles at a given temperature by increasing the ionic strength of the medium or amphiphile concentration (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 (Sarkar et al. 1996; Maiti et al. 1997; Rawat et al. 1997; Rawat and Chattopadhyay 1999) and theoretical (MacKerell 1995) approaches. It is fairly well established now that practically all types of molecule 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 the organization and dynamics of a variety of micelles differing in charge and shape (Rawat et al. 1997; Rawat and Chattopadhyay 1999). In this paper, we have monitored the change in the conformation and dynamics of melittin bound to micelles of various charge types (see

Fig. 1) utilizing fluorescence and circular dichroism (CD) spectroscopy. Our study assumes significance in the overall context of the role of the 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.

Materials and methods

Materials

Cetyltrimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), 3-[(3-cholamidopropyl)dimethyl-ammonio]-1-propanesulfonate (CHAPS), and melittin of the highest available purity were obtained from Sigma (St. Louis, MO, USA). To check for any residual phospholipase A_2 contamination in melittin, phospholipase activity was assayed using ^{14}C-labeled dioleoyl-sn-glycero-3-phosphocholine (DOPC), as described earlier (Argiolas and Pisano 1983). No appreciable activity could be detected this way. Brij 35 was from Pierce (Rockford, IL, USA) and 1,6-diphenyl-1,3,5-hexatriene (DPH) was purchased from Molecular Probes (Eugene, OR, USA). Ultrapure grade acrylamide was from Gibco BRL (Rockville, MD, USA). The purity of the acrylamide was checked from its absorbance using its molar extinction coefficient (ϵ) of 0.23 M^{-1} cm^{-1} at 295 nm and optical transparency beyond 310 nm (Eftink 1991a). Water was purified through a Millipore (Bedford, MA, USA) 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 (ϵ) of 5570 M^{-1} cm^{-1} at 280 nm (Ghosh et al. 1997). The purity of the detergents was checked by measuring their CMC values and comparing with literature CMC. The CMCs of detergents were determined fluorimetrically using a widely used

Fig. 1 Chemical structures of the detergents used

CTAB

CHAPS

Brij 35

SDS
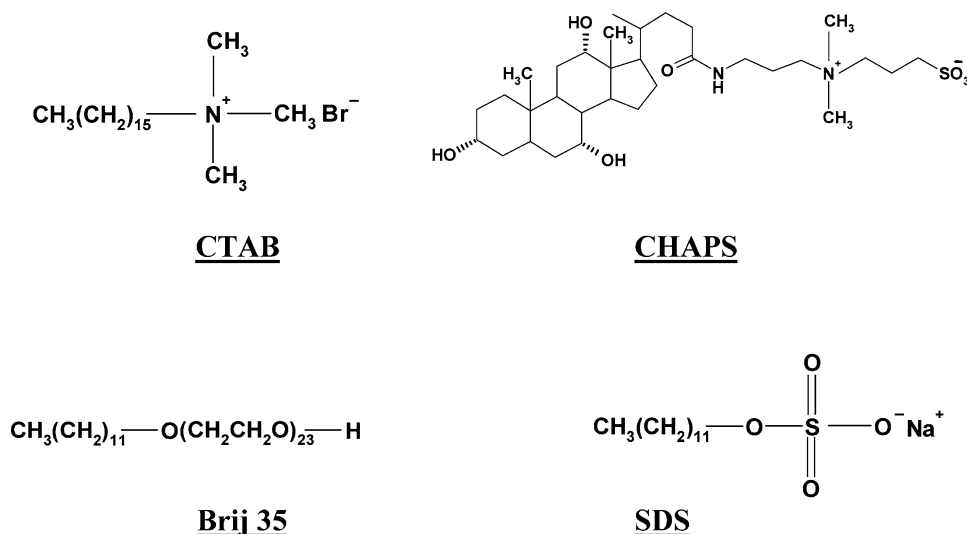
method previously developed by one of us (Chattopadhyay and London 1984), which utilizes enhancement of DPH fluorescence upon micellization.

Sample preparation

Concentrations of detergents used were above the CMC of the respective detergents to ensure that they were in the micellar state. Melittin was added to micellar systems from an aqueous stock solution to give a 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 $\sim$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 the dark before measuring the 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 pathlength 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 from the equation (Lakowicz 1999):

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}} \qquad (1)$$

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{HV}/I_{HH}. All experiments were done with multiple sets of

samples and average values of the polarization are shown in Fig. 3.

Time-resolved fluorescence measurements

Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single-photon counting mode as described previously (Raghuraman and Chattopadhyay 2003). 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:

$$F(t) = \sum_i \alpha_i \exp(-t/\tau_i) \qquad (2)$$

where α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i. The decay parameters were recovered as described previously (Raghuraman and Chattopadhyay 2003). Mean (average) lifetimes, $<\tau>$, for biexponential decays of the fluorescence were calculated from the decay times and preexponential factors using the following equation (Lakowicz 1999):

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_1\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2} \qquad (3)$$

Fluorescence quenching measurements

Acrylamide quenching experiments of melittin fluorescence in micelles and in aqueous solution were carried out by measurement of the 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 the dark for 1 h before measuring the 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 the inner filter effect were made using the following equation (Lakowicz 1999):

$$F = F_{obs}\text{antilog}[(A_{ex} + A_{em})/2] \qquad (4)$$

where F is the corrected fluorescence intensity and F_{obs} is the background subtracted fluorescence intensity of the sample. A_{ex} and A_{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):

$$F_0/F = 1 + K_{SV}[Q] = 1 + k_q\tau_0[Q] \qquad (5)$$

where F_0 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. K_{SV} is equal to $k_q\tau_0$, where k_q is the bimolecular quenching constant and τ_0 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 which was calibrated with (+)-10-camphorsulfonic acid (Chen and Yang 1977). The spectra were scanned in a quartz optical cell with a pathlength of 0.1 cm. All spectra were recorded in 0.2 nm wavelength increments with a 4 s response and a band width of 1 nm. For monitoring changes in secondary structure, spectra were scanned in the far-UV range from 205 to 250 nm at a scan rate of 50 nm/min. Each spectrum is the average of 15 scans with a full scale sensitivity of 50 mdeg. All spectra were corrected for background by subtraction of appropriate blanks and were smoothed, making sure that the overall shape of the spectrum remained unaltered. Data are represented as molar ellipticities and were calculated using the equation:

$$[\theta] = \theta_{obs}/(10Cl) \qquad (6)$$

where θ_{obs} is the observed ellipticity in mdeg, l is the pathlength in cm, and C is the melittin concentration in mol/L.

Results

Figure 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 the 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 detergents 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 a 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 1 shows the fluorescence emission maxima and polarization of melittin incorporated into various types of micelles under a wide range of detergent-to-melittin ratios (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 ratios, 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.

REES of melittin in micelles of various charge types

Red edge excitation shift (REES) represents a powerful approach which can be used to directly monitor the environment and dynamics around a fluorophore in an organized molecular assembly (Chattopadhyay 2003; Raghuraman et al. 2003). A shift in the wavelength of the maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed REES. This effect is mostly observed with polar fluorophores in motionally restricted media such as 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. REES therefore arises from 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. Utilizing this approach, it becomes possible to probe the mobility parameters of the environment itself (which is represented by the relaxing solvent molecules) using the fluorophore merely as a reporter group. Since the bulk solvent in micellar systems is water, the information obtained from REES measurements in such organized systems has its origin in the dynamics of the otherwise "optically silent" water molecules.

The shifts in the maximum of the fluorescence emission[1] 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 Fig. 2. We have previously reported that the fluorescence emission maximum of melittin incorporated in model membranes of DOPC, in which the localization of melittin has been shown to be interfacial, is ~335 nm (Chattopadhyay

[1]We have used the term maximum of fluorescence emission in a somewhat wider sense here. In every case, we have monitored the wavelength corresponding to the maximum fluorescence intensity, as well as the center of mass of the fluorescence emission. In most cases, both these methods yielded the same wavelength. In cases where minor discrepancies were found, the center of mass of the emission has been reported as the fluorescence maximum

Table 1 Binding of melittin in micelles of different charge types

Medium	Detergent to melittin ratio (mol/mol)	Emission maximum (nm)	Fluorescence polarization[a]
CTAB	100–2500	349	0.035
CHAPS	500–5000	339	0.083
Brij 35	400–1000	338	0.097
SDS	100–2000	334	0.103

[a] Calculated using Eq. <equationcite>1</equationcite>. The values shown are the mean of three independent experiments

and Rukmini 1993; Ghosh et al. 1997). Figure 2 shows that upon excitation at 280 nm the fluorescence emission maxima of melittin are 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. 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), from 338 to 343 nm (Brij 35), and from 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
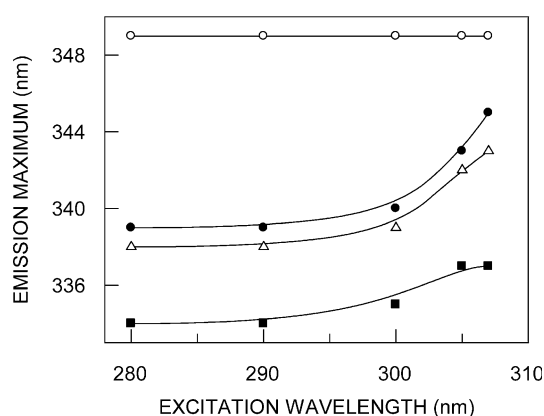

Fig. 2 Effect of changing the excitation wavelength on the wavelength of the maximum emission of melittin in micelles of SDS (*solid squares*), Brij 35 (*open triangles*), CHAPS (*solid circles*) and CTAB (*open circles*). The ratio of melittin to detergent was 1:1000 (mol/mol) for SDS micelles, 1:4000 (mol/mol) for CHAPS micelles, and 1:500 (mol/mol) for Brij 35 and CTAB micelles. The concentration of melittin ranged from 1.6 to 16 μM. See Materials and methods for other details

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 the 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, owing 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 the 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 the presence of 100 mM NaCl, which helps screen the charge and therefore enhances binding (data not shown). However, even under this condition, melittin does not display REES, implying a 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 Fig. 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 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 owing to unfavorable electrostatic interactions. This is further confirmed by an increase in the polarization of melittin in CTAB micelles in the presence of NaCl, which effectively screens the charge, as shown in Fig. 3.

Fig. 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 Fig. 2. See Materials and methods for other details
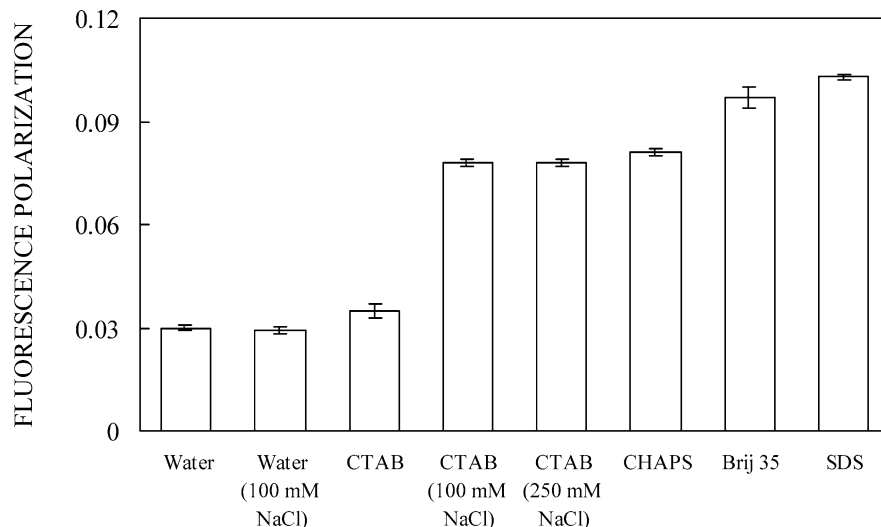
Time-resolved fluorescence measurements of micelle-bound melittin

The fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed (Prendergast 1991). In addition, it is well known that the fluorescence lifetime of tryptophan in particular is sensitive to solvent, temperature, and excited state interactions (Kirby and Steiner 1970; Beechem and Brand 1985). A typical decay profile of the 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 Fig. 4.

The fluorescence lifetimes of melittin in water and micelles of various charge types are shown in Table 2. As seen from the table, all fluorescence decays could be fitted well with a biexponential function. The mean fluorescence lifetimes were calculated using Eq. 3 and are shown in Table 2. As can be seen from the table, the mean fluorescence lifetime of monomeric melittin in aqueous solution is 3.5 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 3.4 ns, which is similar to the mean lifetime value obtained in water. This further shows that melittin does not bind to CTAB micelles owing 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 2.2 ns. The shortening of fluorescence lifetime could be due to steric interaction between Trp19 and Lys23, 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
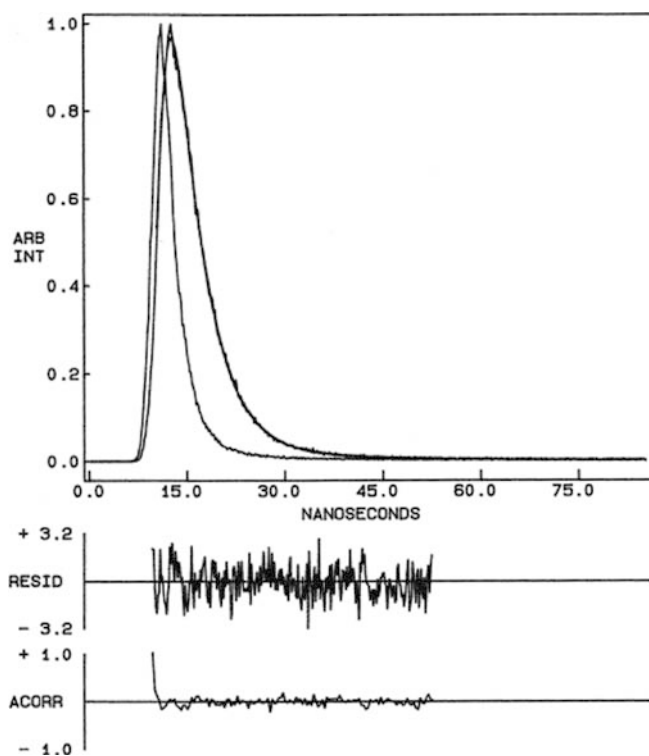

Fig. 4 Time-resolved fluorescence intensity decay of melittin incorporated in CHAPS micelles. The excitation wavelength was at 297 nm, which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 339 nm. The *sharp peak* on the *left* is the lamp profile. The relatively *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 Fig. 2. See Materials and methods for other details

fact, CD measurements show that SDS micelles induce maximum helicity in melittin (see later, Fig. 6). Since Lys23 in melittin has a pK_a 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 Lys23 is believed to be an efficient quencher of

Table 2 Lifetimes of melittin in water and in micelles of different charge types

Medium[a]	α_1	τ_1 (ns)	α_2	τ_2 (ns)	$<\tau>$[b] (ns)
Water	0.62	2.18	0.38	4.50	3.5
CTAB	0.87	2.25	0.13	6.20	3.4
CHAPS	0.88	1.65	0.12	6.30	3.2
Brij 35	0.85	2.33	0.15	5.47	3.3
SDS	0.93	0.95	0.07	5.13	2.2

[a]The ratio of melittin to detergent was 1:500 (mol/mol) for CTAB and Brij 35 micelles, 1:4000 (mol/mol) for CHAPS micelles, and 1:1000 (mol/mol) for SDS micelles. The concentration of melittin ranged from 1.6 to 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
[b]Calculated using Eq. <equationcite>3</equationcite>

Table 3 Apparent rotational correlation times of melittin in water and in micelles of different charge types

Medium	τ_c[a] (ns)
Water	1.0
CTAB	1.2
CHAPS	5.0
Brij 35	9.6
SDS	8.2

[a]Calculated using mean fluorescence lifetimes from Table 2 and using Eq. <equationcite>7</equationcite>. All conditions are as in Table 2. See the Results section for other details

tryptophan fluorescence which is observed as a reduction in fluorescence lifetime (Weaver et al. 1992). This represents a noteworthy example of a cation–π interaction which has an important role in biological interactions (Dougherty 1996). Such preferential shortening of the 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 owing to fast deactivating processes in polar environments (Kirby and Steiner 1970). An additional factor for shortening of the 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.

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 Perrin's equation (Lakowicz 1999):

$$\tau_C = \frac{\langle\tau\rangle r}{r_0 - r} \tag{7}$$

where r_0 is the limiting anisotropy of tryptophan, r is the steady-state anisotropy [derived from the polarization values using $r = 2P/(3-P)$], and $<\tau>$ is the mean fluorescence lifetime as calculated from Eq. 3 (shown in Table 2). The values of the apparent rotational correlation times, calculated this way using a value for r_0 of 0.09 (Weber 1960), are shown in Table 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 the apparent rotational correlation times show that the observed changes in polarization values (Fig. 3) were not due to any lifetime-induced artifacts.

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 1991b). The results of these experiments for quenching of the tryptophan residue of melittin by acrylamide in various micellar systems are plotted in Fig. 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. 5). The quenching parameters obtained by analyzing the Stern–Volmer plots are shown in Table 4. It is apparent from the table that the tryptophan in melittin is most exposed in the 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
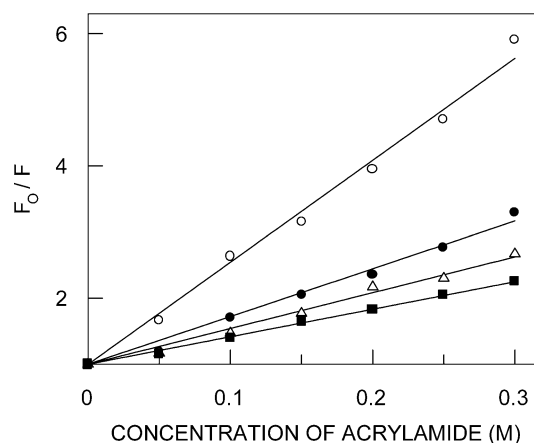

Fig. 5 Representative data for Stern–Volmer analysis of acrylamide quenching of melittin fluorescence in micelles of different charge types. F_0 is the fluorescence in the absence of quencher, F is the corrected fluorescence in the presence of quencher. The excitation wavelength was 295 nm and emission was monitored as in Fig. 3. The ratio of melittin to detergent was 1:500 (mol/mol) for micelles of CTAB (*open circles*), Brij 35 (*open triangles*), and SDS (*solid squares*), and 1:2000 (mol/mol) for micelles of CHAPS (*solid circles*). The concentration of melittin ranged from 1.6 to 24 μM. See Materials and methods for other details

Table 4 Acrylamide quenching of tryptophan fluorescence of melittin in aqueous and micellar systems

Medium[a]	K_{SV}[b] (M^{-1})	k_q ($\times 10^{-9}$)[c] (M^{-1} s^{-1})
Water	18.33 ± 1.2	5.24
CTAB	15.76 ± 1.1	4.64
CHAPS	7.15 ± 0.1	2.23
Brij 35	5.43 ± 0.3	1.65
SDS	4.05 ± 0.5	1.84

[a]The ratio of melittin to detergent was 1:500 (mol/mol) for CTAB, Brij 35, and SDS micelles, and 1:2000 (mol/mol) for CHAPS micelles. The concentration of melittin ranged from 1.6 to 24 μM
[b]Calculated using Eq. <equationcite>5</equationcite>. The quenching parameter shown represents the mean ± standard error of at least three independent measurements, while the quenching data shown in Fig. 5 are from representative experiments. See Materials and methods for other details
[c]Calculated using mean fluorescence lifetimes from Table 2 and using Eq. <equationcite>5</equationcite>. See Materials and methods for other details

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.

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 Fig. 6. It is well established that monomeric melittin in aqueous solution shows essentially a random coil conformation, as reported earlier by us (Ghosh et al. 1997) and others (Bello et al.
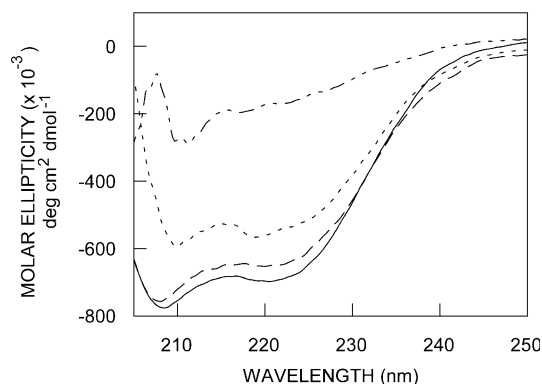

Fig. 6 Far-UV CD spectra of melittin in micelles of CTAB (*long dashed-short dashed line*), CHAPS (*short dashed line*), Brij 35 (*long dashed line*), and SDS (*full line*). The ratio of melittin to detergent was 1:500 (mol/mol) for micelles of CTAB and Brij 35, 1:1000 (mol/mol) for micelles of CHAPS and SDS. The concentration of melittin ranged from 13.3 to 16 μM. See Materials and methods for other details

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 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 Fig. 6), since the micellar organization and association in CHAPS is distinctly different than the other micelles owing 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.

Discussion

Lipid–protein and protein–protein interactions in biological membranes are of vital importance in understanding various cellular processes (Shai 2001; 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 the 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 (Benachir and Lafleur 1995; Monette and Lafleur 1995;

Hincha and Crowe 1996; Ghosh et al. 1997). 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 paper, we have monitored the change in the conformation and dynamics of melittin bound to micelles of various charge types by utilizing fluorescence approaches and CD spectroscopy. Our results show that melittin does not partition into CTAB micelles owing 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 the presence of sodium chloride.

In the 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 (Fig. 2). In the 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, or 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). In addition, the fluorescence lifetime of melittin is modulated in micelles of different charge types. The lowest mean fluorescence lifetime (2.2 ns) is observed in the case of melittin in anionic SDS micelles. We attribute this short lifetime to steric interaction between the closely spaced Trp19 and Lys23 in a helical arrangement (Weaver et al 1992) induced by the micellar surface. This is reinforced by results from CD spectroscopy, which show that SDS micelles induce maximum helicity in melittin.

In summary, we show here that the micellar surface charge can modulate the conformation and dynamics of melittin. These results are relevant to understand the role of the surface charge of membranes in the interaction of membrane-active, amphiphilic peptides with membranes.

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