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Effect of Ionic Strength on Folding and Aggregation of the Hemolytic Peptide Melittin in Solution

Abstract: *Melittin is a cationic, amphipathic, hemolytic peptide composed of 26 amino acid residues. It is intrinsically fluorescent due to the presence of a single tryptophan residue, which has been shown to be crucial for its hemolytic activity. It undergoes a structural transition from a random coil monomer to an α-helical tetramer at high ionic strength. Although the aggregation behavior of melittin in solution is well characterized, dynamic information associated with the aggregation of melittin is lacking. In this paper, we have monitored the effect of ionic strength on the dynamics and aggregation behavior of melittin in aqueous solution by utilizing sensitive fluorescence approaches, which include the red edge excitation shift (REES) approach. Importantly, we demonstrate that REES is sensitive to the self-association of melittin induced by ionic strength. The change in environment experienced by melittin tryptophan(s) is supported by changes in fluorescence emission maximum, polarization, and lifetime. In addition, the accessibility of the tryptophan residue was probed by fluorescence quenching experiments using acrylamide and trichloroethanol as soluble and hydrophobic quenchers, respectively. Circular dichroism studies confirm the ionic strength–induced change in the secondary structure of melittin. Taken together, these results constitute the first report showing that REES could be used as a sensitive tool to monitor the aggregation behavior of melittin in particular and other proteins and peptides in general.* © 2006 Wiley Periodicals, Inc. Biopolymers 83: 111–121, 2006

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INTRODUCTION

Melittin is the principal toxic component in the venom of the European honey bee, *Apis mellifera*. It is a small linear peptide composed of 26 amino acid residues (NH$_2$-GIGAVLKVLTTGLPALISWIKRKRQQ-CONH$_2$) and is known to have a powerful hemolytic activity.[1] It is a cationic peptide in which the amino terminal is composed predominantly of hydrophobic amino acids (residues 1–20), whereas the carboxy terminal end has a stretch of predominantly hydrophilic amino acids (residues 21–26), which give rise to its amphiphilic character. This amphiphilic property of melittin makes it water soluble and yet it spontaneously associates with natural and artificial membranes.[2,3] 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.[2,4] This has resulted in melittin being used as a convenient model to

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monitor 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.[2,5]

Melittin is intrinsically fluorescent due to the presence of a single tryptophan residue, Trp-19, which makes it a sensitive probe to monitor the interaction of melittin with membranes[6–11] and membrane–mimetic systems.[12,13] This is particularly advantageous since there are no other aromatic amino acids in melittin and this makes interpretation of fluorescence data less complicated due to lack of interference and heterogeneity. 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[14] and substitution by leucine.[15] This is further reinforced by studies with single amino acid omission analogues of melittin.[16] 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 adopts predominantly random coil conformation as a monomer in aqueous solution.[17] However, at high ionic strength, pH, or peptide concentration, it self-associates to form an α-helical tetrameric structure.[2,17] These factors strongly suppress the charge of melittin and promote the self-association of melittin monomers into tetramers since melittin has a high charge density (+6 at neutral pH) and aggregation would be prevented by electrostatic repulsion. Importantly, the conformational diversity of melittin (random coil monomer to α-helical tetramer) due to environmental factors presents an opportunity to characterize protein folding under a broad range of conditions without the use of denaturants.[18] The three-dimensional X-ray crystal structure of tetrameric melittin in aqueous solution is available at high resolution (2 Å).[19] The interior of the tetramer is nonpolar due to the packing of hydrophobic residues, which also includes tryptophan, the intrinsic fluorophore of melittin. Interestingly, the hydrophobic residues that form the interior of the melittin tetramer are involved in melittin–solvent interactions.[19] However, the interaction between monomers in the tetrameric form of melittin has been shown to be weaker in solution than that in the crystal structure.[20] A number of studies have characterized the factors responsible for the aggregation behavior of melittin and its analogues in aqueous solution utilizing various techniques.[17,18,21–24] The consensus is that the self-association of melittin in aqueous solution is a complex process and depends on the interplay between peptide concentration and solution properties (ionic strength and pH), which in turn affect the hydrophobic, electrostatic, and helix–dipole interactions at the N-terminus of melittin.[2,24]

Although a number of reports have earlier described the aggregation behavior of melittin in solution, dynamic information associated with the aggregation of melittin is still lacking. Since melittin targets membranes though the aqueous phase, the information about the dynamics of melittin in solution is relevant for its effect on membranes. In this paper, we have monitored the effect of ionic strength on the aggregation behavior of melittin utilizing sensitive fluorescence techniques, which include the red edge excitation shift (REES) approach, and circular dichroism (CD) spectroscopy in a comprehensive manner. REES represents a powerful approach that can be used to directly monitor the environment and dynamics around a fluorophore in a complex biological system.[25,26] We have previously shown that REES serves as a sensitive tool to monitor the organization and dynamics of peptides and proteins in solution[27–29] and when bound to membranes and membrane–mimetic systems.[9–13,30,31] In this paper, we demonstrate for the first time that the information on the dynamics of hydration obtained by REES could be used as a powerful tool to monitor the self-association of melittin in solution. This approach could be useful in analyzing the complex aggregation behavior of peptides and proteins in solution.

EXPERIMENTAL

Materials

Melittin, NaCl, 3-(*N*-morpholino)propanesulfonic acid (MOPS), EDTA, and trichloroethanol (TCE) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The concentration of melittin in aqueous solution was calculated from its molar extinction coefficent (ε) of 5570 $M^{-1}cm^{-1}$ at 280 nm.[9,22] Ultrapure acrylamide was from Invitrogen Life Technologies (Carlsbad, CA, USA). The purity of 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.[32] All of the other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

Sample Preparations

Melittin from an aqueous stock solution was added to 1.5 mL of 10 m*M* MOPS, 5 m*M* EDTA, pH 7.0 buffer containing various concentrations of NaCl ranging from 0 to 2 *M* and mixed well. The concentration of melittin (20 μM) was kept constant in all experiments. We chose this concentration to

avoid inner filter effects. Background samples were prepared in the same way except that melittin was not added to them. 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 (Tokyo, Japan) using 1 cm path length quartz cuvettes. Excitation and emission slits with a nominal bandpass of 5 nm were used for all measurements unless mentioned otherwise. All spectra were recorded using the correct spectrum mode (which is used to measure the spectrum inherent to the sample unaffected by the wavelength characteristic of the instrument and is determined by utilizing the correction function stored through excitation and emission calibrations). 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. Fluorescence polarization measurements were performed at room temperature (23°C) using a Hitachi polarization accessory. Polarization values were calculated from the equation[33]:

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

where I_{VV} and I_{VH} are the measured fluorescence intensities (after appropriate background subtraction) with the excitation polarizer vertically oriented and the 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 polarization are shown in Figure 4. The spectral shifts obtained with different sets of samples were identical in most cases. In other cases, the values were within ±1 nm of those reported.

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 inches of mercury vacuum) and is run at 17–19 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal to noise ratio, 10,000 photon counts were collected in the peak channel. All experiments were performed using excitation and emission slits with a nominal bandpass 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 were routinely transferred to an IBM PC for analysis. Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms:

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

where $F(t)$ is the fluorescence intensity at time t and α_i is a preexponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\Sigma_i \alpha_i = 1$. The decay parameters were recovered and analyzed as described previously.[12] Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and preexponential factors using the following equation[33]:

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

Fluorescence Quenching Measurements

Acrylamide and TCE quenching experiments of tryptophan fluorescence in melittin solutions containing various concentrations of salt were carried out by measurement of fluorescence intensity after serial addition of small aliquots of either a freshly prepared stock solution of 8 *M* acrylamide in water or neat TCE (10.42 *M*) to a stirred sample followed by incubation for 2 min in the sample compartment in the dark (shutters closed). The excitation wavelength used was 295 nm and emission was monitored at 342 nm. Corrections for inner filter effect were made using the following equation[33]:

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

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. Inner filter effects in the case of TCE quenching were negligible. Quenching data were analyzed by fitting to the Stern–Volmer equation[33]:

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

where F_0 and F are the fluorescence intensities in the absence and presence of the quencher (acrylamide or TCE), respectively, $[Q]$ is the molar quencher (acrylamide or TCE) concentration, and K_{SV} is the Stern–Volmer quenching constant. 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 (acrylamide or TCE).
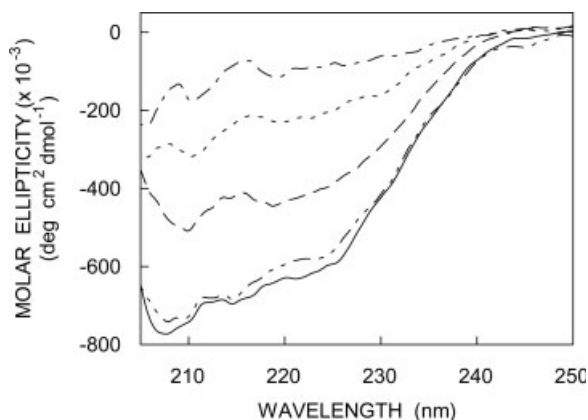
FIGURE 1   Effect of increasing ionic strength on the secondary structure of melittin. Far-UV CD spectra of melittin in buffer (– · – · –) containing varying amounts of NaCl corresponding to 0.4 (········), 0.8 (– – —), 1 (– · · – · · –), and 2 (————) M NaCl. The concentration of melittin used was 20 μM. See Experimental for other details.

Circular Dichroism Measurements

CD measurements were carried out at room temperature (23°C) in a JASCO J-715 spectropolarimeter (Tokyo, Japan) that was calibrated with (+)-10-camphorsulfonic acid. 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 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 10 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:

$$[\theta] = \theta_{obs}/(10C\ell) \tag{6}$$

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

RESULTS

Folding and Aggregation of Melittin in Solution

To monitor the effect of ionic strength on the secondary structure of melittin, we carried out far-UV CD spectroscopy of melittin in aqueous solution containing various amounts of salt (shown in Figure 1). As shown in the figure, melittin in aqueous solution

adopts random coil conformation in the absence of salt as reported previously.[18] Upon increasing the ionic strength, the propensity to form α-helix increases significantly, as shown by increasing helicity in the CD spectra of melittin with increasing salt concentration (see Figure 1). The helical content of melittin reaches a maximum at 1 M NaCl beyond which there is no significant change in the secondary structure of melittin. It is well established that melittin is predominantly in a tetrameric conformation at high salt concentration.[17,18,22,23] This is due to the charge screening effect of ions, which decreases the electrostatic repulsion among melittin monomers, thus stabilizing the melittin tetramer at high salt concentrations.[21,23,34] We therefore interpret the change in CD spectra with increasing ionic strength to be indicative of the folding and aggregation of melittin monomers to tetramer.

Effect of Ionic Strength on Fluorescence of Melittin in Aqueous Solution

The fluorescence emission spectra of melittin in buffers of varying ionic strength are shown in Figure 2a. The maximum of fluorescence emission of melittin in the absence of salt is 349 nm, which is in agreement with the previously reported value.[35] (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 of these methods yielded the same wavelength. In cases in which minor discrepancies were found, the center of mass of emission has been reported as the fluorescence maximum.) This suggests that the tryptophan residue of melittin is exposed to the surrounding aqueous medium. The fluorescence emission maximum exhibits significant blue shift to shorter wavelengths in the presence of increasing concentrations of salt (Figure 2a). The maximum of fluorescence emission of melittin with increasing ionic strength and the corresponding blue shift are shown in Figure 2b. As is evident from the figure, there is a significant change in the fluorescence emission maximum when the salt concentration is varied in the range of 0.2 to 1 M. The overall change in the emission maximum of melittin corresponds to a blue shift of 14 nm (349 to 335 nm) in the presence of up to 2 M salt. In general, tryptophan fluorescence is known to be sensitive to the polarity of its immediate environment.[36] The blue shift of the emission maximum could therefore be attributed to a change in the environment of tryptophan residue(s) caused by the
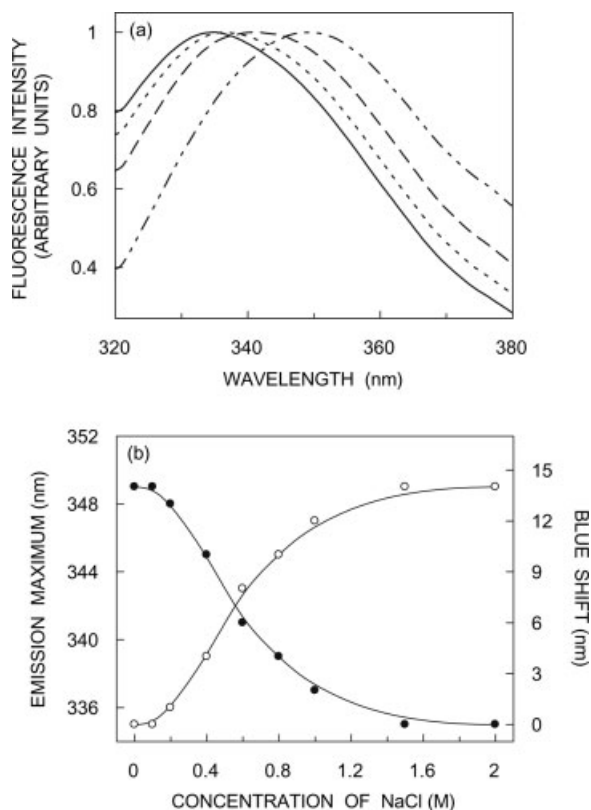
FIGURE 2 (a) Effect of increasing ionic strength on fluorescence emission spectra of melittin in 10 mM MOPS, 5 mM EDTA, pH 7.0 buffer. Spectra shown correspond to the absence (– · · – · · –) and presence of 0.6 (– – –), 1 (· · · ·), and 2 (——) M NaCl. (b) Fluorescence emission maximum (●) and the extent of blue shift in emission maximum (○) of melittin in aqueous solution as a function of increasing NaCl concentration. The spectra are intensity-normalized at the respective emission maximum. The excitation wavelength used was 280 nm and the concentration of melittin was 20 μM. See Experimental for other details.

self-association of melittin to the tetrameric form at high ionic strength, which would decrease the polarity experienced by the tryptophan(s). In the tetrameric form of melittin, it is known that the four tryptophan residues are partially exposed to solvent and are therefore less accessible.[19] In addition, the accompanying transition from random coil to α-helical conformation induced by ionic strength would result in shielding of tryptophan residue(s) from the aqueous polar environment.

Red Edge Excitation Shift of Melittin in Solution

As mentioned earlier, REES represents a powerful approach to directly monitor the environment and dynamics around a fluorophore in a complex biological

system.[25,26] A shift in the wavelength of maximum fluorescence emission toward higher wavelengths, caused by a shift in the excitation wavelength toward the red edge of the absorption band, is termed 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.[25,26,37] REES provides information about the relative rates of water relaxation (reorientation) dynamics and therefore this approach is widely used to monitor the dynamics of hydration in complex biological systems.

The shifts in the maxima of fluorescence emission of melittin with increasing ionic strength are shown in Figure 3a. The magnitude of REES of the sole tryptophan residue of melittin in buffer with increasing ionic strength is shown in Figure 3b. As the excitation wavelength is changed from 280 to 307 nm, the maximum of emission wavelength of the melittin tryptophan changes from 349 to 350 nm in the absence of any added salt and from 335 to 340 nm in the presence of 2 M salt. This corresponds to a REES of 1 and 5 nm for the monomeric and tetrameric forms of melittin, respectively. Such dependence of the emission maximum on excitation wavelength is characteristic of REES. Since monomeric melittin adopts random coil conformation in solution, the relatively small magnitude of REES implies that the tryptophan of melittin is exposed to the bulk aqueous environment where the solvent relaxation is very fast. This situation is comparable to exposed tryptophans in denatured proteins. We have previously shown that such exposed tryptophans generally do not exhibit REES.[27] On the other hand, a REES of 5 nm for tetrameric melittin implies that tryptophan residues of melittin are, on the average, localized in a motionally restricted region of the tetramer. This could be due to the fact that tryptophan residues are in the interior of the melittin tetramer and are in contact with bound water molecules as well as polar residues (as shown in the crystal structure of melittin tetramer),[19] which could contribute to slow solvent relaxation. Interestingly, the magnitude of REES is found to be dependent on the ionic strength of the medium. A significant change in the magnitude of REES is observed upon increasing the concentration of NaCl from 0.1 to 1 M beyond which the magnitude of REES appears to be invariant. This could be attributed to an increase in the motional restriction of solvent molecules around the excited-state tryptophan residue(s) with increasing ionic strength, possibly in response to the structural transition of melittin from monomer to tetramer.
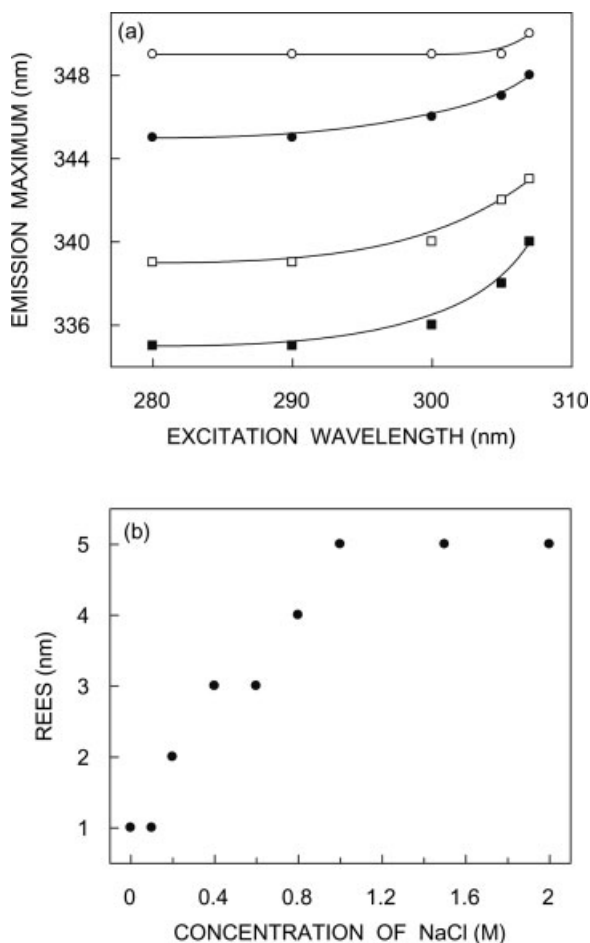
FIGURE 3   (a) Effect of changing excitation wavelength on the wavelength of maximum emission of melittin in aqueous solution corresponding to 0 (○), 0.4 (●), 0.8 (□), and 2 (■) *M* NaCl. (b) Effect of increasing ionic strength on the magnitude of REES of melittin in aqueous solution. The concentration of melittin was 20 *μM* in all cases. See Experimental for other details.

This is suggestive of salt-induced self-association of melittin being coupled to the change in the dynamics of hydration around the tryptophan residue. To the best of our knowledge, this is the first observation demonstrating that REES could be used as a potential tool to monitor the aggregation behavior of an amphiphilic peptide such as melittin in solution.

Fluorescence Polarization and Lifetime Measurements of Melittin in Solution

The steady state fluorescence polarization of melittin in solution with increasing ionic strength is shown in Figure 4. The fluorescence polarization of melittin increases with increasing salt concentration, implying

increased rotational restriction experienced by the tryptophan residue(s) with increasing salt. The increase in the rotational restriction with increasing salt concentration could be attributed to the structural transition of melittin with increasing ionic strength. In addition, the shape and size of the monomer and tetramer forms of melittin are significantly different, which would also contribute to the increased rotational restriction leading to higher polarization.

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed.[38] In addition, it is well known that the fluorescence lifetime of tryptophan in particular is sensitive to solvent, temperature, and excited state interactions.[36,39] The fluorescence lifetimes of melittin in aqueous solution containing varying amounts of salt are shown in Table I. 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 Figure 5. The lifetime of the tryptophan residue of the monomeric melittin in buffer is ∼ 2.9 ns, similar to previously reported values.[13,40,41] As shown in the figure, there is a sharp decrease in the mean fluorescence lifetime of the tryptophan residue(s) of melittin upon increasing the NaCl to 0.8 *M*. In general, a decrease in polarity of the tryptophan environment is known to increase the fluorescence lifetime of tryptophans due to fast deactivating processes in polar environments.[36]
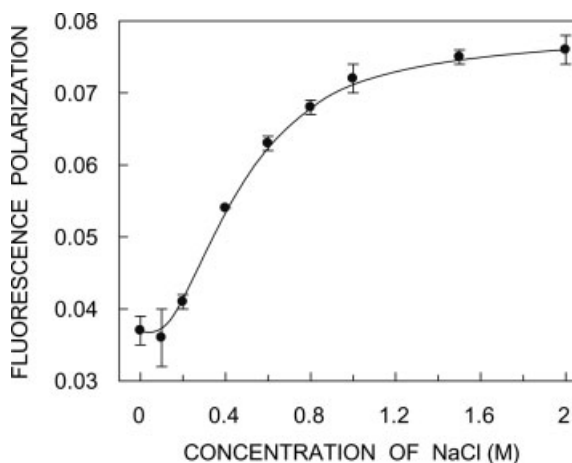

FIGURE 4   Effect of increasing ionic strength on the fluorescence polarization of melittin in aqueous solution. Polarization values were recorded at 342 nm and the excitation wavelength used was 280 nm. All other conditions are as in the legend to Figure 2. The data points shown are the mean ± SD of three independent measurements. See Experimental for other details.

Table I   Effect of Ionic Strength on Fluorescence Lifetimes of Melittin[a]

NaCl (M)	α_1	τ_1 (ns)	α_2	τ_2 (ns)
0	0.24	4.21	0.76	1.99
0.1	0.26	3.80	0.74	1.76
0.2	0.25	3.79	0.75	1.72
0.4	0.29	3.61	0.71	1.56
0.6	0.24	3.41	0.76	1.50
0.8	0.23	3.18	0.77	1.59
1.0	0.19	3.80	0.81	1.74
1.5	0.22	3.69	0.78	1.57
2.0	0.16	4.01	0.84	1.78

[a] The excitation wavelength was 297 nm; emission was monitored at 342 nm. All other conditions are as in the legend to Figure 2. See Experimental for other details.

However, in the present case, the polarity around the tryptophan residue(s) should decrease due to the change in conformation from random coil to α-helical monomer and the self-association of melittin monomers (see Figures 1 and 2). The decrease in the fluorescence lifetime could therefore possibly be due to steric interaction between Trp-19 and Lys-23, which will be in close proximity in a helical arrangement,[42,43] since increased helicity of melittin has been shown to be induced with an increase in salt concentration (see Figure 1). Since Lys-23 in melittin has a pK_a of 8.6,[44] it will be positively charged in aqueous medium at neutral pH. Interestingly, only the protonated form of the amino group of Lys-23 is believed to be an efficient quencher of tryptophan fluorescence.[43] This is an example of cation–π interaction, which has been shown to play an important role in a variety of biological processes.[45] Interestingly, the mean lifetime of tryptophan residues of melittin is slightly increased when the concentration of NaCl reaches above 0.8 M, beyond which there is no significant change in the fluorescence lifetime of melittin. It should be noted that the conformational transition from the monomeric to the tetrameric form of melittin is favored at high salt concentration (1 M NaCl and above). The small increase in lifetime of melittin at high salt concentration (> 0.8 M) could therefore be attributed to the formation of the melittin tetramer in which the tryptophan residues are located in the interior of the tetramer, which is predominantly nonpolar in nature.[19] Overall, these results suggest that the bulk polarity effect as well as the specific cation–π interactions are responsible for the shortening of the fluorescence lifetime of melittin at salt concentrations below 1 M.

To ensure that the observed change in steady state polarization (Figure 4) is not due to any change in lifetime, the average apparent rotational correlation times for the tryptophan residue(s) of melittin in solution were calculated using Perrin's equation[33]:

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

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 $\langle\tau\rangle$; is the mean fluorescence lifetime calculated using Eq. (3) (shown in Figure 5). The values of the apparent rotational correlation times, calculated this way using a value of r_0 of 0.16,[46] are shown in Figure 5. There is a considerable increase in rotational correlation times of tryptophan residue(s) of melittin with increasing ionic strength, which clearly shows that the observed change in fluorescence polarization (Figure 4) is not due to any lifetime-induced artifacts.

The rotational correlation times for monomeric and tetrameric forms of melittin deserve comment. The reported rotational correlation times correspond to the rotational mobility of tryptophan residue(s) of melittin and to not the entire protein in the case of melittin tetramer. The rotational correlation time obtained for the tryptophan residue of tetrameric melittin (~ 1.1 ns) is in excellent agreement with the previously reported value (1.05 ns).[42] Interestingly, considering the volume and shape of the tryptophan residue, the calculated rotational correlation time of 0.53 ns for monomeric melittin is significantly higher that the value expected for the rotational motion of tryptophan alone (0.17 ns).[42] This is not surprising since the backbone of monomeric melittin is flexible
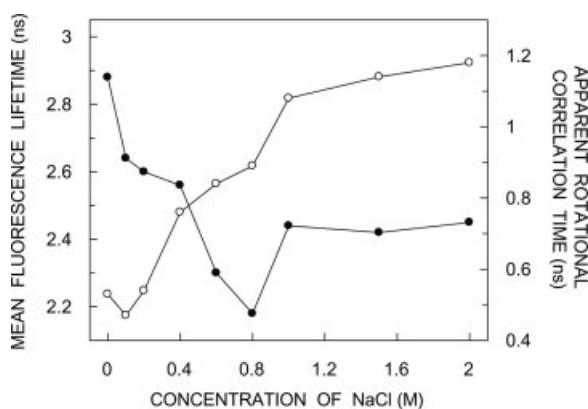

FIGURE 5 Mean fluorescence lifetimes (●) and apparent rotational correlation times (○) of melittin in buffer containing increasing concentrations of NaCl. The excitation wavelength used was 297 nm, and emission was monitored at 342 nm. Mean lifetimes were calculated from Table I using Eq. (3). See Experimental and Results for other details.

and this gives rise to the possibility that the tryptophan residue in monomeric melittin moves collectively with other residues. The calculated rotational correlation time for monomeric melittin does not therefore represent only the rotational motion of tryptophan residue.

Quenching of Melittin Tryptophan Fluorescence

The above results show that the ionic strength-induced self-association of melittin from a random coil monomeric to tetrameric entity is accompanied by a marked change in hydration dynamics (see Figure 3). To comprehensively examine the change in the accessibility of different forms of melittin to solvent molecules induced by change in ionic strength, fluorescence quenching experiments were performed with both aqueous and hydrophobic quenchers, which would effectively quench the exposed and buried tryptophan residue(s), respectively. We carefully chose neutral quenchers so as to avoid any specific interaction with the quencher and melittin since this peptide possesses +6 and +24 charges in its monomer and tetramer forms, respectively.[47]

Acrylamide is a widely used neutral aqueous quencher of tryptophan fluorescence. Acrylamide quenching of tryptophan fluorescence is widely used to monitor tryptophan environments in proteins and peptides.[48] Figure 6a shows representative Stern–Volmer plots of acrylamide quenching of melittin tryptophan(s) with increasing ionic strength. The linearity of the plot with no apparent downward curvature, even at high ionic strength, indicates minimal heterogeneity in tryptophan population. The slope of the Stern–Volmer plot, the Stern–Volmer constant (K_{SV}), is related to the degree of exposure (accessibility) of the melittin tryptophan to the water-soluble quencher. In general, the higher the slope, the greater the degree of exposure, assuming that there is not a large change in fluorescence lifetime (in the absence of quencher). The Stern–Volmer constants obtained by analysis of quenching data with increasing ionic strength are shown in Figure 6b. The K_{SV} value for monomeric melittin in the absence of any added salt was found to be 18 M^{-1}, whereas the corresponding value for the tetramer (1–2 M salt) is $\sim 10\ M^{-1}$. This indicates the accessibility of melittin tryptophan(s) is considerably reduced upon tetramerization. However, interpretation of K_{SV} values is complicated this way due to its intrinsic dependence on fluorescence lifetime [see Eq. (5)]. The bimolecular quenching constant (k_q) is therefore a more accurate measure of the degree of exposure since it takes into account the dif-
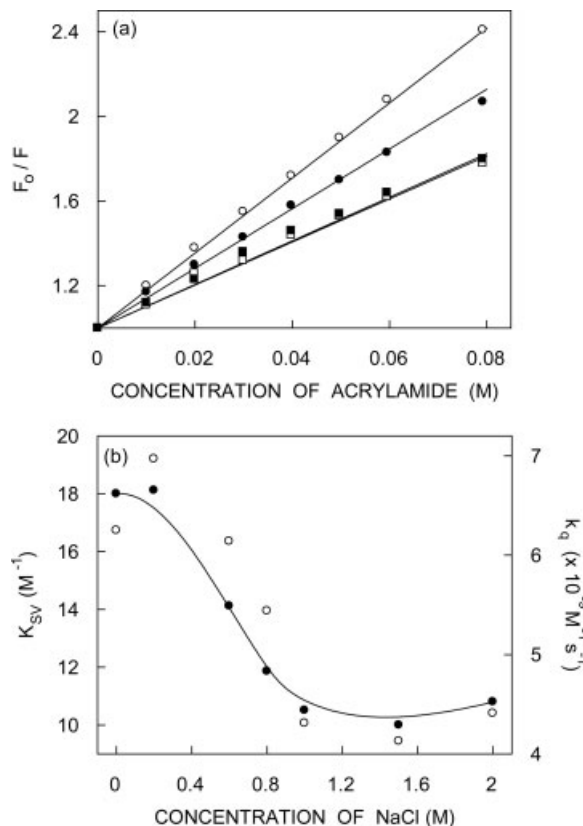

FIGURE 6 (a) Representative data for Stern–Volmer analysis of acrylamide quenching of tryptophan fluorescence of melittin in buffer containing varying amounts of NaCl corresponding to the absence (○) and presence of 0.6 (●), 1 (□), and 2 (■) M NaCl. (b) Stern–Volmer quenching constants, K_{SV} (●), and bimolecular quenching constants, k_q (○), for acrylamide quenching of melittin tryptophan fluorescence as a function of increasing ionic strength. The excitation wavelength was 295 nm and emission was monitored at 342 nm. All other conditions are as in the legend to Figure 2. See Experimental for other details.

ferences in fluorescence lifetime (in the absence of quencher). The k_q values, calculated using mean fluorescence lifetimes from Figure 5 and Eq. (5), are shown in Figure 6b. The k_q values are in overall agreement with K_{SV} values, implying that the conclusions derived by the analysis of Stern–Volmer constants are not influenced by changes in lifetime.

TCE is a hydrophobic neutral quencher of tryptophan fluorescence that is less polar than acrylamide and has been shown to be a more effective hydrophobic quencher than acrylamide.[49,50] The advantage of TCE as a quencher lies in the fact that it can penetrate into the interior of the protein matrix because of its relatively nonpolar nature. The representative Stern–Volmer plots of TCE quenching of melittin tryptophan(s) at different ionic strengths are shown in
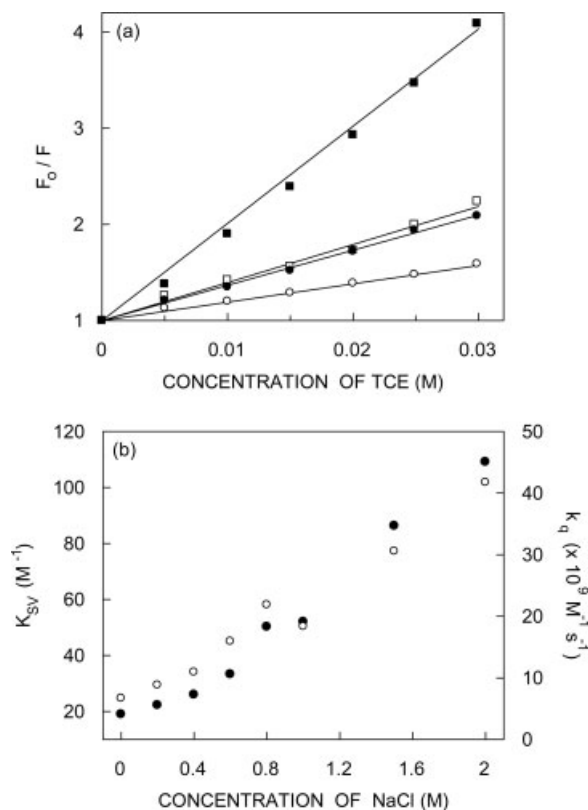
FIGURE 7 (a) Representative data for Stern–Volmer analysis of TCE quenching of tryptophan fluorescence of melittin in buffer containing varying amounts of NaCl corresponding to the absence (○) and presence of 0.6 (●), 1 (□), and 2 (■) *M* NaCl. (b) Stern-Volmer quenching constants, K_{SV} (●), and bimolecular quenching constants, k_q (○), for TCE quenching of melittin tryptophan fluorescence as a function of increasing ionic strength. The excitation wavelength was 280 nm and emission was monitored at 342 nm. All other conditions are as in the legend to Figure 2. See Experimental for other details.

Figure 7a. The K_{SV} value for monomeric melittin in the absence of added salt was found to be 19 M^{-1}. Increasing the salt concentration considerably increases the accessibility of melittin tryptophan(s) as evident from the increased K_{SV} values. This clearly suggests that TCE could quench the fluorescence of tryptophan residue(s) of melittin that is not completely exposed to aqueous environment and is present in the interior of the melittin aggregates. This is further supported by increased k_q values as a function of increasing ionic strength. Taken together, these results provide insight into the accessibility of tryptophan residue(s) during the self-association of melittin with increasing ionic strength and are consistent with the overall idea of loose packing of melittin tetramer in solution compared with the crystal structure of tetrameric melittin.[20,47]

DISCUSSION

Information on the dynamics of protein folding is useful for the elucidation of protein conformational transitions from unfolded to folded structures. In addition, protein–water interactions play an important role in folding, aggregation, stability, and function of proteins in a cellular environment.[51–57] Understanding protein–water interactions would therefore be crucial in preventing the early misfolding and aggregation of peptides and proteins that takes place in several neurodegenerative diseases.[58] In this paper, we have monitored the self-association of melittin in solution induced by change in ionic strength utilizing the intrinsic fluorescence of Trp-19. Melittin is one of the most extensively studied amphiphilic peptides in the understanding of membrane protein structure and dynamics. Melittin is an interesting peptide to characterize the protein folding phenomenon since the structural transition from an unfolded, random coil monomer to a compact, folded α-helical tetramer can be easily achieved by changing environmental factors (such as pH and ionic strength) without the use of chemical denaturants.[18] Further, it has been shown that tetrameric melittin in solution behaves like an ordered molten globule structure, i.e., a molten globule state with significant native-like characteristics.[47]

We show here that ionic strength induces a change in the secondary structure of melittin in solution due to the electrostatic screening of the cationic residues, which in turn promotes the folding and aggregation of melittin. The change in shape and size of melittin as a function of increasing ionic strength due to the folding and aggregation of melittin monomers is documented by the decreased rotational mobility of melittin as shown by fluorescence polarization measurements. The blue shift in the emission maximum of the tryptophan residue of melittin indicates a change in polarity in the immediate environment of tryptophan residue(s) upon increasing the ionic strength of the medium. This is supported by acrylamide and TCE quenching experiments, which show differential accessibility of tryptophan residue(s) to solvent molecules upon ionic strength–induced conformational transition. This conformational transition is accompanied by a change in fluorescence lifetime of melittin, which is attributed to a combination of the bulk polarity effect and the specific cation–π interaction of Trp-19 and Lys-23 in the helical structure formed.

Recently, a molecular dynamics (MD) simulation study has indicated a strong dewetting transition in the formation of melittin tetramer (hydrophobic collapse).[59] In this study, it was observed that Ile-2, one of the hydrophobic residues present in the inner sur-

face of tetramer, plays a central role in the water drying (dewetting) transition in the melittin tetramer. Interestingly, the Ile-2 side chains have previously reported to be in close proximity to Trp-19 in the melittin tetramer.[19,60] To probe the dynamics of hydration during the self-association of melittin induced by ionic strength, we utilized the REES approach. This approach 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.[25,26] The unique feature of REES is that, while all other fluorescence techniques such as fluorescence quenching, energy transfer, and polarization measurements yield information about the fluorophore (either intrinsic or extrinsic) itself, REES provides information about the relative rates of solvent (water in biological systems) relaxation dynamics, which is not possible to obtain by other techniques. Our results show that the magnitude of REES increases considerably as a function of increasing ionic strength when melittin undergoes a conformational transition from a random coil monomer to helical tetrameric melittin. This suggests the presence of water molecules with slow relaxation dynamics in the aggregated state of melittin. It is interesting to note that the observed magnitude of REES for melittin tetramer (5 nm) is similar to that of membrane-bound melittin ($\sim$6 nm).[9,10,30] This suggests that tetrameric melittin tryptophans are localized, on the average, in a restricted environment and the restriction imposed by the relaxing solvent molecules is similar to that observed for melittin localized at the membrane interface. This is in agreement with a recent report that shows the hydration dynamics is similar in tetrameric melittin and membrane-bound melittin.[35]

In summary, our results show that REES is a sensitive parameter to the change in the dynamic hydration profile of melittin upon folding and self-association induced by ionic strength. This is the first report demonstrating that REES could be used as a powerful tool to monitor the aggregation of proteins and peptides in solution. Our results could be potentially useful in studies of aggregation of proteins and peptides that are prone to aggregation under a variety of factors leading to pathogenic conditions.[61]

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