Organization and Dynamics of Melittin in Environments of Graded Hydration: A Fluorescence Approach

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Melittin is a cationic hemolytic peptide composed of 26 amino acid residues. It is intrinsically fluorescent because of the presence of a single tryptophan residue which has been shown to be crucial for its hemolytic activity. We have previously shown that the sole tryptophan of melittin is located in a motionally restricted region in the membrane and the tryptophan environment is modulated in the presence of negatively charged phospholipids. Reverse micelles represent a type of organized molecular assembly which offer the unique advantage of monitoring dynamics of embedded molecules with varying degrees of hydration. We have employed reverse micelles as a membrane−mimetic system to monitor the effect of hydration on the organization and dynamics of melittin. Our results show that fluorescence parameters such as intensity, emission maximum, and polarization of melittin incorporated in reverse micelles of AOT in heptane are dependent on [water]/[surfactant] molar ratio (w_o) of the reverse micelle. Time-resolved fluorescence measurements of melittin in AOT reverse micelles show a gradual reduction in mean lifetime with increasing w_o. More importantly, melittin in reverse micellar environment showed red edge excitation shift (REES) implying that localization of the peptide in reverse micelles results in considerable restriction to the reorientational motion of the solvent dipoles around the excited-state fluorophore. Interestingly, the extent of REES decreased with increasing w_o. Fluorescence polarization of melittin in reverse micellar environments was wavelength-dependent. In addition, increasing hydration causes significant increase in helicity of melittin bound to reverse micelles. Taken together, our results provide information about the dynamics of melittin in environments of graded hydration and are relevant to dynamics of membrane-bound peptides under conditions of differential hydration which are more difficult to analyze experimentally.

Introduction

Melittin, the principal toxic component in the venom of the European honey bee, *Apis mellifera*, is a cationic hemolytic peptide.[1,2] It is a small linear peptide composed of 26 amino acids (NH_2-GIGAVLKVLTTGLPALISWI-KRKRQQ-$CONH_2$) in which the amino-terminal region (residues 1−20) is predominantly hydrophobic whereas the carboxy-terminal region (residues 21−26) is hydrophilic because of the presence of a stretch of positively charged amino acids. The amphiphilic property of this peptide makes it water soluble and yet it spontaneously associates with natural and artificial membranes.[3−7] 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.[4,5,8] 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 also induces voltage-dependent ion channels across planar lipid bilayers and causes bilayer micellization and membrane fusion.[3−5,9]

Melittin adopts predominantly random coil conformation as a monomer in aqueous solution.[5,10] 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.[5,10] Interestingly, melittin adopts an α-helical conformation when bound to membranes or micelles.[5,11−13] Despite the availability of crystal structure of tetrameric melittin in aqueous solution with high resolution,[14] the structure of the membrane-bound form is not yet resolved. 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,[15−17] signal peptides,[10,18,19] and the envelope glycoprotein gp41 from the human immunodeficiency virus (HIV).[20,21] Furthermore, understanding of melittin−membrane interaction assumes greater significance because of the recent finding that melittin mimics the N-terminal of HIV-1 virulence factor Nef1-25.[22]

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Melittin is intrinsically fluorescent because of the presence of a single tryptophan residue, Trp-19, which makes it a sensitive probe to study the interaction of melittin with membranes and membrane−mimetic systems.[12,23−27] This is particularly advantageous since there are no other aromatic amino acids in melittin and this makes interpretation of fluorescence data less complicated because of 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,[28] and substitution by leucine.[29] This is further reinforced by studies with single amino acid omission analogues of melittin.[30] 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. We have previously monitored the microenvironment experienced by the sole tryptophan in melittin when bound to membranes utilizing the wavelength-selective fluorescence approach (see below). Our results show that the tryptophan residue is located in a motionally restricted region in the membrane[23,25] 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.[23]

Hydration plays a key role in cell structure and function and is crucial for lipid−protein interactions in membranes.[31] Water plays a crucial role in determining the structure and dynamics, and in turn the function of proteins.[32,33] It is estimated that a threshold level of hydration (less than 0.4 g of water per gram of protein) is required to fully activate the dynamics and function of globular proteins.[34−36] In addition, it has become increasingly evident that water molecules mediate lipid−protein interactions[31,37,43] and hence the function of membrane proteins.[38,39] Any alteration in the degree of hydration, particularly at the protein−lipid interface in cell membranes, could potentially lead to modifications of protein structure that could in turn modify its function.[40,41] Further, it has been established that there is a "bound" hydration shell surrounding the headgroup of membrane phospholipids which plays an important role in membrane stability.[42] In this paper, we have examined the effect of hydration on melittin−membrane interactions by using reverse micelles formed by AOT (sodium bis(2-ethylhexyl) sulfosuccinate) in heptane as a membrane−mimetic system.

Reverse micelles are relatively simple yet versatile systems and represent a unique kind of self-organized molecular assembly. They provide an attractive model system for biomembranes since they mimic several important and essential features of biological membranes although lacking much of the complexity associated with them.[44,45] The general principles underlying their formation are common to other related assemblies such as micelles, bilayers, liposomes, and biological membranes.[46−49] The polar headgroups in reverse micelles are directed toward the aqueous interior of these aggregates, while their hydrocarbon tails project into the surrounding bulk nonpolar solvent. The highly structured yet heterogeneous water molecules in reverse micelles represent interesting models for water molecules present in biological systems such as membranes, which are more difficult to analyze experimentally. The physical and chemical properties of the entrapped water are markedly different from the properties of bulk water but similar in several aspects to those of biological interfacial water as found in membranes or protein interfaces.[50−53] The interfacial water is crucial for the induction of secondary structure in peptides and proteins when bound to surfaces such as membranes or micelles as well as for variation of their local internal motion. Both experimental[50−52] and theoretical[54] approaches have shown that the key structural parameter of reverse micelles is the [water]/[surfactant] molar ratio (w_o), which determines the micellar size as well as the unique physicochemical properties of the entrapped water. Reverse micelles therefore represent a type of organized molecular assembly that offer the unique advantage of monitoring dynamics of embedded molecules with varying degrees of hydration. A wide range of physicochemical properties of the micellar water such as micropolarity, dielectric constant, microviscosity, water activity, freezing point, proton transfer efficiency, and the hydrogen-bonding potential of the aqueous inner core can be experimentally varied with w_o therefore providing a unique and versatile reaction medium. The reverse micellar water pool has been shown to substantially influence the conformational states and dynamics of biopolymers solubilized in it.[55] Because of their optical transparency and well-defined size (which are advanta-

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geous for spectroscopic measurements because of lack of scattering artifacts), reverse micelles represent a suitable model system for studying peptides and proteins in a controlled water-restricted environment.[56−58]

In this paper, we have employed a combination of the wavelength-selective fluorescence approach and circular dichroism spectroscopy to monitor the effect of varying degrees of hydration on the dynamics of melittin in reverse micellar environments. Wavelength-selective fluorescence comprises a set of approaches based on the red edge effect in fluorescence spectroscopy which can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system.[59−62] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of absorption band, is termed red edge excitation shift (REES).[59−64] This effect is mostly observed with polar fluorophores in motionally restricted environments 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.[59−66] REES arises because of slow rates of solvent relaxation (reorientation) around an excited state fluorophore, which is dependent on 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. This makes the use of REES in particular and the wavelength-selective fluorescence approach in general very useful since hydration plays a crucial modulatory role in a large number of important cellular events such as protein folding, lipid−protein interactions, and ion transport.[31,38,67,68] An in-depth discussion of the photophysical framework for REES and wavelength-selective fluorescence approach is provided in recent reviews.[61,62]

We have previously shown that REES and related techniques (wavelength-selective fluorescence approach) serve as a powerful tool to monitor organization and dynamics of probes and peptides bound to membranes,[23,25,69−75] and membrane−mimetic media such as micelles,[76,77] and reverse micelles.[78] In addition, we have previously used the wavelength-selective fluorescence approach to analyze the organization and dynamics of tryptophans in the soluble hemolytic protein α-toxin[74] and the cytoskeletal proteins tubulin[79] and spectrin.[80]

Application of the wavelength-selective fluorescence approach to monitor dynamics of molecules embedded in reverse micellar systems represents a useful approach since the water content in reverse micelles can be controlled precisely providing the unique opportunity of monitoring the dynamics of molecules with varying degrees of hydration.[78] In this paper, we have examined the effect of hydration on the dynamics of melittin−membrane interactions by using melittin incorporated in reverse micelles formed by AOT in heptane which serves as a membrane−mimetic system. Our results provide novel information about the dynamics of melittin in environments of graded hydration and are relevant to dynamics of membrane-bound peptides under conditions of differential hydration which are more difficult to analyze experimentally.

Experimental Section

Materials. Melittin of the highest available purity and AOT were obtained from Sigma Chemical Co. (St. Louis, MO). Heptane and methanol used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA) Milli-Q system and used throughout. The purity of AOT was confirmed by good agreement of its UV absorption spectrum with previously reported spectrum.[44]

Preparation of Melittin-Incorporated Reverse Micelles. Reverse micelles of AOT containing melittin were prepared without addition of any cosolvent as follows. Melittin in methanol (48 nmol) was dried under a stream of nitrogen while being warmed gently (∼35 °C). After further drying under a high vacuum for at least 4 h, 1.5 mL of 50 mM AOT in heptane was added, and each sample was vortexed for 3 min and then sonicated in a Laboratory Supplies (Hicksville, NY) bath sonicator for 10 min. Appropriate amounts of water were subsequently added to make reverse micellar dispersions of different [water]/[surfactant] molar ratio (w_o). The optical density of the fluorescent samples at the excitation wavelength was low (generally <0.2) in all cases. Background samples were prepared the same way except that the melittin was not added to them. Samples were kept in the dark for 12 h before doing experiments and all experiments were done at room temperature (23 °C).

The molar ratio of peptide to surfactant was carefully chosen to give optimum signal-to-noise ratio with minimal perturbation to the micellar organization and negligible interprobe interactions. The final peptide concentration in the reverse micelles was 32 μM while the concentration of AOT was 50 mM in all cases. This corresponds to a final molar ratio of peptide to surfactant of 1:1562 (mol/mol). At such a low peptide to surfactant molar ratio, not more than one peptide molecule would be present per reverse micelle on an average which rules out any peptide aggregation effects, especially keeping in mind the aggregation number of AOT of ∼50−170 in the range of w_o between 5 and 15.[81]

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 band-pass of 5 nm were used for all measurements. All spectra were recorded using the correct spectrum mode. Background intensi-

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ties 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:[82]

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

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 equal to I_{HV}/I_{HH}.

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. This machine uses a thyratron-gated nanosecond flash lamp filled with nitrogen as the plasma gas (16 ± 1 in. of mercury vacuum) and is run at 22−25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal-to-noise ratio, 5000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits with a nominal band-pass of 4 nm or less. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam thereby avoiding any possible photodamage of the fluorophore. The data stored in a multichannel analyzer was routinely transferred to an IBM PC for analysis. Intensity decay curves so obtained were fitted as a sum of exponential terms:

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

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 experimentally observed decay parameters were analyzed as described earlier.[78] Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation:[83]

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

Global analysis of the lifetime data was performed as described earlier[77] keeping the lifetimes constant and allowing the amplitides to vary.[84] The software used for the global analysis was obtained from Photon Technology International (London, Western Ontario, Canada).

Circular Dichroism (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.[85] The spectra were scanned in a quartz optical cell with a path length of 0.1 cm. All spectra were recorded in 0.2 nm wavelength increments with a 4 s response and a bandwidth 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 remains unaltered. Data are represented as molar ellipticities and were calculated using the equation:

$$[\theta] = \theta_{obs}/(10Cl) \quad (4)$$

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

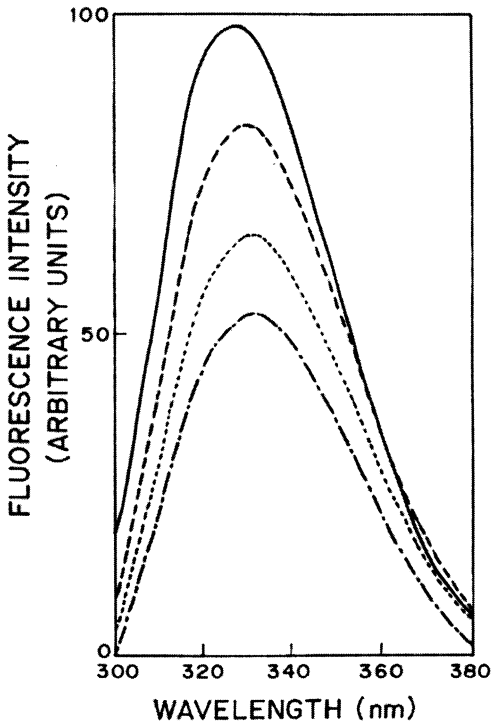

Figure 1. Effect of increasing amounts of added water on fluorescence emission spectra of melittin in AOT/heptane reverse micelles. Fluorescence emission spectra are shown as a function of [water]/[surfactant] molar ratio (w_o) in order of decreasing intensity corresponding to w_o = 0 (—), 6 (----), 10 (·····), and 15 (—·—·—·). The excitation wavelength used was 280 nm. The ratio of melittin to surfactant (AOT) was 1:1562 (mol/mol) and the concentration of melittin was 32 μM in all cases. See Experimental Section for other details.

Results

The fluorescence emission spectra of melittin in AOT/heptane reverse micelles as a function of increasing [water]/[surfactant] molar ratio (w_o) are shown in Figure 1. The maximum of fluorescence emission of melittin in AOT/heptane reverse micelles without any added water (w_o = 0) is 325 nm which is significantly blue shifted relative to the emission maximum in buffer[25] (~353 nm) or in membranes[23,25] (~335 nm). This blue shift in emission maximum indicates the nonpolar nature of the site of localization of the tryptophan residue of melittin in the reverse micellar assembly. However, the fluorescence emission maximum of melittin undergoes a progressive red shift of 7 nm (from 325 to 332 nm) when the water content is increased, that is, when w_o is increased from 0 to 15 (Figure 1). The shift is more pronounced till w_o = 8 beyond which no significant change in the fluorescence emission maximum is observed. This red shift in emission maximum is accompanied with a marked reduction (~40%) in peak fluorescence intensity when w_o is increased from 0 to 15 (see Figure 2). Taken together, these results clearly show that there is an increase in the polarity around the tryptophan of melittin in AOT reverse micelles because of an increase in water content. This is in agreement with

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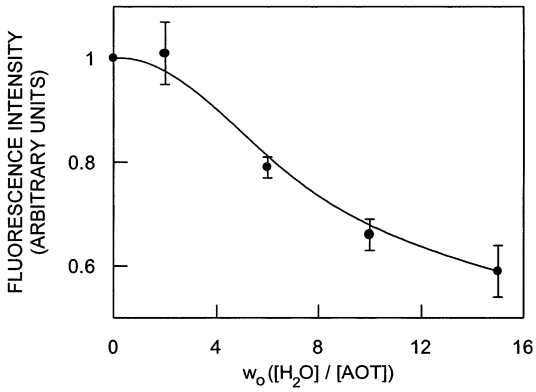

Figure 2. Effect of increasing water content on the fluorescence intensity of melittin in reverse micelles of AOT in heptane. Fluorescence intensity was monitored at 328 nm and is plotted as a function of w_o. The data points shown are the means of three independent measurements. The error bars represent the standard error. All other conditions are as in Figure 1. See Experimental Section for other details.

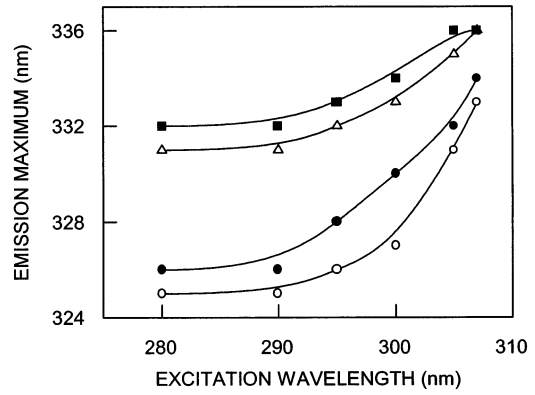

Figure 3. Effect of changing excitation wavelength on the wavelength of maximum emission of melittin in AOT/heptane reverse micelles corresponding to $w_o = 0$ (○), 2 (●), 6 (△), and 10 (■). All other conditions are as in Figure 1. See Experimental Section for other details.

a previous report in which it was shown that the changes in fluorescence emission maximum and intensity of tryptophan octyl ester (TOE), an important model for membrane-bound tryptophan,[71] in AOT/heptane reverse micelles exhibit a similar pattern.[86]

Figure 3 shows the shifts in the maxima of fluorescence emission[87] of melittin when bound to AOT/heptane reverse micelles as a function of excitation wavelength. As the excitation wavelength is changed from 280 to 307 nm, the emission maxima of melittin bound to reverse micelles of varying w_o display shifts toward longer wavelengths in all [water]/[surfactant] molar ratios (w_o). The emission maxima are shifted from 325 to 333 nm (in reverse micelles with no added water, i.e., $w_o = 0$), 326 to 334 nm ($w_o = 2$), 331 to 336 nm ($w_o = 6$), and 332 to 336 nm ($w_o = 10$), which correspond to REES of 4−8 nm in all cases. It is possible that there could be further red shift when melittin is excited beyond 307 nm. We found it difficult to work in this wavelength range because of low signal-to-noise ratio

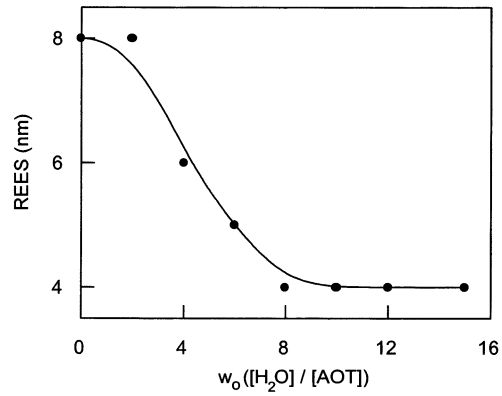

Figure 4. Effect of increasing amounts of water on the magnitude of red edge excitation shift (REES) of melittin in reverse micelles of AOT in heptane. REES data obtained from Figure 3 is plotted as a function of [water]/[surfactant] molar ratio (w_o). All other conditions are as in Figure 1. See Experimental Section for other details.

and artifacts due to the solvent Raman peak that sometimes remained even after background subtraction. Such dependence of the emission maximum with changes in excitation wavelength is characteristic of the red edge excitation shift. This implies that the sole tryptophan of melittin is localized in a motionally restricted environment. It is known from previous studies that when bound to membranes, the tryptophan residue of melittin is localized in the motionally restricted interfacial region of the membrane[23,25,88] and exhibits a similar red edge effect.[23,25] Thus, the observation of REES for melittin bound to reverse micelles would directly imply that the interfacial region of the reverse micelle offers considerable restriction to the reorientational motion of the solvent molecules (dipoles) around the excited-state fluorophore. The interfacial region of reverse micelles is associated with bound water with characteristic dynamics.[50−54] Since REES arises because of the rate of reorientational motion of solvent molecules, these results therefore assume significance in the context of recent reports of slow (∼ns) water relaxation in reverse micelles.[89−92]

The magnitude of REES obtained from Figure 3 as a function of w_o is shown in Figure 4. As the water content of the reverse micellar system increases, the magnitude of REES decreases gradually until $w_o = \sim 8$ is reached. At $w_o > 8$, REES attains a more or less steady value and becomes less sensitive to further addition of water into the system. The shift in emission maximum also attains a more or less steady value at this range of w_o (see Figure 1). This essentially means that there is a reorganization of the water molecules in the reverse micellar assembly upon increasing w_o from 0 to 8. This is in excellent agreement with earlier reports in which it was shown that water relaxation rates in reverse micelles become faster with an increase in w_o.[90] This suggests that the overall motional restriction experienced by the reorienting solvent molecules is reduced as more water is added to the reverse micelles. Similar results have previously been obtained for amphiphilic probes such as hemicyanine dye[93] or fluorescent phospholipid[78] incorporated in reverse

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(87) 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 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 emission has been reported as the fluorescence maximum.

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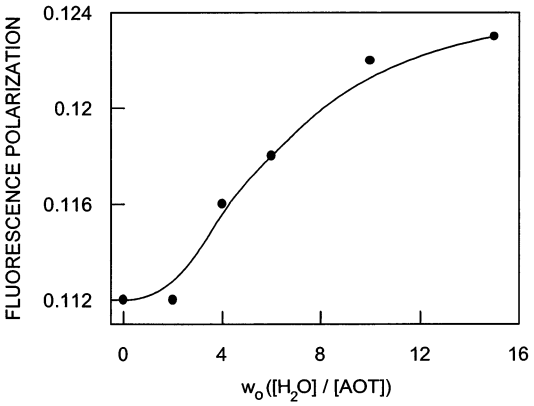

Figure 5. Effect of increasing hydration on the fluorescence polarization of melittin in AOT/heptane reverse micelles. Polarization values were recorded at 328 nm and the excitation wavelength used was 280 nm. All other conditions are as in Figure 1. See Experimental Section for other details.

micelles. Melittin in bulk water shows REES of ~1 nm under the same experimental conditions (not shown). To the best of our knowledge, our present observation constitutes the first report demonstrating that REES is sensitive to the changing dynamic hydration profile of an amphiphilic peptide. This can potentially offer a way to monitor dynamics of peptides in various states of hydration.

Interestingly, we report here REES of 8 nm for melittin in the so-called "dry micelles" ($w_o = 0$). This could be attributed to the interaction of polar headgroups of AOT with the excited state tryptophan of melittin which would result in motional restriction giving rise to REES. This indicates that the peptide experiences a motionally restricted environment even in the absence of hydration.

Figure 5 shows the steady-state polarization of melittin in AOT/heptane reverse micelles as a function of increasing amounts of water. The fluorescence polarization of melittin increases with increasing hydration implying increased rotational restriction experienced by the tryptophan residue with increase in w_o. The increase in the rotational restriction with increasing w_o could be rationalized as follows. At low values of w_o, the water molecules can form hydrogen bonds with the negatively charged polar headgroups of AOT[52] as well as to the melittin tryptophan. With increase in w_o, an increasing number of water molecules form hydrogen bonds with the melittin tryptophan giving rise to higher values of polarization (also see below).

Fluorescence polarization is also known to be dependent on excitation wavelength in motionally restricted media.[59] A plot of steady-state polarization of melittin incorporated in reverse micelles as a function of excitation wavelength is shown in Figure 6 for various values of w_o. The observed increase in polarization value, with a typical sharp dip at 290 nm, toward the red edge of the excitation band, is characteristic for peptides and proteins containing tryptophans localized in a restricted environment.[23,70] This strengthens our earlier conclusion that the tryptophan residue of melittin is localized in a motionally restricted environment in AOT reverse micelles. In agreement with Figure 5, higher polarization values are displayed by samples at higher w_o. This clearly shows the increased motional restriction of melittin as the water content of the system is increased.

In addition, we monitored the change in fluorescence polarization of melittin in AOT reverse micelles as a function of emission wavelength (see Figure 7). There is

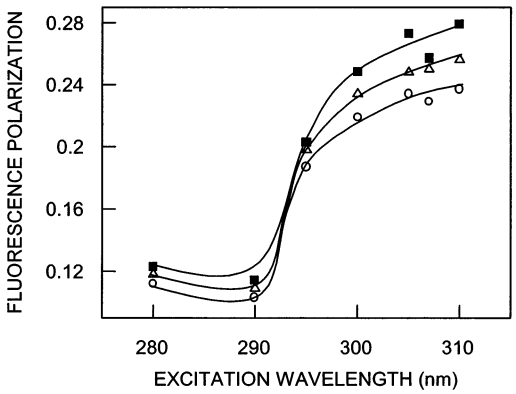

Figure 6. Fluorescence polarization of melittin in AOT/heptane reverse micelles corresponding to $w_o = 0$ (○), 6 (△), and 15 (■) as a function of excitation wavelength. Polarization values were recorded at 328 nm. All other conditions are as in Figure 1. See Experimental Section for other details.

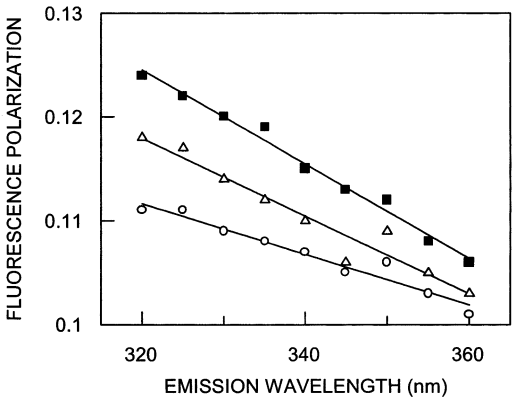

Figure 7. Fluorescence polarization of melittin in AOT/heptane reverse micelles corresponding to $w_o = 0$ (○), 6 (△), and 15 (■) as a function of emission wavelength. The excitation wavelength used was 280 nm. All other conditions are as in Figure 1. See Experimental Section for other details.

a considerable reduction in fluorescence polarization upon increasing the emission wavelength irrespective of the presence of water in reverse micelles and this decrease is more pronounced in higher [water]/[surfactant] molar ratios (w_o). The lowest polarization is observed toward longer wavelengths (red edge) where emission from the relaxed fluorophores predominates. Taken together, these results support that the tryptophan residue of melittin in AOT reverse micelles experiences increased motional restriction upon increasing the water content of the system.

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed.[94] In addition, it is well known that fluorescence lifetime of tryptophan in particular is sensitive to solvent, temperature, and excited-state interactions.[95,96] A typical decay profile of tryptophan residue of melittin incorporated in AOT/heptane reverse micelles with its biexponential fitting and the various statistical parameters used to check the goodness of the fit is shown in Figure 8.

The fluorescence lifetimes of reverse micelle-bound melittin for varying degrees of hydration are shown in Table 1. As seen from the table, all fluorescence decays could be fitted well with a biexponential function. To obtain a comprehensive picture of these lifetime changes, the

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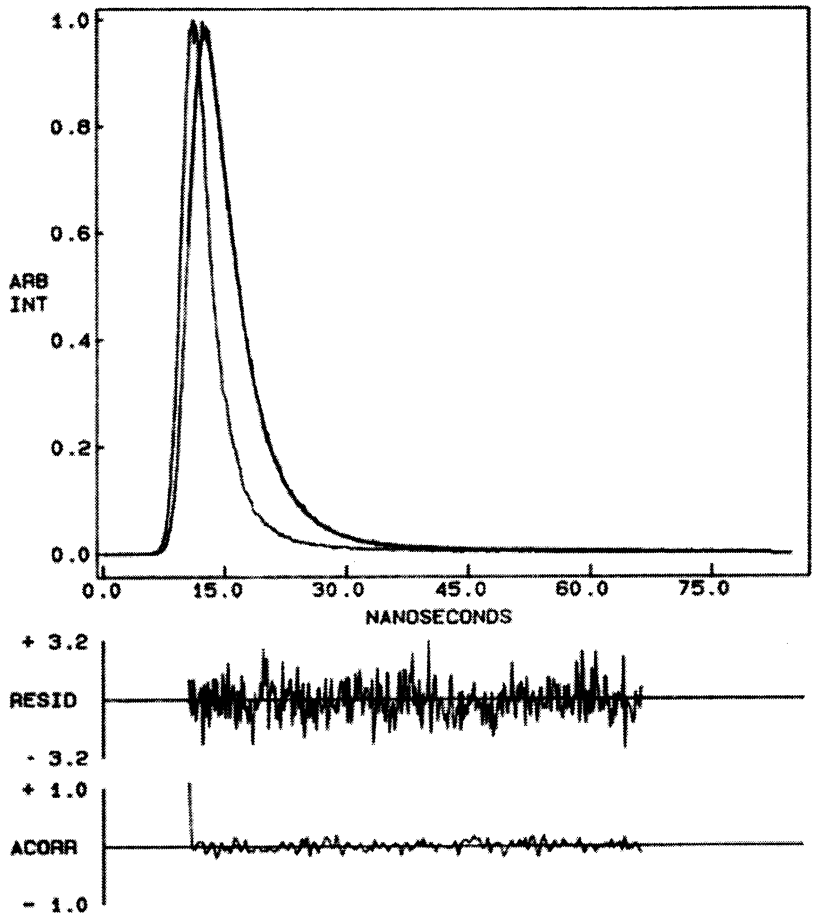

Figure 8. Time-resolved fluorescence intensity decay of melittin in reverse micelles of AOT in heptane. Excitation wavelength was at 297 nm which corresponds to a peak in the spectral output of the nitrogen lamp. Emission was monitored at 328 nm. The sharp peak on the left is the lamp profile. The relatively 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 1. See Experimental Section for other details.

Table 1. Lifetimes of Melittin in AOT/Heptane Reverse Micelles as a Function of w_o[a]

[water]/[surfactant] molar ratio (w_o)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
0	0.40 (0.52)[b]	3.99 (3.78)	0.60 (0.48)	2.44 (1.82)
2	0.45 (0.53)	3.98 (3.78)	0.55 (0.47)	2.04 (1.82)
4	0.28 (0.49)	4.31 (3.78)	0.73 (0.51)	2.50 (1.82)
6	0.41 (0.49)	3.93 (3.78)	0.59 (0.51)	2.09 (1.82)
8	0.36 (0.41)	3.90 (3.78)	0.65 (0.59)	2.03 (1.82)
10	0.23 (0.36)	4.24 (3.78)	0.77 (0.64)	2.15 (1.82)
12	0.10 (0.28)	5.05 (3.78)	0.90 (0.72)	2.23 (1.82)
15	0.30 (0.28)	3.69 (3.78)	0.70 (0.72)	1.45 (1.82)

[a] The excitation wavelength was 297 nm; emission was monitored at 328 nm. [b] Numbers in parentheses are the results of global analysis.

same set of fluorescence decays were subjected to global analysis. The decays were all assumed to be biexponential (on the basis of the results from discrete analysis), with lifetime components that were assumed to be linked among the data files and whose relative contribution (preexponential factors) was allowed to vary. The results of the global analysis are shown in parentheses in Table 1. The fittings of the set of decay profiles analyzed by the global method are presented as a pseudo-three-dimensional plot of intensity versus time versus increasing file number in Figure 9. This figure also shows the weighted residuals corresponding to each of these fittings. The normalized global χ^2 value obtained was 1.1.

The mean fluorescence lifetimes of melittin in AOT/heptane reverse micelles were calculated using eq 3 and are plotted as a function of w_o in Figure 10 for both discrete and global analysis. As shown in the figure, there is a continuous decrease in mean lifetime of melittin with increasing water content in the reverse micelles irrespective of the method of analysis (discrete or global). Thus when w_o is varied from 0 to 15, ~20% reduction in fluorescence lifetime of melittin is observed. The shortening of lifetime could possibly be attributed to a combination of two factors. In general, an increase in polarity of the tryptophan environment is known to reduce the lifetime of tryptophans because of fast deactivating processes in polar environments.[95] With increase in w_o, the polarity of the tryptophan environment would increase because of increased water content. This would result in a reduction in lifetime. More specifically, the shortening of the lifetime could be due to steric interaction between Trp-19 and Lys-23 which will be in close proximity in a helical arrangement[97] since increased helicity of melittin has been shown to be induced with an increase in w_o (see Figure 12). Since Lys-23 in melittin has a pK_a of 8.6,[98] 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 seen as a reduction in fluorescence intensity and lifetime (see Figures 2 and 10).[97] This is an example of

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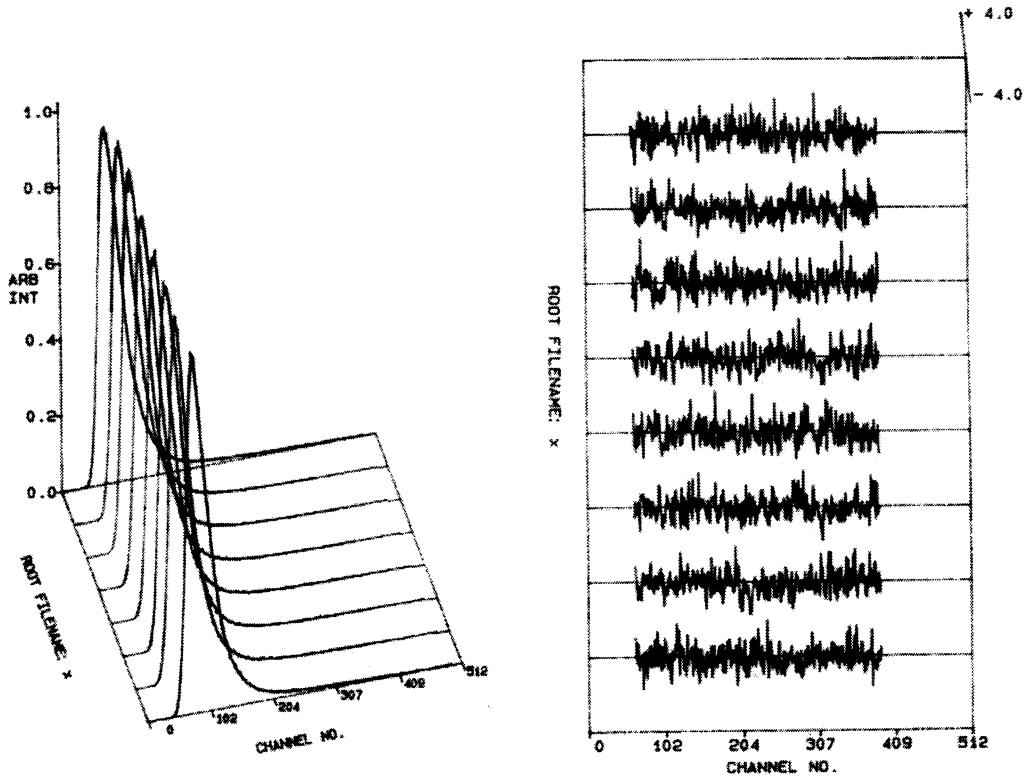

Figure 9. Global fittings and corresponding weighted residuals of the set of decay profiles of melittin in AOT/heptane reverse micelles obtained as a function of increasing [water]/[surfactant] molar ratio (w_o). All other conditions are as in Figure 1. See Experimental Section for other details.

cation−π interactions which have been shown to play an important role in biology.[99,100]

To ensure that the observed change in steady-state polarization (see Figure 5) as a function of w_o is not due to any change in lifetime with w_o (Figure 10), the apparent (average) rotational correlation times for the tryptophan residue of melittin in AOT/heptane reverse micelles were calculated using Perrin's equation:[83]

$$\tau_c = \frac{\langle\tau\rangle\, r}{r_o - r} \tag{5}$$

where r_o is the limiting anisotropy of tryptophan, r is the steady-state anisotropy (derived from the polarization values using $r = 2P/(3 - P)$), and $\langle\tau\rangle$ is the mean fluorescence lifetime as calculated from eq 3. The values of the apparent rotational correlation times, calculated this way using a value of r_o of 0.09,[101] are shown in Figure 11. There is a considerable increase in rotational correlation times of tryptophan residue of melittin with increasing hydration in AOT reverse micelles, which clearly shows that the observed change in polarization values (Figure 5) were not due to any lifetime-induced artifacts.

To investigate the effect of increasing hydration on the secondary structure of melittin, we carried out far-UV circular dichroism (CD) spectroscopy with varying w_o. The CD spectra of melittin in AOT/heptane reverse micelles in varying degrees of hydration are shown in Figure 12. It is well established that melittin in aqueous solution shows essentially random coil conformation as reported earlier by us[23] and others.[10] Our results show a dramatic

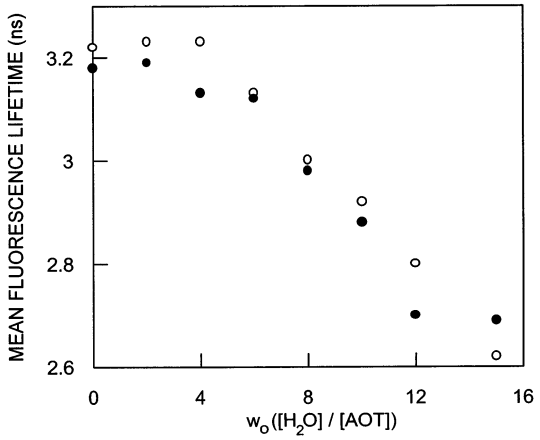

Figure 10. Effect of increasing amounts of water on mean fluorescence lifetime of melittin in AOT/heptane reverse micelles obtained by discrete (○) and global (●) lifetime analysis. The excitation wavelength used was 297 nm, and the emission was set at 328 nm. All other conditions are as in Figure 1. See Experimental Section for other details.

increase in helicity of melittin upon increasing the water content till $w_o = 6$ beyond which there is no significant change in the helical content of the peptide. The presence of helical conformation of melittin when bound to reverse micelles has been reported earlier by other groups.[12,102−104] However, there have been fewer reports monitoring secondary structure changes at very low values of w_o (<6). Our results clearly show that under conditions of low w_o, increasing hydration causes an increase in helicity of

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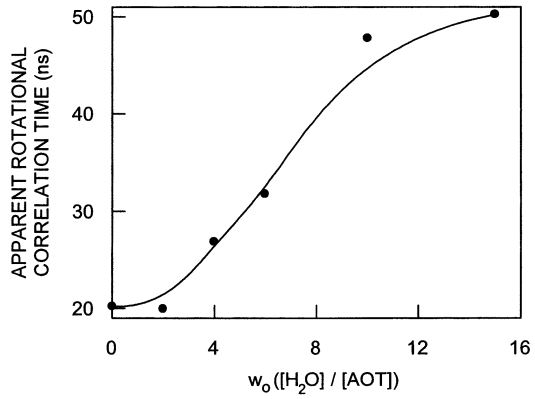

Figure 11. Effect of increasing water content on the apparent rotational correlation times of melittin in AOT/heptane reverse micelles. All the conditions are as in Figure 1. See text for other details.

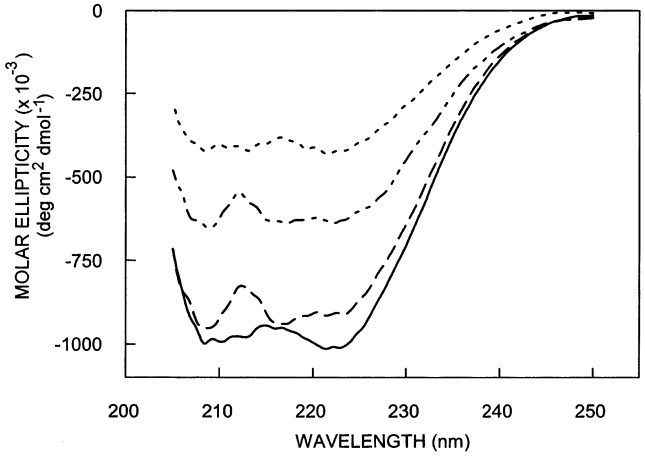

Figure 12. Effect of increasing hydration on the far-UV CD spectra of melittin in AOT/heptane reverse micelles corresponding to $w_o = 0$ (- - -), 2 (– - - – - -), 6 (— —), and 15 (—). All other conditions are as in Figure 1. See Experimental Section for other details.

melittin incoporated in AOT/heptane reverse micelles. This is further supported by our lifetime results (discussed above) in which a shortening of lifetime is observed (because of steric interaction between Trp-19 and Lys-23 which will be in close proximity in a helical arrangement) with increasing hydration in melittin incorporated in AOT reverse micelles.

Discussion

Knowledge of dynamics of hydration at the molecular level is of considerable importance in understanding the cellular structure and function since water plays a crucial role in the formation and maintenance of organized molecular assemblies such as proteins and membranes in a cellular environment.[31,33,35,36,67,68,105] In addition, water plays a crucial role in mediating lipid−protein interactions thereby controlling the functionality of membrane proteins.[31,38,43,105] For example, it was shown earlier using neutron diffraction of small peptides in membrane bilayers that there was a direct association of water with the peptides, possibly with a tryptophan residue.[106] It was suggested that such an effect, if enhanced by larger peptides, would be important for the insertion of helices into bilayers and for helix−helix interactions involving hydrogen bonding. Very recently, it has been shown that water molecules localized in the vicinity of highly conserved residues and in the retinal pocket of rhodopsin regulate the activity of rhodopsin and other G-protein-coupled receptors by mediating intermolecular interaction.[39]

Biological and model membranes (liposomes) and membrane−mimetic systems such as micelles are not appropriate for exploring the effect of hydration on the stability and function of proteins since the controlled variation of water content is difficult in these systems. This makes reverse micelles the system of choice in hydration studies. Reverse micelles are relatively simple yet versatile systems and represent ideal systems to address a variety of problems in biology including their application in reaction media[107] and in protein biotechnology.[108] For example, reverse micelles are used to study solvation dynamics of proteins confined to the micellar water pool.[109] A particular advantage of trapping a protein in reverse micelles is that its tumbling rate is altered resulting in well-resolved NMR signal which is otherwise difficult to obtain with large proteins.[110] Reverse micelles therefore represent an attractive model system for biomembranes since they mimic several important and essential features of biological membranes although lacking much of the complexity associated with biological membranes. Reverse micelles offer the unique advantage of monitoring the dynamics of bound molecules with varying degrees of hydration by varying the [water]/[surfactant] molar ratio (w_o) which is difficult to achieve with complex systems such as membranes. In addition, the hydration profile of fully hydrated reverse micellar interface has been shown to be similar to those reported for phospholipid vesicles in the liquid crystalline (fluid) state.[111]

It has been shown that three types of water populations (pools) coexist in reverse micelles. These are bound, trapped, and free water.[51,52,89] The crucial parameter is the [water]/[surfactant] molar ratio (w_o) which determines the relative proportions of these three types of water pools. The properties of water in reverse micelles of AOT at low w_o values are rather different from those of bulk water.[50−53] Even at higher water content ($w_o = 50$), the apparent microviscosity is 6−9 times greater than that of free aqueous solutions.[112] The highly structured yet heterogeneous water molecules in reverse micelles therefore represent interesting models for water molecules present in biological systems such as membranes which are more difficult to analyze experimentally.

The application of wavelength-selective fluorescence approach in combination with circular dichroism spectroscopy to monitor the effect of hydration on the dynamics and conformation of melittin has been the focus of this report. As mentioned earlier, melittin has a single tryptophan residue which makes it a convenient peptide for studying its dynamics under restricted hydration conditions. This makes interpretation of fluorescence data less complicated because of lack of heterogeneity that is often encountered while analyzing fluorescence from multitryptophan proteins and peptides. For this reason, single tryptophan proteins are popular for monitoring

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solvation dynamics.[113,114] These studies have pointed out that the rate of solvation dynamics depends crucially on the location of the sole tryptophan in the protein.

We show here that melittin, when incorporated into reverse micelles of AOT with varying amounts of water content, exhibits wavelength-selective fluorescence effects. Our results show that fluorescence parameters such as intensity, emission maximum, and REES of melittin incorporated in AOT reverse micelles depend on [water]/[surfactant] molar ratio (w_o). More importantly, the extent of REES decreased with increasing w_o indicating that REES is sensitive to changing hydration and it is possible to detect the differences in water dynamics that is accompanied with increasing water content. The rotational mobility of melittin is considerably reduced upon increasing hydration as shown by increasing fluorescence polarization values and rotational correlation times. In addition, time-resolved fluorescence measurements of melittin in AOT/heptane reverse micelles show a significant reduction in the mean fluorescence lifetime with increasing w_o which could be correlated to the increased helicity of melittin. Taken together, these results imply that melittin, when bound to AOT reverse micelles, is in motionally restricted interfacial region of reverse micelles. In addition, our results show that hydration significantly affects the dynamics and conformation of melittin bound to reverse micelles.

As mentioned earlier, REES is based on the change in fluorophore−solvent interactions in the ground and excited states brought about by a change in the dipole moment of the fluorophore upon excitation and the rate at which solvent molecules reorient around the excited-state fluorophore. Further, since the ubiquitous solvent for biological systems is water, the information obtained in such cases will come from the otherwise "optically silent" water molecules. The unique feature about REES is that while all other fluorescence techniques (such as fluorescence quenching, energy transfer, polarization measurements) yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation which is not possible to obtain by other techniques. Since the dynamics of hydration is related with functionality of proteins, wavelength-selective fluorescence approach in general, and REES in particular, could prove to be a novel and extremely powerful tool to probe organization and dynamics of peptides and proteins under varying degrees of hydration. Whether the observed effects of hydration on dynamics of melittin associate with the hemolytic activity of melittin poses an interesting question and needs to be explored.

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