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EFFECT OF THE STATE OF ASSOCIATION OF MELITTIN AND PHOSPHOLIPIDS ON THEIR RECIPROCAL BINDING

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In solutions of increasing ionic strength, the molecular weight of melittin varies from 2840 (monomeric melittin) to 11200. This polymerization, concomitant with an important change in conformation (Talbot, J.C., Dufourcq, J., De Bony, J., Faucon, J.F. and Lussan, C. (1979) FEBS Lett. 102, 191–193), is accompanied by a significant alteration in the partial specific volume of the molecule. The binding of melittin to phospholipids (phosphatidylserine, lysolecithin, dihexanoyl-, dioctanoyl- and lysolauroylphosphatidylcholine) depends on the state of association of the toxin and on the critical micelle concentration of lipids. No interaction is observed between monomeric melittin and free lipids, whereas tetrameric melittin can bind free lipids to form mixed micelles. At phospholipid concentrations above the critical micelle concentration, melittin in any state of self-association can bind lipids. The mixed micelles formed at saturation appear to be independent of the initial state of association of melittin.

Introduction

Melittin is one of the best-known amphipathic peptides interacting with membranes and it has been used to attempt to understand lipid-protein interactions. Most of the known features of melittin have been reviewed by Habermann [1], but many contradictory data and interpretations remain. Many problems must first be solved in order to understand the mechanism by which lysis occurs. It has already been shown that melittin can exist in solution both as a tetramer [2–6] and as a monomer [2,5,7], but no accurate value of molecular weight for the assumed monomeric form has yet been found. The value of 3500 [2] may be explained by incomplete dissociation of the toxin in the concentrated solution used for chromatography, and the values of 800–2200 [7] have been explained as the consequence of the non-ideal nature of the petide.

As the targets for melittin action are biomembranes, we propose to examine its mechanism of binding to phospholipids and to determine the characteristics of the resulting complexes. Previous studies [8,9] in this field generally dealt with monomeric melittin (due to the experimental conditions used), whereas the in vivo state of the toxin may well be tetrameric, considering its usual concentration in bee venom and the ionic strength of most intra-cellular media.

In this paper, we first try to resolve the dispute over the presence of monomeric melittin in solution by the use of two independent techniques, sedimentation equilibrium and fluorescence polarization. The effect of various salts on monomer-tetramer equilibrium is also presented. Secondly, the different binding behaviour of melittin to phospholipids, related to the state of polymerization of the peptide, and the nature and association of lipids as micelles or bilayers is also presented. Finally, we propose a scheme which summarizes the various existing complexes.

Materials and Methods

Melittin (research grade) was purchased from Serva Feinbiochemica (Heidelberg, F.R.G.). Lysolauroylphosphatidylcholine was from Sigma (St. Louis MO, U.S.A.) and bovine phosphatidylserine from Lipid Products (Nutfield, U.K.). Dioctanoyl- and dihexanoylphosphatidylcholine from Serdary Research Laboratories (London, Ontario, Canada) were purified by silica gel chromatography with F 254 fluorescent dye (Merck, Darmstadt, F.R.G.) in chloroform/methanol/water (65:25:4, v/v). Lysolecithin was prepared in this laboratory by the action of phospholipase A_2 on purified egg lecithin, according to the method of Hubbel and McConnell [10].

Experiments were generally carried out at 25°C with 20 μM melittin in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA. We used 2 M NaCl only for studies on the binding of tetrameric melittin to lipids. Fluorescence measurements and optical rotatory dispersions were performed as previously described [2].

Values of critical micelle concentration change according to the ionic strength of buffers. Therefore, measurements were made under the same experimental conditions using 8-anilinonaphthalenesulfonate as a fluorescent probe. The following values were obtained: 28 μM for lysolecithin, and 8 μM for lysolecithin in the presence of 2 M NaCl, 560 μM for lysolauroylphosphatidylcholine and 140 μM for this phospholipid with 2 M NaCl. The critical micelle concentrations of dioctanoyl- and dihexanoylphosphatidylcholine have been published by Bonsen et al. [11] and Tausk et al. [12].

Intrinsic fluorescence-polarization studies were done with an apparatus built in this laboratory according to the scheme of Weber and Bablouzian [13]. The degree of polarization (P) depends on the size of fluorescent species and can be related to molecular weights (M_r), assuming values for the partial specific volume ($\bar{V}$). The following relationship can be derived from the Perrin equation [14]:

$$\frac{V_{NaCl}}{V} = \frac{(P^{-1} - P_0^{-1})(P_{0,NaCl}^{-1} - 1/3)}{(P_0^{-1} - 1/3)(P_{NaCl}^{-1} - P_{0,NaCl}^{-1})} \frac{\eta}{\eta_{NaCl}} \frac{\tau_{NaCl}}{\tau}$$

$$= \frac{M_{NaCl}\bar{V}_{NaCl}}{M\bar{V}} \qquad (1)$$

with V, molar volume; P_0, degree of fundamental polarization; η, viscosity; τ, fluorescence lifetime, in the absence of NaCl; and subscripts 'NaCl' with 2 M NaCl at the same temperature and melittin concentration. The lifetime of melittin varies with NaCl concentration (Lakowicz, J.R., personal communication) so that $\tau_{NaCl}/\tau = 0.80$. The viscosity of water is 0.891 cP at 25°C and the calculated value for 2 M NaCl is 1.043 cP. The effect of the buffer can be ignored. It is assumed that the shape of the toxin molecule is not significantly different from a sphere, so that the extrapolation of the linear changes of P^{-1} versus T/η observed upon addition of glycerol leads to a single value of 0.116 for P_0, with or without 2 M NaCl. Eqn. 1 thus reduces to:

$$\frac{V_{NaCl}}{V} = \frac{P^{-1} - P_0^{-1}}{P_{NaCl}^{-1} - P_0^{-1}} \frac{\eta}{\eta_{NaCl}} \frac{\tau_{NaCl}}{\tau}$$

$$= 0.683 \frac{P^{-1} - 8.62}{P_{NaCl}^{-1} - 8.62} \qquad (2)$$

Sedimentation-equilibrium experiments were performed in an MSE Centriscan ultracentrifuge at 35000 rev./min at 20°C with 50 μM melittin in the presence or absence of 1.5 M NaCl. Molecular weights (M_r) were obtained from the equation:

$$M_r = \frac{2RT}{\omega^2(1 - \bar{v}\rho)} \frac{d \ln c}{d r^2} \qquad (3)$$

where R is the gas constant; T, absolute temperature; ω, angular velocity; $\bar{v}$, partial specific volume of the solute; ρ, density of the solvent; c, solute concentration and r, distance from the axis of rotation. To derive quantitative values for M_r, it is necessary to know ρ for the solvents and $\bar{v}$ of melittin. Pycnometric measurements at 20°C gave a value of 1 g · ml^{-1} for the buffer and 1.061 g · ml^{-1} for the same buffer with 1.5 M NaCl. $\bar{v}$ was calculated according to McMeekin et al. [15] from the amino acid composition of melittin [16], and gave a value of 0.782 ml · g^{-1}. This value has been corrected to 0.785 ml · g^{-1} to take into account the average deviation observed between calculated and measured values [17].

Results

Effect of salts on the quaternary structure of melittin

Increasing the NaCl concentration up to 2 M produces a red shift of the absorption spectrum from 279.5 to 281.5 nm and a hyperchromic effect of 9% on the maximum of the spectrum (Fig. 1). The sharp contiguous band centered at 287.5 nm is also shifted 2 nm and enhanced.

Fig. 2. shows the change in the fluorescence emission of melittin induced by salt. It can be seen that divalent anions (HPO_4^{2-}, SO_4^{2-}) are much more effective than chloride ions, whereas the difference between the effects of divalent (Ca^{2+}, Ba^{2+}) and monovalent (Na^+, K^+) cations is weaker. This suggests that ionic strength is not the essential parameter but facilitates self-association of melittin by neutralizing repulsive electrostatic forces between positive monomers of melittin.

Sedimentation-equilibrium experiments give linear plots of ln$c$ versus r^2 (see Materials and Methods), which allow an accurate determination of molecular weights. The values obtained are 11200 ± 400 (S.D.) at high ionic strength, and 2300 ± 130 (S.D.) without salt. The initial fluorescence-polarization values shown in Fig. 6 allow us to calculate the ratio between the volumes of tetrameric and probably monomeric melittin (see Materials and Methods). A value of only 3.71 is obtained. Taking the value of 11200 published by others and obtained here by us for the molecular weight of the tetramer, this leads to a molecular weight of 3020 in the absence of salt.

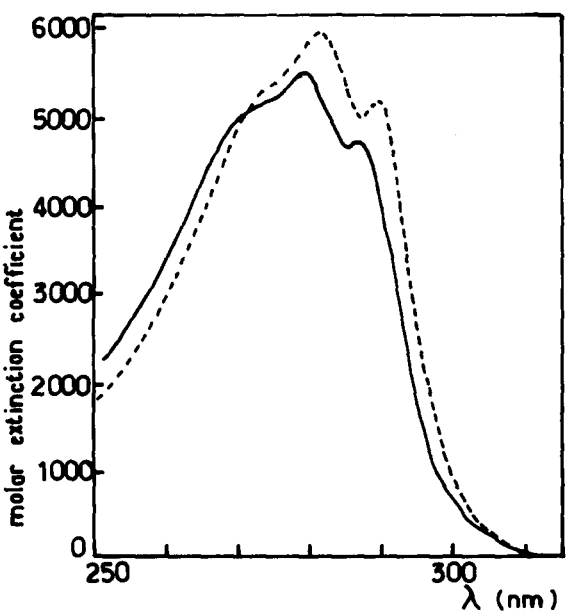

Fig. 1. Absorption spectrum of monomeric melittin in the absence of salt (full line) and tetrameric melittin in the presence of 2 M NaCl (dotted line) in 20 mM Tris-HCl buffer (pH 7.5).

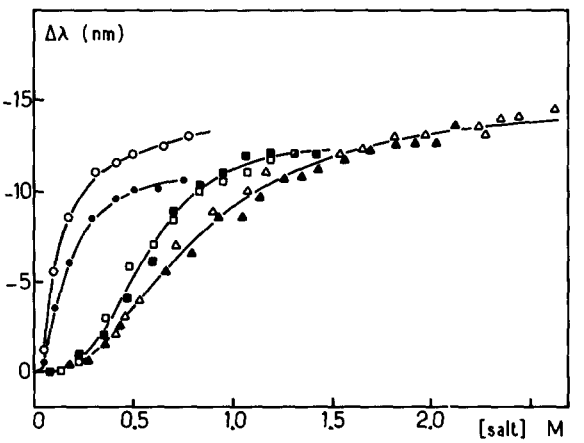

Fig. 2. Salt-induced shifts of the wavelength of the maximum of fluorescence emission of 20 μM melittin in 20 mM Tris-HCl buffer (pH 7.5) at 25°C: ○, Na_2SO_4; ●, Na_2HPO_4; □, $BaCl_2$; ■, $CaCl_2$; △, NaCl; ▲, KCl.

Conformational changes of melittin on binding to lipids

The binding of melittin to lipids can be observed by optical rotatory dispersions with clear

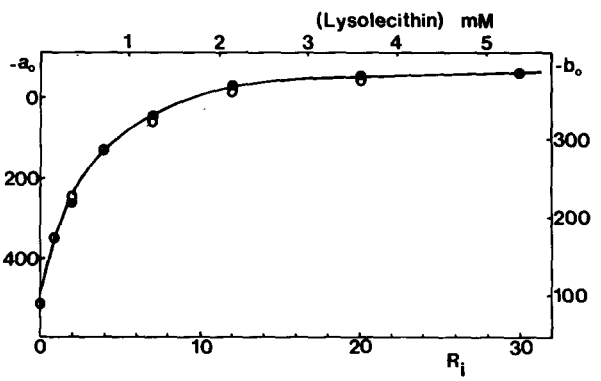

Fig. 3. Conformational change of monomeric melittin on binding to lysolecithin at 20°C. 180 μM melittin in 20 mM Tris-HCl buffer (pH 7.5). R_i, lipid to protein molar ratio. ●, Moffitt parameter a_0; ○, Moffitt parameter b_0.

TABLE I

SECONDARY STRUCTURE OF BOUND MELITTIN COMPARED WITH FREE MONO OR TETRAMERIC MELITTIN AND THE STRUCTURE DERIVED FROM CHOU AND FASMAN CALCULUS [2]

	Percentage		
	α-Helix	β-Sheet	Random coil
Monomeric melittin [2]	12	0	88
Tetrameric melittin [2]	65	15	20
Bound melittin	67	19	14
Calculated from Chou and Fasman [19]	58	0	42

solutions of lysolecithin. Fig. 3 shows the observed changes on the Moffitt parameters a_0 and b_0. The final structure, enriched in α helix (Table I), is very similar to that observed in the presence of salt and attributed to tetrameric melittin; this explains why no significant changes can be observed when tetrameric melittin binds to lipids.

Comparison of monomer and tetramer in their binding to lipids

The effect of the initial association state of melittin on its binding to lipids has been studied by fluorimetry. Binding curves of monomeric melittin to phosphatidylserine bilayers or lysolecithin micelles are monotonous (Fig. 4a) with a 17-nm blue shift (Fig. 4b), while the curve of the emission of fluorescence at 335 nm of tetrameric melittin exhibits a well, followed by an increase of the signal, with only a 2-nm blue shift. The final states seem to be independent of the initial degree of association of melittin.

Effect of chain lengths and critical micelle concentration

The effect of the state of association of lipids on melittin binding cannot be studied with natural phospholipids because of their very low critical micelle concentrations. Since the same kind of binding is observed with negatively charged phospholipid bilayers and zwitterionic lysophospholipid micelles, these studies have been done with solutions of various lysolecithin micelles or very short bicatenar lecithin micelles (in C_8 or C_6)

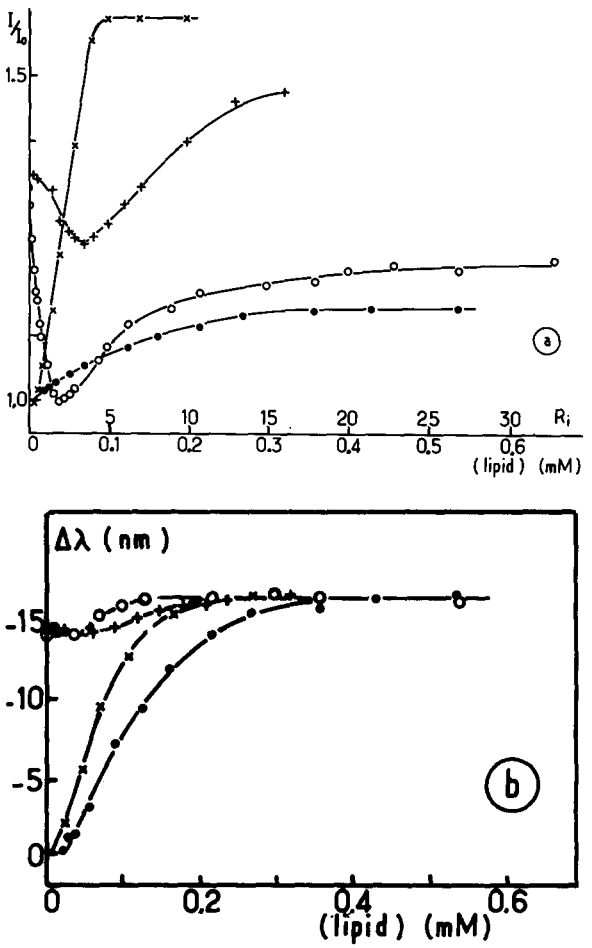

Fig. 4. Fluorescence-emission changes on binding to phospholipid bilayers or micelles of 20 μM melittin in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA, at 25°C. Binding to bovine phosphatidylserine bilayers without NaCl (×) (monomeric melittin), with 2 M NaCl (+) (tetrameric melittin); binding to lysolecithin micelles, without NaCl (●) (monomeric melittin), with 2 M NaCl (○) (tetrameric melittin). R_i, lipid to protein molar ratio. (a) Fluorescence intensity I at 335 nm related to the emission of free monomeric melittin I_0. (b) Shift of the maximum of emission.

which present the advantage of remaining stable and clear after addition of melittin. As observed in Fig. 4a, the binding of melittin monomers is quite different from that of tetramers (Fig. 5). Fig. 5a and b shows the importance of critical micelle concentration on the binding of monomeric melittin, and on the size of the well observed in the binding curves of tetrameric melittin. The toxin always binds to lipids above their critical micelle concentrations, but monomeric melittin cannot

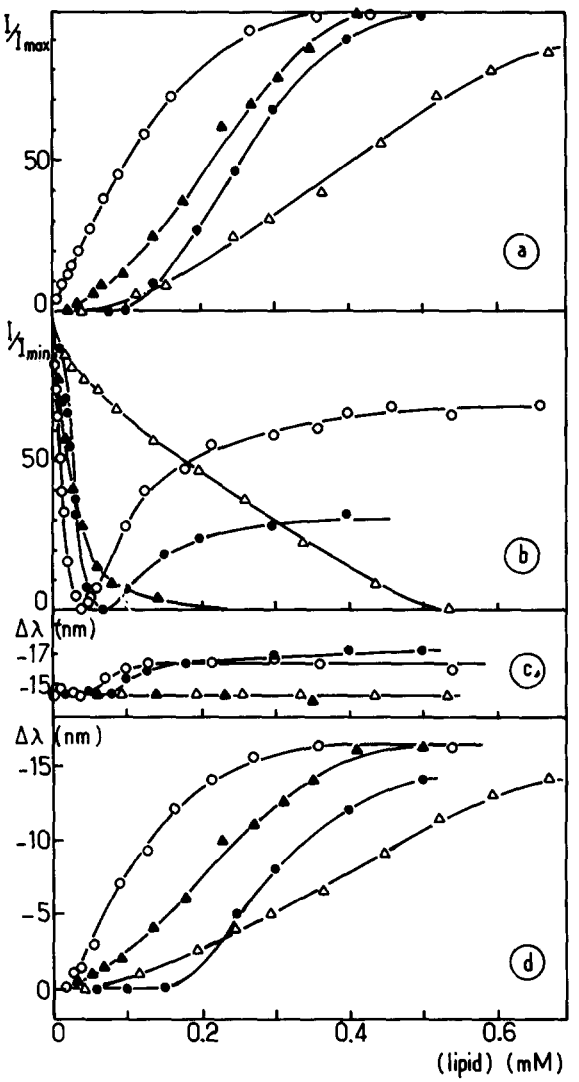

Fig. 5. Effect of critical micelle concentrations on the binding of melittin to various phospholipids. 20 μM melittin in 20 mM Tris-HCl buffer (pH 7.5)/1 mM EDTA, at 25°C. ○, lysolecithin; ●, lysolauroylphosphatidylcholine; ▲, dioctanoylphosphatidylcholine; △, dihexanoylphosphatidylcholine. (a) Fluorescence emission I of monomeric melittin at 335 nm related to the maximum emission I_{max} at 335 nm obtained with each lipid (no NaCl). (b) Fluorescence emission I of tetrameric melittin at 335 nm related to the minimum emission I_{min} at 335 nm obtained with each lipid (2 M NaCl). (c) Shift of the maximum of emission of tetrameric melittin (2 M NaCl), related to the maximum of emission of free monomeric melittin. (d) Shift of the maximum of emission of monomeric melittin (no NaCl).

bind to monomers of lipids, whereas tetrameric melittin interacts with lipids below their critical micelle concentrations. Note (Figs. 5c and 5d) that no change in the maximum emission wavelength is observed in this range. Qualitatively, the higher the critical micelle concentration, the later the beginning of enhanced fluorescence.

Polarization of fluorescence of tryptophan 19

The binding of melittin to lysolauroylphosphatidylcholine has also been studied by polarization of fluorescence (Fig. 6). At low ionic strength with monomeric melittin, no interaction occurs up to 100 μM lysolipid; then, binding takes place in the range of 100–600 μM, as can be seen in Figs. 5a and 5c. At high ionic strength with tetrameric melittin, the degree of polarization increases even in the range of the well and without any shift in wavelength, indicating a swelling of the tetramer, below the critical micelle concentration. The preceding experiments were carried out by adding lipids to 20 μM melittin. The experiment shown in Fig. 6 with lysolauroylphosphatidylcholine, always held above its critical micelle concentration in the presence of 2 M NaCl and melittin added up to 400 μM, underlines the role of the state of associa-

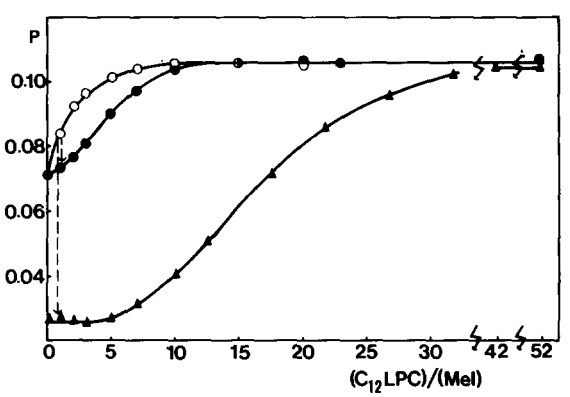

Fig. 6. Binding of melittin (Mel) to lysolauroylphosphatidylcholine (C_{12} LPC) measured by polarization of intrinsic fluorescence P. Effect of critical micelle concentration. 20 mM Tris-HCL buffer (pH 7.5) at 25°C. Excitation wavelength: 280 nm. ▲, 20 μM monomeric melittin (no NaCl) with increasing amounts of lipid; ●, 20 μM tetrameric melittin (2 M NaCl) with increasing amounts of lipid; ○, 400 μM lipid (above its critical micelle concentration) with increasing amounts of tetrameric melittin, with 2 M NaCl. →, test of reversibility by dilution with buffer (long arrow) or with buffer containing 2 M NaCl (short arrow), down to 20 μM melittin and lipid under its critical micelle concentration. The state of association of lipids before addition to the reaction mixture does not affect the results.

tion of lipids in binding to melittin. The reversibility of this binding curve has been checked by dilution with buffer with (or without) 2 M NaCl, down to 20 μM melittin and a lipid concentration below its critical micelle concentration.

Discussion

An apparent discrepancy exists between molecular weights previously obtained for what was assumed to be monomeric melittin [2,5,7] and our values of 2300 from sedimentation-equilibrium measurements and 3020 from polarization of fluorescence. These values are based on a constant $\bar{v}$ of 0.785 $ml \cdot g^{-1}$, but Terwilliger et al. [18] observed that the hydrophobic side-chains of protomers in tetrameric melittin are interdigited, so that the partial specific volume of free monomers should be greater than that of tetramers. Assuming a value of 0.827 $ml \cdot g^{-1}$ for free monomers would give good agreement between our two independent experiments, i.e., 2860 from sedimentation-equilibrium and 2870 from polarization measurements, very close to the molecular weight of one peptidic chain of melittin [19]. The partial specific volume of tetrameric melittin thus obeys McMeekin's calculus, but this method, based on classical globular proteins, cannot be applied to very small 'proteins' such as the peptidic chain of melittin. We have already mentioned a similar problem [2] in the validity of predicted secondary structures [20]. A very high value of $\bar{v}$ is consistent with a random-coiled peptide which is likely to be more extended than a structured protein like the tetramer [21]. The conformation of bound melittin, derived from optical rotatory dispersions, undergoes an increase in helical content similar to that of most membrane proteins [22–25]. It is consistent with the circular dichroism data of Vogel [26] on dimyristoylphosphatidylcholine, of Dawson et al. [27] and Lauterwein et al. [28] on detergents. This secondary structure is very close (as observed by Brown and Wüthrich and co-workers [28,29] on dodecylphosphocholine micelles) to that measured for the tetramer which could form a hydrophobic core resembling the aliphatic part of micelles or bilayers.

The binding of monomeric and tetrameric melittin to lipids is quite different, although final states seem to be very similar. The effect of critical micelle concentrations shows that monomeric melittin needs an interface to interact with lipids, whereas this is not necessary for the tetramer. The slight blue shift observed on tetramer binding may indicate that tryptophan residues are more buried in micelles than in the tetramer. Below the critical micelle concentration, as previously observed by Lauterwein et al. [28] with detergents, a quenching is observed without any change in wavelength. This indicates that an interaction of a different kind is taking place (the fluorescence is always enhanced above the critical micelle concentration). A binding of lipid monomers accompanied by depolymerization of melittin leading to free lipo-monomeric melittin could explain this decrease of fluorescence. This should produce a decrease in the size of fluorescent particles and a subsequent decrease in the degree of polarization. No such decrease is observed in Fig. 6. On the contrary, the degree of polarization always increases, even below the critical micelle concentration, so that the hypothesis of a depolymerization must be discarded. All these results could be explained by a swelling of tetramers induced by monomeric lipids concomitant with the gradual building of mixed micelles, the final state resembling that obtained with monomeric melittin. In tetramers swollen with lipids, no change of the hydrophobicity of the tryptophan environment seems to be observed (no change in emission wavelength) and the quenching

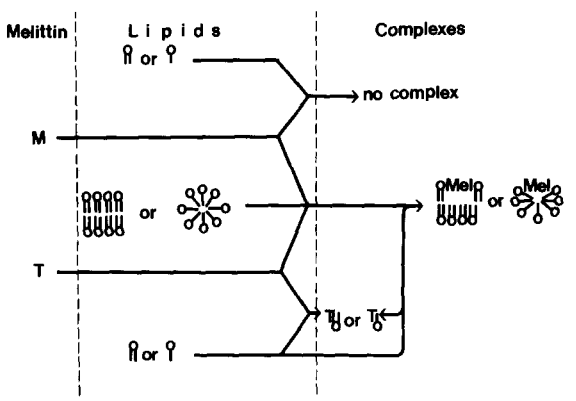

Fig. 7. Melittin binding scheme summarizing the different possibilities described in this paper. or , lipid monomer; M, monomeric, melittin; T, tetrameric melittin; Mel, bound state of melittin.

of fluorescence may be the consequence of a local change of conformation, created by lipids. Differences in lipid organization (bilayers or micelles) due to the length and number of aliphatic chains, or in the charges brought by polar heads, do not qualitatively change the mode of binding of melittin. Therefore, a general scheme (Fig. 7) can be proposed in which bound melittin (Mel) is probably monomeric, since no difference can be observed after the binding of the two species. This study confirms the importance of the state of association of melittin for its binding to organized or monomeric lipids, but leaves still unresolved [28,30–35] the association state of bound melittin.

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