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MELITTIN-PHOSPHOLIPID INTERACTION STUDIED BY EMPLOYING THE SINGLE TRYPTOPHAN RESIDUE AS AN INTRINSIC FLUORESCENT PROBE

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The rotational correlation time of melittin, obtained from the nanosecond anisotropy of the emission from its single tryptophan residue, has been found to increase considerably in phosphate solution relative to that in aqueous solution, consistent with protein aggregation. The steady-state fluorescence spectra as well as the absorption spectra in phosphate solution exhibit a very good degree of similarity with those of the protein bound to egg phosphatidylcholine (PC) and distearoylphosphatidylcholine (DSPC) bilayer liposomes. The value of the second-order rate constant for dynamic quenching, $k_q = 1.4 \cdot 10^9$ $M^{-1} \cdot s^{-1}$, by acrylamide in 0.5 M phosphate solution is comparable to those for the protein-phospholipids complexes ($1 \cdot 10^9$ and $0.7 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ for egg PC and DSPC, respectively). Similarities are also found in the nanosecond properties. There is a much stronger and quite similar dependence of the fluorescence spectra on time in the nanosecond range and of the fluorescence decay times on the emission wavelength in both cases as compared to the case in aqueous solution. These observations support the notion that melittin binds to the phospholipids in an aggregated form. The results suggest that the reduction in the k_q values of bound melittin relative to that in aqueous solution and the blue shift of the fluorescence spectrum (from 352 to 337 nm) are brought about by shielding of the tryptophan residue from the solvent through a combination of protein aggregation and enhancement of its α-helical content (suggested by published CD data). The magnitude of the k_q values for bound melittin, however, is still relatively high implying the occurrence of rather frequent encounters between the tryptophan residue and the hydrophilic acrylamide molecules. Thus, the residue is found not to penetrate deep into the phospholipid bilayer.

Introduction

Melittin, the major protein component of bee venom, has a strong hemolytic activity [1]. The nature of its interaction with membranes, a prerequisite for elucidating the mode of its biological action, has been the subject of several studies [2–10], but a clear picture has yet to emerge. The protein is amphipathic with its amino acid residues 1–20 being predominantly hydrophobic and residues 21–26 being hydrophilic. It contains only one tryptophan residue, located at position 19, that can be advantageously employed as an intrinsic fluorescent probe. Moreover, complications arising from emission from multiple emitters are avoided due to the absence of any tyrosine residues. Thus, the melittin-lipid complex is of interest not only because of its relevance to the biological effects of the protein but also because it represents a useful protein-lipid model.

Previous studies [2,5,6] reported a blue shift in the fluorescence spectral maximum upon binding to phospholipids relative to that in aqueous solution, implying an increase in the hydrophobicity of

the residue. The state of aggregation of the protein in solution was investigated by chromatographic [11] and steady-state fluorescence polarization techniques [12]: it was inferred that the protein is monomeric in aqueous solution whereas it aggregates in sodium chloride solution, apparently as a tetramer. From nanosecond and steady-state fluorometric data we recently [13] confirmed that inference for potassium phosphate and presented evidence supporting the notion that melittin aggregates when it binds to egg phosphatidylcholine bilayer liposomes. The present study extends that work and reports data on fluorescence quenching by acrylamide that provide information on the extent of penetration of the tryptophan residue into the bilayer.

Materials and Methods

Samples of bee venom melittin, obtained from Sigma and ICN Biochemicals, gave identical results. Chromatographically pure egg phosphatidylcholine (PC) and L-α-distearoylphosphatidylcholine (DSPC) were obtained from Sigma. Acrylamide, Tris, dibasic potassium phosphate and sodium chloride were analytical reagent grade products of Fisher. Chromatographically pure tryptophan was obtained from P.L Biochemicals, while propylene glycol was obtained from Baker.

The buffer used in all preparations was 10 mM Tris (pH 7) prepared in triply distilled water. All measurements were made in aerated samples at room temperature except in the case of DSPC above its transition temperature for which a temperature of 61°C was employed. The preparation of the liposomes was done according to published procedures [13–16]. A 20 mg/ml lipid suspension in 10 mM Tris buffer (pH 7) was prepared. The suspension was vigorously stirred from 20 min and then sonicated for 20 min using a Heat Systems-Ultrasonics 20 kHz model W-375 sonifier equipped with 0.5 inch tip at 50% full power. Finally, it was centrifuged at $65000 \times g$ for 30 min. All procedures were carried out under nitrogen. Melittin was incubated for 30 min in the phospholipid solution at room temperature for PC and at 61°C for DSPC (above its transition temperature of 51°C [17]). Steady-state and nanosecond fluorometric techniques did not detect any fluorescence from liposomes unlabelled with melittin.

The fluorescence spectra were taken with a spectrofluorometer previously described [18] and were corrected for the variation of the sensitivity of the photomultiplier-monochromator combination with emission wavelength by employing a standard lamp. Excitation was at 280 nm. The emission bandwidth was 3 nm. The melittin concentration was 0.04 mg/ml. Acrylamide was found to have no significant effect on the shape or on the wavelength of spectral maximum for the cases studied.

Absorption spectra were taken with a Cary 14 spectrophotometer using 1 cm light path cuvettes and a melittin concentration of 0.21 mg/ml. For the case of DSPC melittin was found to cause a significant decrease of light scattering both below and above the phase transition temperature of the phospholipid apparently because of reduction of the size of the liposomes. It was, therefore, found necessary to apply a correction to all fluorescence data, which was calculated by scaling the lines at approx. 315 and 367 nm (observed when exciting at 280 nm) so that a smooth fit to the fluorescence spectrum was obtained at those two wavelengths. Due to that effect, it was not possible to obtain the absorption spectrum in the case of DSPC, as the light transmission of the reference channel differed significantly from that of the sample channel. In the case of PC melittin was found to cause no significant reduction in the amount of scattered light. It should be noted in this regard that other studies [19,20] have also reported a dependence of the reduction of light scattering by other proteins on the nature of the lipid.

For fluorometric titrations involving acrylamide a melittin concentration of 0.17 mg/ml was used which gave an absorbance $\simeq 0.05$ at 305 nm (the wavelength used for excitation). Acrylamide has negligible absorption at the wavelength. The titrations were performed by adding increments from a stock acrylamide solution to the melittin solution. In the case of the liposomes, the stock acrylamide solution was prepared in the liposome solution. Similarly, in the case of salt solutions, the stock acrylamide solution was prepared in the presence of the appropriate salt concentration. A dilution correction was applied to the data.

Nanosecond fluorescence measurements were

made with a nanosecond fluorometer employing a boxcar averager previously described [21]. A Pomfret interference filter of 6 nm f.w.h.m. * and 298 nm peak wavelength was used for excitation. The emission was viewed through a 7–37 Corning filter. A Bausch and Lomb 0.25 m high intensity monochromator of 9.6 nm bandwidth was employed for measurements involving the dependence of the fluorescence decay time on the emission wavelength and for time-resolved spectral measurements (not corrected for the variation of the response of the instrument with emission wavelength); the latter were made by fixing the time-window of the boxcar averager relative to the peak of the exciting light pulse and scanning the monochromator. For emission anisotropy measurements a 3M 105-UV-WRMR polarizer was placed in the excitation light path and a Polaroid HNP'B polarizer in the emission path. For all nanosecond measurements, a melittin concentration of 0.14 mg/ml was used. The data analysis was performed by using a nonlinear least-squares computer program. The quality of the fit was judged by the form of the residuals and of the autocorrelation function of the residuals plotted vs. channel number. The emission anisotropy, $r(t)$, defined as $r(t) = (I_V(t) - CI_H(t))/(I_V(t) + 2CI_H(t))$, was found in all measurements to have the form $r(t) = r_0 \exp(-t/\phi)$, where ϕ is the rotational correlation time and r_0 is the zero-point emission anisotropy; in this expression C is a correction factor calculated by combining nanosecond with steady-state emission anisotropy data [34]. The function $I_V(t) + 2CI_H(t)$, which represents the total fluorescence decay, was first analyzed and then the obtained parameters were used for the analysis of the $I_V(t) - CI_H(t)$ data.

Results

1. Steady-state fluorescence spectra and nanosecond emission anistrotropy of melittin in phosphate solution

The fluorescence spectrum of the tryptophan residue of melittin is seen from Fig. 1 to be strongly dependent on the concentration of dibasic potassium phosphate. As little as 0.02 M phosphate causes a detectable change in the spectrum. With increasing phosphate concentration, the spectrum shifts progressively to the blue relative to that in Tris, which peaks at 352 nm. For 1 M phosphate, the spectral maximum is at 336 nm and shows no significant further blue shift upon increasing further the salt concentration. It should be noted that the fluorescence spectrum of free tryptophan was found not to be affected to any significant extent by the salt. Thus, the blue spectral shifts imply that in the presence of salt the protein undergoes a structural rearrangement that results in shielding of the tryptophan residue to some extent from the aqueous environment.

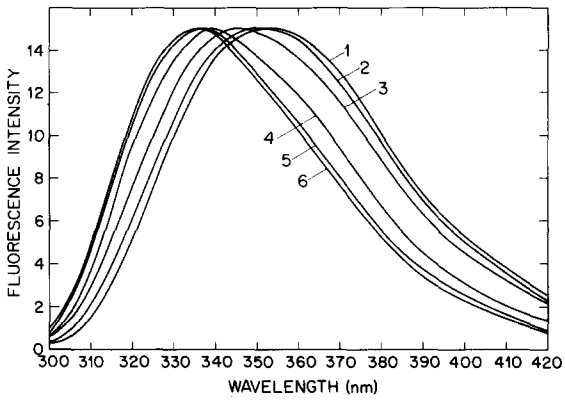

Fig. 1. Effect of dibasic potassium phosphate on the fluorescence spectrum of the single tryptophan residue of melittin. The potassium phosphate concentrations were as follows: (1) 0 (Tris); (2) $2 \cdot 10^{-2}$ M; (3) $4 \cdot 10^{-2}$ M; (4) 0.15 M; (5) 0.5 M; and (6) 1 M. The spectra were normalized at their maxima. The buffer was 10 mM Tris with its pH readjusted to 7 after the addition of salt.

Nanosecond emission anisotropy measurements have yielded values for the rotational correlation time, ϕ, and the zero-point emission anisotropy, r_0, shown in Table I. It is seen that the value of ϕ for that phosphate concentration is 3.5 ns, which is much greater than the value of 1.1 ns in Tris. Also shown in Table I is the value of 3.7 ns for 0.15 M phosphate, which we reported previously [13]. As is discussed below, (see Discussion), the increase in the ϕ values in the presence of phosphate points toward protein aggregation in agreement with the conclusion of chromatographic [11] and steady-state fluoresence polarization [12] studies carried out in NaCl solutions. A recent NMR study [22]

* f.w.h.m., full width half maximum.

TABLE I

EMISSION ANISOTROPY PARAMETERS OF MELITTIN IN SOLUTION AND WHEN BOUND TO LIPOSOMES

ϕ is the rotational correlation time and r_0 is the zero-point emission anisotropy. PC and DSPC stand for egg phosphatidylcholine and distearoylphosphatidylcholine, respectively. The buffer employed in all cases was 10 mM Tris and the pH was adjusted to 7.

Solution conditions	ϕ (ns) *	r_0^*
Tris	1.1	0.185
0.15 M K_2HPO_4	3.7	0.164
0.5 M K_2HPO_4	3.5	0.168
0.15 M NaCl	2.8	0.134
1 M NaCl	2.1	0.206
Bound to PC	10.1	0.185
Bound to DSPC	12.5	0.053
Bound to DSPC (61°C)	4.9	0.108

* The standard deviations in the values of ϕ and r_0 are about $\pm 7\%$ and $\pm 4\%$, respectively.

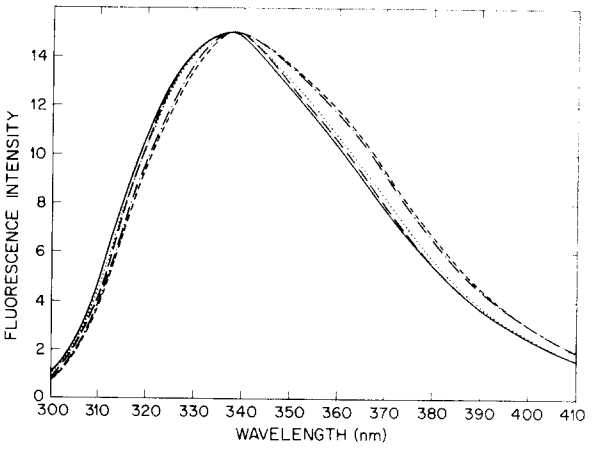

Fig. 2. Fluorescence spectra of the tryptophan residue of melittin in: ———, bound to distearoylphosphatidylcholine (DSPC); · · · · · ·, bound to DSPC at 61°C; — — —, 0.5 M dibasic potassium phosphate; · – · – ·, bound to egg phosphatidylcholine (PC); and – – – – –, 0.15 M dibasic potassium phosphate. The buffer was 10 mM Tris (pH 7).

has reported aggregation of mellitin in phosphate solution, but the protein concentrations employed were greater by a factor of approx. 100 than the highest concentration employed in the present study. That study also reported aggregation of melittin in the absence of salt in both acidic and alkaline solutions.

2. Steady-state fluorescence spectra and nanosecond emission anisotropy of melittin bound to phospholipids

Fig. 2 shows that the fluorescence spectra of melittin bound to PC and DSPC are blue-shifted relative to that in Tris. The spectrum for DSPC exhibits a small blue shift relative to that for PC. Upon going above the phase transition of DSPC, the spectrum shows only a very small red shift as compared to that below the transition. Also shown in Fig. 2 are the fluorescence spectra in 0.15 M and 0.5 M potassium phosphate solution, which are seen to match rather well those for PC and DSPC, respectively. These findings imply a degree of similarity in the environments of the fluorescent residue in the salt solutions and when bound to the phospholipids.

The values of the rotational correlation time, ϕ, are seen from Table I to be much larger than that in Tris due to increased rigidity of the environment of the chromophore as a result of binding to the phospholipids. At 61°C (above the DSPC transition temperature, T_t, of 51°C [17]), the value of ϕ is much smaller than that below T_t apparently as a result of enhanced flexibility of the binding site in the liquid-crystalline state of the phospholipid. Fig. 3 shows the emission anisotropy data for PC.

The values of the zero-point emission anisotropy, r_0, are seen from Table I to be lower than that of 0.24, which we obtained for free tryptophan in the highly viscous medium of propylene glycol at −60°C. This implies the occurrence of a relaxation process on the subnanosecond time scale, observed also in other systems (see, for example, Ref. 23). The depolarization can be considered to arise from reorientation of the transition dipole in a cone of half-angle θ given by the following expression [24]

$$r_0/r_m = \cos^2\theta(1+\cos\theta)^2/4 \qquad (1)$$

where $r_m = 0.24$ is the maximum anisotropy value observed in a highly viscous environment. The values of θ obtained from Eqn. 1 are 23°, 23°, 54° and 40° for Tris, PC and DSPC below and above its T_t, respectively. As the nature of the depolariz-

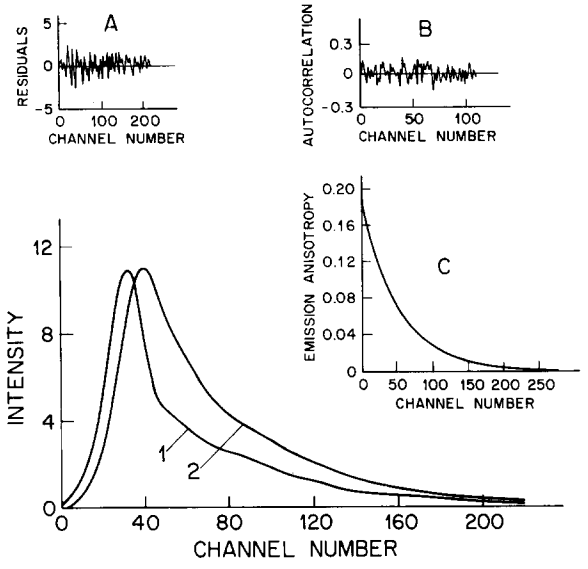

Fig. 3. Nonlinear regression analysis of the nanosecond fluorescence polarization data for the tryptophan residue of melittin bound to PC. Curve 1 represents the exciting light pulse profile. Curve 2 represents the plot of the experimental data for $I_V(t) - CI_H(t)$ and coincides with the convoluted curve; $I_V(t)$ and $I_H(t)$ are the vertical and horizontal fluorescence components, respectively, for vertically polarized exciting light and C is a correction factor calculated by combining nanosecond with steady-state emission anisotropy data [34]. Insert A: Deviations between experimental and convoluted data at each channel. Insert B: Autocorrelation function of the residuals. The lack of any specific trend in A and B implies a satisfactory fit of the experimental data. Insert C: Emission anisotropy plot which is single exponential with zero-point value $r_0 = 0.185$ and rotational correlation time $\phi = 10.1$ ns. Channel width = 0.190 ns. More details are given under Materials and Methods. The buffer was 10 mM Tris (pH 7).

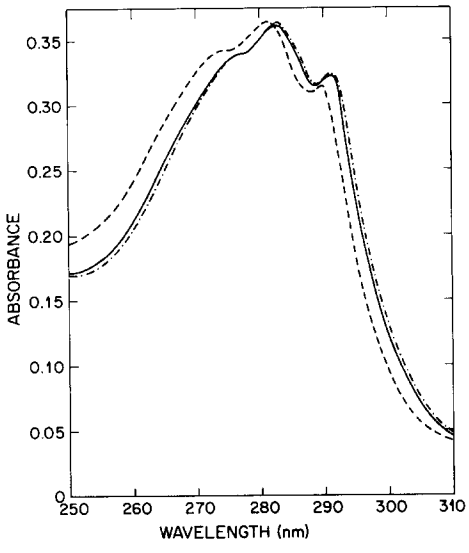

Fig. 4. Absorption spectra of the tryptophan residue of 0.21 mg/ml melittin in: ------, 10 mM Tris; ———, bound to PC; and ·-·-·, in 0.5 M dibasic potassium phosphate. The spectrum in 0.15 M potassium phosphate (not shown) virtually coincides with that for melittin bound to PC. The buffer was 10 mM Tris (pH 7).

ing process is not known, the reduction in θ above T_t cannot be commented upon.

3. Absorption spectra

The absorption spectra of melittin in phosphate solution and when bound to PC liposomes are, like the fluorescence spectra, very similar. Fig. 4 shows that they exhibit a red shift of approx 2 nm relative to the spectrum in Tris.

4. Nanosecond decay times and time-resolved emission spectra

Understanding the nanosecond properties of free tryptophan is a prerequisite for employing the tryptophan residue effectively as a fluorescent probe. Reports in the literature regarding the nature of the fluorescence decay profiles of tryptophan in aqueous solution are conflicting. Whereas all previous studies had reported the decay to be a single exponential function of time, two recent studies reported it to be a double exponential. Rayner and Szabo [25] found two decay components having decay times of 3.14 and 0.51 ns for emission wavelengths up to 370 nm that were independent of the excitation wavelength. For 380 nm, however, the decay was found to be single exponential. Fleming et al. [26] reported decay times of 5.4 and 2.1 ns by using a picosecond laser and observing the emission at wavelengths greater than 370 nm. Thus, their findings disagree with those of Rayner and Szabo [25] who reported a single exponential in that spectral region. A third study by Alpert et al. [27], undertaken to clarify the picture, used two different synchrotron facilities as well as a phase fluorometer. Three different combinations of emission filters were used transmitting in the wavelength, λ, regions $\lambda > 320$, $\lambda > 360$ and $320 < \lambda < 400$ nm. In all cases the decay was found to be single ex-

TABLE II

FLUORESCENCE DECAY PARAMETERS OF MELITTIN AS A FUNCTION OF EMISSION WAVELENGTH

When two decay components are present, τ_1 and τ_2 represent the decay times and A_1/A_2 the ratio of the amplitudes. Decay times are in ns; the standard deviation in their values was typically ±0.05 ns. PC and DSPC stand for egg phosphatidylcholine and distearoylphosphatidylcholine, respectively. The buffer employed in all cases was 10 mM Tris and the pH was adjusted to 7.

Solution conditions	Emission wavelength (nm) [a]					
	330			340	360	390
	τ_1	τ_2	A_1/A_2	τ	τ	τ
Tris	3.01	–			3.10	3.24
0.15 M K_2HPO_4	2.48	0.61	1.48	2.28	2.57	2.95
0.5 M K_2HPO_4	2.80	0.92	0.58	2.11	2.52	2.81
0.15 M NaCl	2.62	–			2.92	3.10
1 M NaCl	2.25	–		2.44	2.59	3.03
Bound to EPC [b]				2.20	2.42	3.14
Bound to DSPC [b]				3.19	3.43	3.64
Bound to DSPC (61°C) [b]				2.09	2.26	2.60

[a] We have shown [21] that the Amperex 56 TUVP photomultiplier employed in our nanosecond fluorometer shows negligibly small dependence of its transit time on the emission wavelength; thus, no artifacts are introduced in the values of the decay time as a function of emission wavelength.

[b] The form of the decay profile at 330 nm for melittin bound to phospholipids could not be determined due to the relatively high proportion of scattered light interfering with the fluorescence signal.

ponential and independent of the exciting wavelength. Thus, these results contradict those of the two aforementioned studies [25,26].

In more recent developments, the research groups of Ref. 26 reported [35] that the 5.4 ns decay component they had reported earlier was due to a photoproduct. In their latest study [35] they reported data that are in agreement with those of Rayner and Szabo [25]. Eftink and Ghiron [36], however, concluded that the modified Stern-Volmer plots for quenching of the fluorescence of tryptophan by acrylamide could not be fitted by using the values of the decay times reported by Rayner and Szabo. For a satisfactory fit, the value of the decay time at short emission wavelengths has to be shorter by approx. 7% than that at long wavelengths; our decay time data are in agreement with that inference (see below).

In the present study, the decay profiles of tryptophan in Tris (pH 7) were found to be single exponential and to exhibit a weak dependence on the emission wavelength. Decay times of 2.52, 2.63 and 2.81 ns were obtained for the emission wavelengths of 325, 350 and 380 nm, respectively. That the nondetection of a subnanosecond component is not due to a limitation imposed by instrumental resolution is seen from the fact that such a component was resolved for melittin in phosphate solution (see below and Table II). The fluorescence spectrum for free tryptophan was found to be weakly dependent on time in the nanosecond range * as was previously reported [25].

For melittin in Tris, the fluorescence decay profile of its single tryptophan residue was found in the present study to be single exponential and to exhibit, as in the case of free tryptophan, only a slight emission wavelength-dependence; decay times of 3.01, 3.10 and 3.24 ns were obtained for 330, 360 and 390 nm, respectively.

For melittin in 0.15 M potassium phosphate solution the decay profile was found to be double exponential at 330 nm with decay times of 2.48 and 0.61 ns. The subnanosecond component was

* This is the result of the weak dependence of the decay time on the emission wavelength. The decay profiles appear exponential due to the closeness of the values of the decay times obtained across the emission spectrum.

found to have a contribution of 40%. The profile and the data analysis are shown in Fig. 5. Similarly, for 0.5 M phosphate decay times of 2.80 and 0.92 ns were obtained for that emission wavelength. The contribution of the short component was 63% in that case. At 340 nm and longer emission wavelengths, however, the decays were found to be single exponential functions of time and to depend rather strongly on the emission wavelength in both cases: e.g., for 0.15 M phosphate values of 2.28 and 2.95 ns were obtained for 340 and 390 nm, respectively. These features for emission wavelengths ≥ 340 nm were also observed for the decay times of melittin bound to PC and DSPC both below and above the transition temperature of the latter phospholipid. It was not possible, however, to determine the form of the decay for the emission wavelength of 330 nm due to the relatively high proportion of light scattered by the phospholipids which interfered with the fluorescence signal at that wavelength. Decay parameters for the different cases studied are listed in Table II.

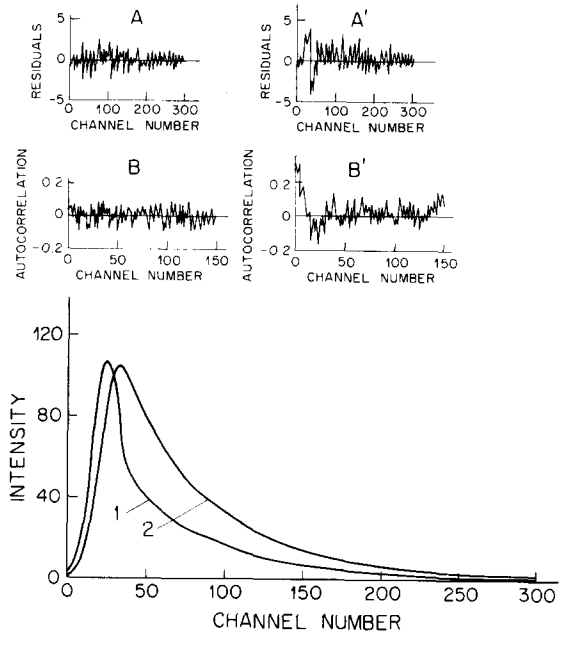

Fig. 5. Nonlinear regression analysis of the fluorescence decay data for the tryptophan residue of melittin in 0.15 M dibasic potassium phosphate monitored at the emission wavelength of 330 nm. Curve 1 represents the exciting light pulse profile. Curve 2 represents the plot of the experimental decay profile which coincides with the convoluted profile. Insert A: Deviations between experimental and convoluted data at each channel. Insert B: Autocorrelation function of the residuals. The lack of any specific trend in A and B implies a satisfactory fit of the experimental data. Channel width = 0.154 ns. The form of the decay is double exponential with decay times $\tau_1 = 2.48$ ns and $\tau_2 = 0.61$ ns and ratio of amplitudes $A_1/A_2 = 1.48$. A' and B' correspond to A and B but for single exponential analysis. Their forms in that case imply a nonsatisfactory fit. The buffer was 10 mM Tris (pH 7).

The time-resolved fluorescence spectra for both free tryptophan and melittin in Tris were found to be very similar and to exhibit small spectral with time on the nanosecond scale following pulsed excitation (as was previously reported for free tryptophan [25]). Phosphate solutions and liposomes at room temperature exhibit spectral shifts which are very similar but more pronounced than those in Tris. The spectra for PC are shown in Fig. 6. The shifts with time for DSPC at 61°C (above its T_t) were found to be reduced relative to those at room temperature.

5. Fluorescence quenching by acrylamide

Fluorescence quenching of the tryptophan residue was undertaken in order to determine the

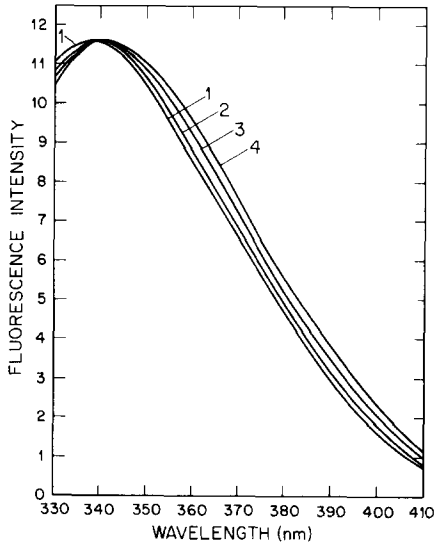

Fig. 6. Time-resolved fluorescence of the tryptophan residue of melittin bound to PC. Time values are relative to the peak of the exciting light pulse which is taken as zero time. (1) −2 ns; (2) −1 ns; (3) +3 ns; and (4) +5 ns. The spectrum did not exhibit any further shift upon increasing the time beyond 5 ns. The spectra were normalized at their maxima. The buffer was 10 mM Tris (pH 7). Similar shifts were observed in 0.15 M potassium phosphate solution (data not shown).

degree of its exposure to the aqueous environment for both melittin in solution and when bound to the phospholipids. Eftink and Ghiron [28] employed acrylamide (a nonionic, polar quencher) in studies involving several proteins in solution. Fig. 7 shows Stern-Volmer plots for melittin in Tris, 0.5 M phosphate and when bound to PC. It is seen that the plots exhibit an upward curvature which implies that quenching takes place not only by collisions between the quencher and the excited residue but also by a static process. The latter involves quenching by acrylamide molecules which were in very close proximity with the residue at the moment of excitation.

Stern-Volmer kinetics, which follow the relation

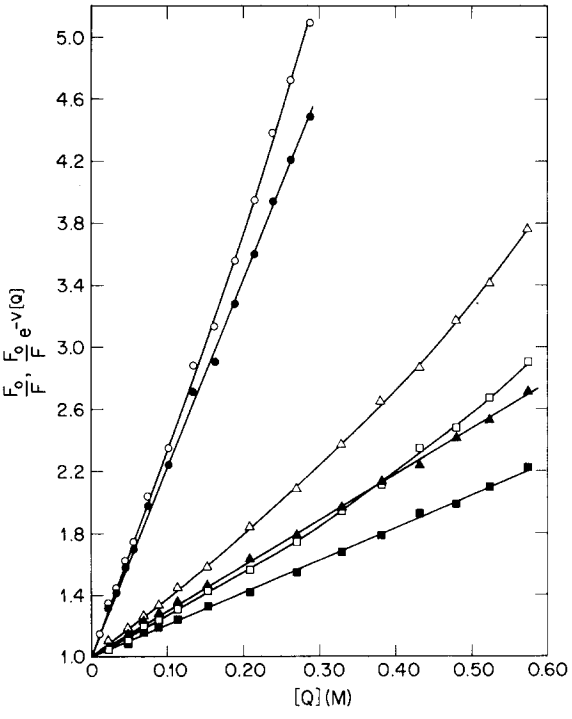

Fig. 7. Fluorescence quenching data for the tryptophan residue of melittin by acrylamide. F_0 and F represent the fluorescence intensities in the absence and presence of the molar concentration [Q] of acrylamide, respectively. Open symbols refer to Stern-Volmer plots, F_0/F vs [Q], whereas filled symbols refer to modified plots $(F_0/F)\exp(-V[Q])$ vs. [Q], where V is the static quenching constant. ○——○, Tris; ●——●, modified plot for Tris; △——△, 0.5 M dibasic potassium phosphate; ▲——▲, modified plot for 0.5 M dibasic potassium phosphate; □——□, bound to PC; and ■——■, modified plot for PC. The buffer was 10 mM Tris (pH 7) in all cases.

$F_0/F = 1 + K_{SV}[Q]$, are applicable only for the case of dynamic (excited-state) quenching. Here, F_0 and F are the fluorescence intensities in the absence and presence of the quencher of molar concentration [Q], respectively, and K_{SV} is the Stern-Volmer quenching constant. When static quenching makes a contribution, however, Weller [29] showed that the modified equation

$$(F_0/F)\exp(-V[Q]) = 1 + K_{SV}[Q] \qquad (2)$$

must be employed. The parameter V is the static quenching constant which may be considered a measure of the accessibility of the tryptophan residue in its ground electronic state to the solvent.

With the aid of a least-squares computer program, V was varied so that the best fit of the experimental data to expression 2 was obtained. The resulting linear plots for melittin in Tris, in 0.5 M phosphate solution, and when bound to PC are also shown in Fig. 7. The values of the parameters V, K_{SV}, and k_q for all studied cases are listed in Table III. The parameter k_q is the second-order rate constant for dynamic quenching and is calculated from $k_q = K_{SV}/\tau_0$, where τ_0 is the fluorescence decay time of the residue in the absence of acrylamide*. The value of $6.2 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ for k_q, reported in the present study for free tryptophan, agrees very well with that of $5.9 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ reported by Eftink and Ghiron [30]. The values of V, 1.2 and 1.5 M^{-1}, respectively, are in rather good agreement. It is also seen from Table III that the value of k_q for melittin in Tris is quite high implying a high degree of exposure of the tryptophan residue to the aqueous environment. The values of k_q for melittin in phosphate solutions and when bound to the phospholipids (Table III) imply some degree of shielding of the residue from the solvent.

6. *Optical properties in NaCl solution*

Sodium chloride has also been found to affect the optical properties of the tryptophan residue of melittin. For 0.15 M in Tris (pH 7) a blue shift of the fluorescence spectral maximum by about 1 nm

* The probability of fluorescence quenching per collision between an excited tryptophan molecule and an acrylamide molecule is close to unity [30].

relative to that in Tris was observed. Higher concentrations of salt caused larger shifts with 1 M yielding a limiting shift of the maximum to 336 nm. These observations agree with those reported by Talbot et al. [11]. Similarly, the absorption spectra in 0.15 M and 1 M NaCl were found to be similar with those in Tris and 0.15 M potassium phosphate, respectively. It is, therefore, seen that, although greater concentrations of NaCl are needed for inducing a given spectral shift, the two salts exhibit similar behavior as far as their effects on the steady-state fluorescence spectrum are concerned. This trend is also seen in the values of the excited-state dynamic quenching rate constant, k_q. Table III shows that the value for 0.15 M NaCl is very close to that in Tris, whereas that in 1 M NaCl is identical with the value in 0.15 M potassium phosphate.

The rotational correlation time, ϕ, is seen from Table I to increase from 1.1 ns in Tris to 2.8 and 2.1 ns in 0.15 M and 1 M NaCl, respectively. This trend in qualitatively similar with that observed in the case of potassium phosphate. The time-resolved fluorescence spectra were also found to exhibit spectral shifts with time on the nanosecond time scale in the presence of the two salts which were comparable (data not shown). The fluorescence decay times are seen from Table II to exhibit also a similar emission wavelength-dependence for the two salts. A difference, however, is observed (Table II) in the form of the decay profile at 330 nm which is monoexponential in NaCl but double exponential in potassium phosphate (the possible origin of the subnanosecond component for the latter salt is discussed briefly below).

Finally, the values of the ground-state quenching constant V (Table III) are compared. Solutions in NaCl are seen to have the highest values as compared to all the other solutions as well as the melittin-liposome complexes studied. For 0.15 M salt the V value is very similar with that for free tryptophan. Thus, the conformation of the protein in that case appears to be rather extended posing very little steric hinderance to the approach of acrylamide to the tryptophan residue in its ground electronic state. Indeed, a CD study [7] reported that the protein possesses very low helical content in 0.15 M NaCl. Following this argument through, one would expect low values of V in phosphate solution (which we did observe) and a high value in Tris (which we did not observe) on the basis of the results of CD studies which found that the α-helical content of melittin is high in the former case [10] and low in the latter [31]. This apparent

TABLE III

ACRYLAMIDE QUENCHING PARAMETERS FOR MELITTIN

V, K_{SV} and k_q are the static quenching constant, Stern-Volmer quenching constant and rate constant of dynamic quenching, respectively. The value of the decay time, τ_0, in the absence of acrylamide used in the calculation of k_q was obtained from Table II at the wavelength closest to the maximum of the steady-state fluorescence spectrum. PC and DSPC stand for egg phosphatidylcholine and distearoylphosphatidylcholine, respectively. The buffer employed in all cases was 10 mM Tris and the pH was adjusted to 7. For free tryptophan (in Tris) the following values were obtained: $V = 1.20\ M^{-1}$, $K_{SV} = 16.2\ M^{-1}$, $\tau_0 = 2.63$ ns and $k_q = 6.2 \cdot 10^9\ M^{-1} \cdot s^{-1}$. The standard deviation in the values of V is about $\pm 8\%$. The standard deviation in the values of k_q is about $\pm 7\%$.

Solution conditions	$V\ (M^{-1})$	$K_{SV}\ (M^{-1})$	τ_0 (ns)	$k_q\ (M^{-1} \cdot s^{-1})\ (\times 10^{-9})$
Tris	0.44	12.1	3.10	3.9
Tris at 61°C [a]	0.35	7.9	1.41	5.6
0.15 M K_2HPO_4	0.52	4.2	2.28	1.8
0.5 M K_2HPO_4	0.57	2.9	2.11	1.4
0.15 M NaCl	1.02	11.9	2.92	4.1
1 M NaCl	0.72	4.4	2.44	1.8
Bound to PC	0.46	2.1	2.20	1.0
Bound to DSPC	0.26	2.2	3.19	0.7
Bound to DSPC (61°C)	0.50	8.2	2.09	3.9

[a] There was no denaturation of the protein at 61°C as evidenced by the reversibility in its optical properties upon cooling to room temperature.

inconsistency may be explained by noting that the quenching data yield information on the accessibility of the tryptophan residue to the solvent, not on the overall structure of the protein.

Discussion

The present work reports a remarkable effect of potassium phosphate on the optical properties of the single tryptophan residue of melittin. As little as $2 \cdot 10^{-2}$ M phosphate has a detectable effect on the steady-state fluorescence spectrum (Fig. 1). Upon increasing the phosphate concentration, progressively larger blue spectral shifts are observed implying enhanced shielding of the fluorophore from the aqueous environment. The possibility that the salt-induced conformational change is accompanied by aggregation was explored by studying the depolarization of fluorescence on the nanosecond time scale. As can be seen from Table I, the rotational correlation time, ϕ, increases from 1.1 ns in Tris to 3.5 ns in 0.5 M phosphate. Also shown is the value of 3.7 ns in 0.15 M phosphate which we reported previously [13]. It should be noted in this regard that a CD study [10] reported an increase of the α-helical content of melittin in phosphate solution relative to that in aqueous solution, in which the protein is in an extended conformation [31]. It would, therefore, be expected that ϕ will decrease in the presence of phosphate as a result of the enhanced compactness of the macromolecule. Thus, the observed increase in ϕ can only be explained if aggregation of the protein is invoked. The values of the rotational time for an unhydrated sphere of molecular weight 2800 are calculated from

$$\phi = \frac{M \eta \bar{v}}{kTN} \tag{3}$$

to be approx. 0.8 and 3.2 ns for the monomer and the tetramer, respectively. Thus, the protein appears to aggregate as a tetramer in phosphate solution. In Eqn. 3, M is the molecular weight, η is the viscosity of water $= 8.9 \cdot 10^{-3}$ P, $\bar{v}$ is the protein specific volume $\simeq 0.75$ cm^3/g, k is Boltzmann's constant $= 1.38 \cdot 10^{-16}$ ergs/K, $T = 298$ K and $N = 6.02 \cdot 10^{23}$ molecules/mol. The larger shifts of the time-resolved fluorescence spectra with time on the nanosecond time scale and the greater dependence of the fluorescence decay time on the emission wavelength for phosphate solutions than for Tris solution (Table II) are also in line with this inference, as some degree of heterogeneity in the optical properties of the fluorophores of the aggregate may be present. The data for NaCl solutions (see section 6) also support aggregation. The differences observed in some of the optical properties of the protein in the two salts, discussed in section 6, suggest that ionic strength effects may not be of major importance; instead, specific electrostatic effects appear to operate. The interaction may be envisioned as involving electrostatic attraction between the basic lysine or argenine residues of the C-terminal part of melittin and the negatively charged groups of the salts. Protein-protein electrostatic repulsion would, consequently, be diminished facilitating protein aggregation through hydrophobic interactions. The present inference regarding melittin aggregation in phosphate solution parallels that reached for NaCl solution from chromatographic [11] and steady-state fluorescence polarization [12] data.

The subnanosecond component in the decay profile at 330 nm in phosphate solution (Table II) may arise from proximity of one or more tryptophan residues of the aggregate to quenching chemical groups of the macromolecule. Alternatively, the tryptophan residues of two polypeptides may be in close proximity to each other forming a weakly fluorescent dimer. That would enhance the hydrophobicity of their environment, which is consistent with the short-wavelength emission from the quenched tryptophans.

A comparison between the steady-state fluorescence spectra of melittin bound to PC and DSPC (both below and above its phase transition temperature, T_t) with those in 0.15 and 0.5 M phosphate solution, respectively, reveals a very good degree of similarity (Fig. 2). There is also a very good agreement between the absorption spectra (Fig. 4). The values of the rate constant, k_q, for dynamic quenching by acrylamide (Table III) are also comparable in the two cases and much lower than that in Tris (further discussion of the quenching data is given below). Thus, the extent of shielding of the tryptophan residue from the solvent in the presence of phosphate appears to be similar to that for

the case of melittin bound to the phospholipids. There is also a stronger and quite similar dependence of the fluorescence spectra on time on the nanosecond scale in phosphate solution and when the protein is complexed to the phospholipids than in Tris solution. These observations support the notion that mellitin binds to the phospholipids in an aggregated form. The magnitude of ϕ, however, cannot be used to estimate the number of protein molecules participating in the oligomeric aggregate, as the partial immobilization of the protein upon binding to the phospholipid gives rise to an increase in ϕ. Interestingly, a recent NMR study [22] reported that the conformation of aggregated melittin is similar to that in detergent micelles and inferred that the protein is in an aggregated form in that case.

We now discuss the acrylamide quenching data (Table III). The value of the dynamic quenching rate constant, k_q, for the tryptophan residue of melittin in Tris is comparable to that for free tryptophan, implying a high degree of exposure of the residue to the solvent. The fact that an upward curvature is still exhibited by the Stern-Volmer quenching plots for melittin in the presence of salt or when bound to the phospholipids (Fig. 7) implies that all the tryptophans of the aggregate are quenched by acrylamide to a similar extent or that only one tryptophan is emitting. This is consistent with the observation that the fluorescence spectrum does not exhibit a shift in the presence of acrylamide. In those cases k_q represents an effective rate constant pertaining to the aggregate [28]. The magnitude of the k_q values obtained for protein bound to the phospholipids (e.g., $k_q = 1 \cdot 10^9$ $M^{-1} \cdot s^{-1}$ for PC) implies the occurrence of rather frequent encounters between the hydrophilic acrylamide molecules and tryptophan during the lifetime of its excited singlet electronic state. These results imply that the tryptophan residue of melittin does not penetrate deep into the phospholipid bilayer. This finding is consistent with the fact that the tryptophan residue is located close to the hydrophilic part of the protein. It is of interest to note in this regard that, on the basis of the blue shift of the fluorescence spectrum of the tryptophan of melittin when bound to phospholipids relative to that in aqueous solution, previous workers [2,5,6] suggested that the residue penetrates into the hydrophobic core of the bilayer. As the studies of Eftink and Ghiron [28] clearly showed for a variety of other proteins, however, there is no linear relationship between the spectral maximum and the degree of exposure of the residue to the solvent. The latter may be overestimated as a result of interactions occurring between the residue and neighboring polar groups of the protein that red-shift the spectrum. On the basis of the present data and recent CD data [10], we attribute the blue shift to an increase in the hydrophobicity of the environment of the residue brought about by shielding from the solvent through a combination of protein aggregation and enhancement of its α-helical content. This also explains the reduction in the k_q values relative to that in Tris (Table III). Interestingly, a recent hydrogen-exchange study [22] reported considerable enhancement of solvent-shielding of the backbone amide protons in aggregated melittin as compared to monomeric melittin.

At 61°C, above the T_t of DSPC, k_q increases by a factor of about 5.6 relative to that at room temperature and reaches the room-temperature value in Tris (Table III). The increase in k_q cannot be explained simply on the basis of temperature effects on the diffusion rate, for the enhancement factor in k_q for Tris upon going to 61°C is only about 1.4 (Table III). Instead, it implies greater accessibility of the residue to the solvent above T_t than below it, consistent with the greater frequency of thermally-induced structural fluctuations in the liquid-crystalline state of the bilayer. The lower value of ϕ in that case (Table I) can be also understood in the same context.

The inital step in the melittin-phospholipid interaction may involve electrostatic attraction [6,7,32] between the lipid phosphate group and basic amino acid residues (lysine or argenine of the C-terminal part). Interestingly, a differential scanning calorimetry study [32] reported that the hydrophilic fragment 20–26 decreased the heat of the phase transition, mimicking the intact melittin, whereas the hydrophobic fragment 1–19 was ineffective in that respect. That study estimated that the C-terminal part of melittin prevents ten phosphatidylcholine molecules from participating in the cooperative phase transition. The protein-lipid electrostatic attraction will cause partial neutrali-

zation of the protein positive charge and diminish interprotein electrostatic repulsion, which, in turn, will facilitate hydrophobic interactions leading to protein aggregation. In the second step of the interaction, the predominantly hydrophobic part of melittin may penetrate into the bilayer giving rise to further stabilization of the protein-phospholipid complex. The model of Dawson et al. [7] proposes that the depth of protein penetration is less than 10 Å. Not surprisingly, capacitance measurements [33] reported that the protein does not span the bilayer.

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