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Restricted mobility of the sole tryptophan in membrane-bound melittin

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In spite of numerous studies, there appears to be no consensus regarding the orientation and aggregation state of membrane-bound melittin. We report here the restricted environment of the sole tryptophan residue in membrane-bound melittin using environment-induced effects on the rates of solvent relaxation. When incorporated into unilamellar vesicles of dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), melittin exhibits a red edge excitation shift (REES) of 5 nm. In addition, fluorescence polarization of melittin in the membrane shows both excitation and emission wavelength dependence. Taken together, these observations indicate that the tryptophan residue of melittin is located in a motionally restricted region in the membrane.

Restricted tryptophan environment; Membrane-bound melittin; Red edge excitation shift; Fluorescence polarization

1. INTRODUCTION

Melittin, the principal toxic component in the venom of the honey bee, *Apis mellifera*, is a small linear peptide composed of 26 amino acids and is known to have a powerful hemolytic activity [1]. It is a cationic peptide with a large hydrophobic region (residues 1–20) and a stretch of predominantly hydrophilic amino acids (residues 21–26) at the carboxy-terminal end of the molecule 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 (for reviews, see [2–4]). Such a sequence of amino acids, together with the amphiphilic nature, is characteristic of many membrane-bound peptides and putative transmembrane helices of membrane proteins [4]. This has resulted in melittin being used as a simple model to study lipid–protein interactions in membranes [2–14]. In addition to its hemolytic activity, melittin induces voltage-dependent ion channels across planar lipid bilayers, bilayer micellization and membrane fusion [2–4]. However, in spite of a number of studies [8,14–26], there appears to be no consensus regarding the orientation and aggregation state of membrane-bound melittin. Melittin has a single fluorescent residue, Trp-19, and has no tyrosine. This makes the tryptophan a sensitive intrinsic probe to study the interaction of melittin with membranes. In this paper, we have monitored the microenvironment experienced by this tryptophan residue in membrane-bound melittin utilizing the red edge excitation shift effect.

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 the red edge excitation shift (REES). This effect is mostly observed with polar fluorophores in motionally restricted media, such as very viscous solutions or condensed phases [27–31]. The origin of REES lies in 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 re-orientate around the excited state fluorophore. If the rate of solvent re-orientation is comparable to, or slower than, the fluorescence lifetime, a different average population of fluorophores is sub-selected with each excitation wavelength, resulting in the red edge effect. We have recently shown that highly organized molecular assemblies, such as membranes, offer a motionally restricted environment for membrane-bound probes, and reported that the probe, NBD-PE, when incorporated in model membranes, exhibits REES [32]. In the present study, we report that the tryptophan residue in membrane-bound melittin exhibits such red edge effects. Our study represents one of the first attempts to probe dynamics of the tryptophan environment of a membrane-bound peptide using the red edge effect. In particular, we show that in the case of membrane-bound melittin, the sole tryptophan residue is localized in a region of the membrane which offers considerable resistance to solvent re-orientation.

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Abbreviations: DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DOPC, dioleoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; NBD-PE, *N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; REES, red edge excitation shift; TLC, thin-layer chromatography.

2. MATERIALS AND METHODS

Melittin of the highest available purity and DMPC were obtained from Sigma Chemical Co. (St. Louis, MO, USA). DOPC was purchased from Avanti Polar Lipids (Birmingham, AL, USA). Its purity was checked by TLC on silica gel plates in chloroform/methanol/water (65:35:5, v/v/v). DOPC gave one spot with a phosphate-sensitive spray and on subsequent charring [33]. Its concentration was determined by phosphate assay subsequent to total digestion by perchloric acid [34]. DMPC was used as a standard to assess lipid digestion. The concentration of melittin in solution was calculated from its molar extinction coefficient value of 5,570 $M^{-1} \cdot cm^{-1}$ at 280 nm [35]. To check for phospholipase A_2 contamination in melittin, phospholipase activity was assayed using ^{14}C-labeled DOPC obtained from Amersham International [36]. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

Unilamellar vesicles (ULV) of DOPC containing 2% (mol/mol) melittin were prepared by drying 640 nmol of DOPC under a stream of nitrogen and then under a high vacuum for at least 3 h. The lipids were swollen by adding 1.5 ml of 10 mM sodium phosphate/150 mM NaCl, pH 7.0 buffer containing 1 mM EDTA, and samples were vortexed for 3 min to disperse the lipid. The lipid dispersions were then sonicated for 10 min (in bursts of 1 min, while being cooled in ice) using a Branson B-30 sonifier. The sonicated samples were centrifuged at 5,000 rpm for 15 min to remove titanium particles. To incorporate melittin into membranes, a small aliquot containing 12.8 nmol melittin was added from a stock solution in water to the pre-formed vesicles and mixed well. Samples were kept in the dark for 16 h before measuring fluorescence. Background samples were prepared in the same way except that melittin was not added to them.

Fluorescence measurements were performed with a Hitachi F-4010 spectrofluorometer using 1 cm pathlength quartz cuvettes. Slits with a nominal bandpass of 3 nm were used for measurement of emission maxima as a function of excitation wavelength. For polarization measurements, excitation and emission slits with a nominal bandpass of 5 nm were used. All experiments were done at room temperature. Background intensities of samples in which melittin was omitted were subtracted from sample fluorescence to cancel out any contribution due to scattering artifacts. Polarization measurements were performed using a Hitachi polarization accessory. Polarization values were calculated as described previously [32]. All experiments were done with multiple sets of samples. The spectral shifts obtained with different sets of samples were identical in most cases. The polarization values reported in Figs. 2 and 3 are the average of several measurements, and all individual data points are within 2% of the reported values.

3. RESULTS

The purity of melittin was checked by phospholipase assay on vesicle samples containing radiolabeled phospholipids [36]. Although no phospholipase A_2 activity could be detected for shorter incubations, some activity was found upon prolonged incubation. This activity was found to be totally inhibited by the addition of 1 mM EDTA. Experiments were thus performed using buffers containing 1 mM EDTA in order to avoid any possible artifacts caused by phospholipase contamination. After completion of the fluorescence experiments, lipids were checked for degradation by TLC on silica gel plates as described above (see section 2). Even after keeping the samples for a few days at room temperature, no lysoPC could be detected.

Fig. 1 shows the fluorescence emission spectra of melittin in phosphate buffer and in ULVs of DOPC. As

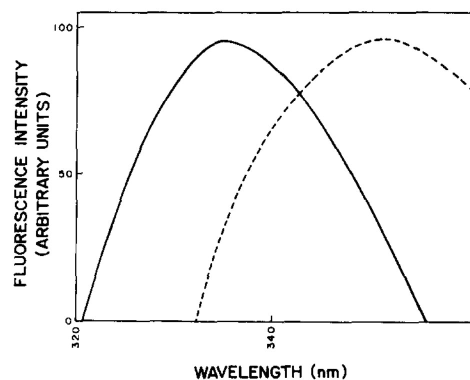

Fig. 1. Fluorescence emission spectra of melittin in 10 mM sodium phosphate/150 mM NaCl, pH 7.0 buffer containing 1 mM EDTA (—), and in unilamellar vesicles of DOPC (----) with a melittin-to-DOPC ratio of 1:50 (mol/mol). The spectra are intensity normalized at the emission maximum. The concentration of melittin in buffer was 8.5 μM. See section 2 for other details.

shown in the figure, the emission maximum of melittin in buffer is around 352 nm, indicating that melittin is essentially present in a monomeric form and that the tryptophan is completely exposed to the aqueous environment. Upon incorporation into DOPC vesicles, the emission maximum is shifted to 335 nm. This blue shift of the emission maximum is due to a decrease in the polarity of the surrounding matrix [2,8,19]. This clearly indicates that the sole tryptophan of melittin which is exposed to water in monomeric melittin is incorporated into the membrane.

Fig. 2 shows the change in emission maxima of melittin bound to DOPC vesicles as a function of excitation wavelength. As the excitation wavelength is changed from 280 to 310 nm, the emission maximum is shifted from 335 to 340 nm, which corresponds to a REES of 5 nm. Such dependence of the emission maximum on the excitation wavelength is characteristic of the red edge effect and indicates that the tryptophan in membrane-bound melittin is in a region of the membrane which offers considerable restriction to the re-orientational motion of solvent dipoles around the excited-state tryptophan.

In addition to the shift in emission maximum on red edge excitation, fluorescence polarization is also known to be dependent on excitation wavelength in viscous solutions [32,37]. The excitation polarization spectra of melittin in DOPC vesicles and in water are shown in Fig. 3. The polarization of membrane-bound melittin changes with excitation wavelength, with a sharp increase occurring towards the red edge of the absorption band. Such an increase in polarization upon red edge excitation has previously been reported for other tryptophan-containing peptides and proteins in media of reduced mobility [38]. This is further indicative of the motional restriction experienced by the tryptophan of membrane-bound melittin. The polarization of mono-

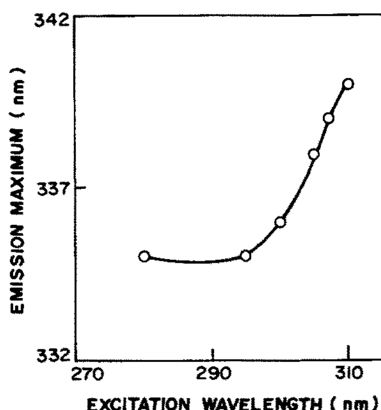

Fig. 2. Effect of changing excitation wavelength on the wavelength of maximum emission for melittin bound to DOPC vesicles. Melittin to DOPC ratio was 1:50 (mol/mol). See section 2 for other details.

meric melittin in water, on the other hand, is more or less independent of the excitation wavelength.

Fig. 4 shows the variation in polarization of melittin in DOPC vesicles and in water as a function of emission wavelength. While monomeric melittin in water does not show any significant variation in polarization over this emission range, there is a considerable reduction in polarization with increasing emission wavelength in the case of membrane-bound melittin. Polarization is lowest towards the red edge where the 'solvent-relaxed' emission predominates. Similar observations have previously been reported for melittin in reversed micelles [39] and other fluorophores in membranes [32,40,41].

4. DISCUSSION

A number of earlier studies [42,43] have dealt with tryptophan environments of melittin in aqueous media. The present study focuses on the restriction imposed on the sole tryptophan residue of melittin *in its membrane-bound form*. Our results indicate that when incorporated into DOPC vesicles, the tryptophan in melittin is local-

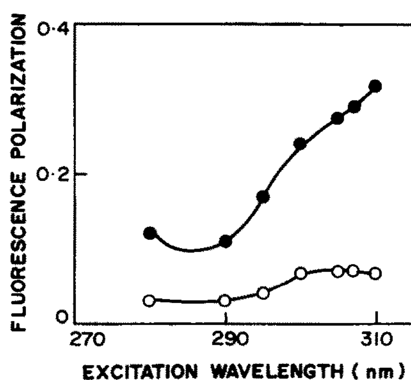

Fig. 3. Fluorescence polarization of melittin as a function of excitation wavelength in water (○), and when bound to DOPC vesicles (●). Polarization values were recorded at 335 nm. The concentration of melittin in water was 8.5 μM. See section 2 for other details.

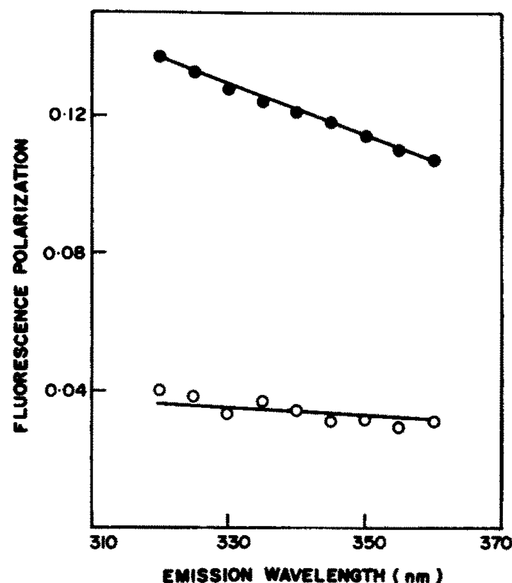

Fig. 4. Fluorescence polarization of melittin as a function of emission wavelength in water (○), and when bound to DOPC vesicles (●). The excitation wavelength was 280 nm. All other conditions are as in Fig. 3.

ized to a motionally restricted region of the membrane. The fluorescence properties of melittin are ideal for such studies because it has only one tryptophan, and all the complications of fluorescence from multi-tryptophan proteins or peptides are thus avoided. Moreover, since melittin has no tyrosine, there is no interference from tyrosine fluorescence, and a broad range of excitation wavelengths can be used.

REES of proteins and peptides containing a single tryptophan has been previously reported by Demchenko [44]. In this study, it was reported that REES effects were not significant for either short wavelength-emitting proteins (emission maxima < 325 nm), or for long wavelength-emitting proteins (emission maxima > 341 nm). Appreciable excitation wavelength dependence of emission spectra was found for proteins which exhibit fluorescence maxima in the intermediate range of emission wavelengths (325–341 nm), since the dipolar re-orientation of solvent molecules in such cases is comparable to, or slower than, their fluorescence lifetime. Membrane-bound melittin, with its emission maximum at 335 nm, thus falls into this category of proteins. Our results show that it exhibits a REES of 5 nm in accordance with the above classification.

We have previously noted that the ability of a membrane-bound fluorophore to exhibit REES could depend on a number of factors, including its location in the membrane [32,45]. Several previous studies have commented on the location of the tryptophan residue in membrane-bound melittin using NMR [46] and fluorescence quenching techniques [2,8,10,15,18,19]. All these studies indicate that the tryptophan residue is localized

to a shallow position (depth) near the membrane interface. Preliminary experiments from our laboratory, using the parallax method [47] and utilizing quenching by spin-labeled phosphatidylcholines with the spin-label (nitroxide) group attached to positions 5 and 12 of the fatty acyl chain, show more quenching with the PC having the spin-label at the 5 position. This further supports the above results and indicates that the tryptophan residue in membrane-bound melittin is in a shallow interfacial location in the membrane where motional restriction of various types could be expected to slow down the solvent re-orientation rate. This region of the membrane is characterized by unique motional and dielectric characteristics and is known to participate in charge interactions and hydrogen bonding [32]. These structural features which slow down the rate of solvent re-orientation have previously been recognized as typical features that give rise to significant red edge effects [48]. In fact, the motional restriction experienced by the tryptophan residue of membrane-bound melittin could possibly be due to hydrogen bonding between the indole rings of the tryptophan and interfacial water molecules (or neighboring lipid carbonyls). It is interesting to note that the presence of water at the peptide-lipid interface for membrane-bound peptides has recently been demonstrated [49], and it has been postulated that such bound water molecules could play a modulatory role in the structure and function of membrane-bound peptides and proteins. These results are thus relevant to ongoing analyses of the location and orientation of membrane-bound melittin.

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