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BBA Report

Kinetics of melittin binding to phospholipid small unilamellar vesicles

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We have used the decrease in the fluorescence intensity of the single tryptophan residue of bee venom melittin at long emission wavelengths that accompanies binding of the peptide to phospholipid small unilamellar vesicles to determine the rate of binding through the use of stopped-flow fluorometry in the millisecond range. We have found the rate to depend on the degree of saturation of the lipid acyl chains as well as on the physical state of the bilayer, the net electric charge of the polar headgroups, and the lipid-to-melittin molar ratio *R*. For zwitterionic lipids (i) the binding process is found to exhibit negative cooperativity, and (ii) the rate-limiting step appears to be penetration of the protein into the hydrophobic region of the bilayer. For negatively charged lipids the results show that binding is a very fast process that seems to be electrostatic in nature.

The interaction of melittin, a major protein component of bee venom [1], with phospholipid vesicles has been used in several studies as a model for protein–lipid interactions. The protein fuses lipid vesicles [2–4] and exerts profound effects on the conformation and motions of the lipid acyl chains [5–7]. In all of the studies published to date, however, melittin was incubated with the lipid vesicles for some time before measurements were made, and consequently, their results pertain to the interaction of the protein with fused vesicles. In the present work we have investigated this interaction on the millisecond time scale, over which melittin-induced fusion is negligible [4]. We have done this by using stopped-flow fluorometry with the single tryptophan residue of melittin as the fluorescent probe. We examine the role of the physical state of the bilayer, the lipid-to-melittin molar ratio, the degree of saturation of the lipid acyl chains, and the electric charge of the lipid headgroups, in the kinetics of binding of melittin to phospholipid small unilamellar vesicles.

Below, we describe the model which we used for the analysis of the fluorescence data. Consider the binding of melittin, at an initial concentration c_0, to vesicles. We have

$$c_o = c_b(t) + c_f(t) \tag{1}$$

where $c_b(t)$ and $c_f(t)$ are the concentrations of bound and free protein, respectively, at time t. For relatively high lipid-to-melittin molar ratios, R, the equilibrium is expected to be shifted toward the melittin-lipid complex. The rate at which $c_f(t)$ changes with time is then

$$\frac{dc_f(t)}{dt} = -k \cdot c_f(t) \tag{2}$$

where k is the rate of binding. From Eqns. 1 and 2 (and the initial condition that $c_f(t) = c_o$ at $t = 0$), we have

$$c_f(t) = c_o \exp(-kt), \quad c_b(t) = c_o[1 - \exp(-kt)] \tag{3}$$

The method we use here utilizes the shift of the fluorescence spectral maximum of the single tryptophan residue of melittin from about 352 nm in an aqueous environment to about 337 nm when bound to lipids. At any emission wavelength there will be a contribution γ_f from the free melittin fluorescence intensity I_f and a contribution γ_b from the bound melittin fluorescence

Abbreviations: DMPC, dimyristoylphosphatidylcholine; dimyristoleoyl-PC, dimyristoleoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid; R, lipid-to-melittin molar ratio; T_m, lipid phase transition temperature.

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intensity I_b, i.e. the total fluorescence intensity I will be given by

$$I = \gamma_f I_f + \gamma_b I_b \tag{4}$$

We consider dilute solutions (absorbance ≤ 0.05) so that the fluorescence intensity is proportional to the concentration. Thus from Eqn. 4 we have

$$I(t) \propto [\gamma_f q_f \varepsilon_f c_f(t) + \gamma_b q_b \varepsilon_b c_b(t)] \tag{5}$$

where q and ε are the fluorescence quantum yield and the molar absorption coefficient for free melittin (subscript f) and for bound melittin (subscript b). Thus Eqn. 5 combined with Eqn. 3 yields

$$I(t) \propto [(\gamma_f q_f \varepsilon_f - \gamma_b q_b \varepsilon_b) \exp(-kt) + \gamma_b q_b \varepsilon_b] \tag{6}$$

We previously [8] established that $q_f \varepsilon_f$ is not very different from $q_b \varepsilon_b$. Thus, the relative transmissions of the filter through which the fluorescence is observed for free melittin and for bound melittin mainly determine whether the preexponential factor in Eqn. 6 is positive or negative. We chose the Corning 0-51 cut-off filter that has a higher transmission for free than for bound melittin. (This filter has a 50% transmission at about 383 nm.) This results in a positive preexponential factor. Thus, as melittin binds to the lipid, the fluorescence intensity of the single tryptophan residue of melittin will decrease with time and will reach a plateau at long times when most of the melittin will be bound. It should be noted that we previously found [4] that melittin-induced vesicle fusion takes place over a much longer time scale (minutes) than that over which melittin binding was observed in the present study.

Fig. 1 shows the plot for binding of melittin to DMPC vesicles at their transition temperature, T_m, of 21°C for $R = 90$. It is seen that the data are consistent with the kinetic model which is described by Eqn. 6, i.e. the tryptophan fluorescence decreases exponentially and reaches a plateau at long times. A nonlinear least-squares

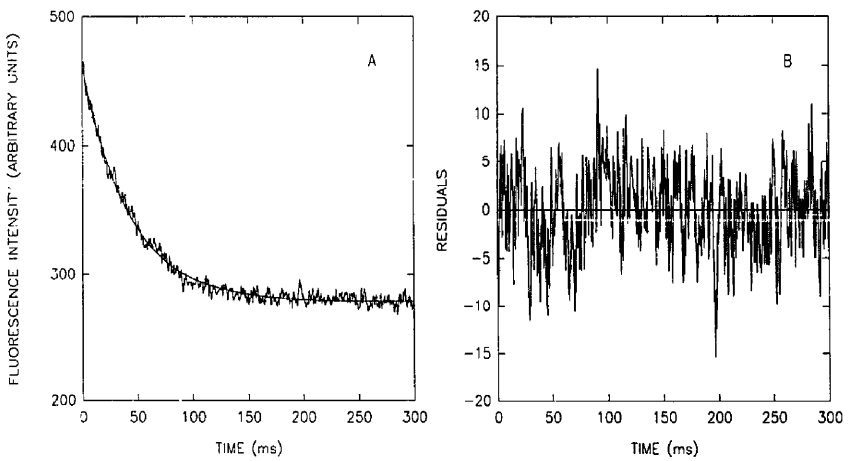

Fig. 1. Stopped-flow fluorometric study of the binding of melittin to DMPC small unilamellar vesicles at the lipid phase transition temperature of 21°C for a lipid-to-melittin molar ratio R of 90. Panel A: Plot of the decrease in the fluorescence intensity of the single tryptophan residue of melittin that accompanies binding of the protein to vesicles. The full line is the nonlinear least-squares fit of Eqn. 6 to the data that yields a value of 0.025 ± 0.002 ms^{-1} for the rate k of melittin binding. (This value is the average of those obtained from at least three experiments.) Panel B: Plot of the deviations (residuals) between experimental data and fitted curve; the lack of a specific trend implies a satisfactory fit to the data. The buffer was 50 mM Tris, 1 mM EDTA (pH 7.6) prepared in triply distilled water. DMPC, as well as all the other lipids used in the present study, of a purity greater than 99%, were obtained from Avanti Polar Lipids (Alabaster, AL). EDTA, analytical grade, was obtained from Fisher Scientific (Pittsburg, PA). Melittin was a product of Sigma (St. Louis, MO). We included in the buffer 1 mM EDTA in order to inactivate any phospholipase A_2 present in the melittin sample; melittin purified in our laboratory as we previously described [6] gave identical results. Small unilamellar vesicles were prepared as previously described [4]. The vesicles were always maintained above T_m prior to measurements; under these conditions they are stable [20]. Stopped-flow measurements were made on a modified Aminco-Bowman DW-2 UV/Vis spectrophotometer previously described [4], with the addition of an Analog Devices RTI 815 A/D converter to digitize the data. One chamber was filled with a 0.118 mM melittin solution. (For this concentration, melittin is known to be monomeric in solution [14].) The other was filled with a lipid solution of the appropriate concentration to yield the desired lipid-to-melittin molar ratio, R, upon mixing with the melittin solution. (For the conditions which obtain in this figure, the lipid concentration used was 7.2 mg/ml.) A Corion interference filter with peak transmission at 280 nm and a bandwidth of 7 nm was used for excitation. Fluorescence was viewed with a Corning 0-51 cut-off filter that has a 50% transmission at about 383 nm. For the sample optical path length of 2 mm, the absorbance of melittin at 280 nm after mixing was about 0.05. This ensures proportionality between the fluorescence signal and the melittin concentration. The sample temperature was controlled to within ±0.1C° with a bath circulator. The dead time of the fluorometer imposed by the mixing of the two solutions is about 3 ms.

TABLE I

Rate of binding k of melittin to lipid vesicles

Lipid/melittin molar ratio (R)	k(ms^{-1})			
	DMPC			dimyristoleoyl-PC
	16°C	21°C (T_m)	33°C	33°C ($> T_m$)
90	0.036	0.025	0.095	0.189
135	0.045	0.036	0.096	too fast
180	0.070	0.045	0.099	too fast

fit of Eqn. 6 to the data yields an average value of 0.025 ms^{-1} for the rate of binding k. We also studied the dependence of k on temperature for three values of R (Table I). It is seen that for all cases k is minimal at T_m and maximal above T_m. The increase in k below T_m (Table I) may be the consequence of the unusual faceted or polygonal structure of the vesicles in that temperature region [9,10]; it is conceivable that the protein exhibits a preference for the disordered boundaries between the facets, the number of which increases as the temperature is lowered. Fig. 2 shows the temperature-dependence of the binding constant for $R = 90$. The regions adjacent to T_m in the plot can be approximated by straight lines whose slopes yield −90 kcal/mol and 70 kcal/mol for the values of the activation energy below and above the transition temperature, respectively. Above T_m, however, there is a second region of lower activation energy, about 20 kcal/mol; this behavior may be the result of the asymmetry of the thermal transition profile [11]. It is also seen from Table I that, for a particular temperature, k decreases with a decrease in R, except above T_m. (A dependence of k on R is also exhibited by the binding of melittin to a derivative of DMPC that has a double bond on each acyl chain, see below.) It could be argued that this effect is due to the successive reduction in the lipid concentration as lower values of R are obtained, so that binding would then take place at successively slower rates. [We note that the same melittin concentration (0.118 mM) was used in all of our measurements.] We have calculated, however, that the melittin-vesicle encounter time for all the concentrations used here is in the microsecond range and is therefore not observable with our stopped-flow fluorometer. Consequently, the kinetics of the 'binding' we observe should be concentration-independent. We confirmed this by showing that, in experiments in which both the lipid and the melittin solutions were diluted by a factor of ten before they were mixed, the values of the rate of binding were identical with those for undiluted solutions. This implies that the melittin 'binding' we observe is not a bimolecular reaction, and that therefore the change in the rate constant with R is not due to a change in the concentration of the reactants. The origin of the reduction in k as R decreases appears instead to be due to negative cooperativity in the binding process. That is to say, as a population of melittin molecules binds in a random time sequence, the molecules that bind at earlier times may shield charges on the lipid headgroups and/or induce changes in the bilayer which render subsequent binding less probable. In this respect, the value of k should not be considered as being a single one but rather an apparent one and to stem from a distribution of rates. Interestingly, at 33°C binding to DMPC vesicles is not cooperative as deduced from the finding that at this temperature k is virtually independent of R (Table I).

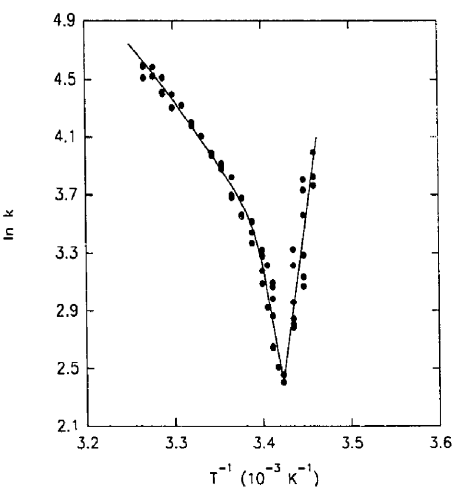

Fig. 2. Arrhenius plot of the dependence on temperature T of the rate of binding, k, of melittin to DMPC small unilamellar vesicles for a lipid-to-melittin molar ratio R of 90. The standard deviation in the values of k is estimated to be about ±7%. Linear fits to the regions adjacent to the transition temperature, T_m, gave values of −90 and 70 kcal/mol for the activation energies of binding below and above T_m, respectively. Also present further above T_m is a second region, a linear fit to which gave an activation energy of about 20 kcal/mol. The minimum is reached at a temperature of about 19.5°C, which is close to T_m. The temperature was not lowered below 16°C in order not to facilitate extensive vesicle aggregation. Such aggregation for dipalmitoylphosphatidylcholine small unilamellar vesicles at a much lower temperature relative to their T_m than was the case for the present study, was shown [20] to take a much longer time period than the 10 min of temperature equilibration used in making the present measurements. More experimental details are given in the caption to Fig. 1.

We investigated the importance of the degree of saturation of the acyl chains in the binding process by using dimyristoleoyl-PC (an unsaturated derivative of DMPC with a double bond at positions 9,10 on each acyl chain). As can be seen from Table I, at 33°C and for $R = 90$ the rate of melittin binding is much larger than that for the corresponding saturated lipid DMPC, 0.189 ms^{-1} vs. 0.095 ms^{-1}. This difference is very pronounced for $R = 135$ and $R = 180$: for dimyrist-

oleoyl-PC this rate of binding is too fast to measure with our fluorometer. This means that k is greater than about 0.3 ms^{-1}. (This value corresponds to a relaxation time = $1/k$ of about 3 ms which is the limit of resolution of the fluorometer.)

We next studied binding to negatively charged phospholipids. For dimyristoylphosphatidylserine at pH 7.6, for which this lipid carries one negative charge, we found the binding to be too fast to measure for below, at, and above the phase transition temperature of 38°C [12].

These results have important implications regarding the nature of the forces involved in the binding process. The first step in the binding process most probably involves electrostatic attraction between the positive charges of the C-terminus of the protein and the negative charges of the lipid polar headgroups. This appears to be a very fast process as we observed for the negatively charged dimyristoylphosphatidylserine. A steady-state fluorescence polarization study [13] found that for negatively charged lipids binding is independent of the physical state of the bilayer, which suggests that it is external. The shift in the fluorescence spectral maximum to shorter wavelengths that accompanies binding to charged lipids would then stem from a conformational change in the protein that results in shielding of the tryptophan residue from the aqueous environment. The observed large increase in k for unsaturated acyl chains (Table I), which are more flexible than the saturated ones, suggests that for zwitterionic lipids the rate-limiting step is the partial penetration of the protein into the hydrophobic region of the bilayer. The observed increase in k above T_m for DMPC (Table I) is in accord with this conclusion. Further work will include binding to other phospholipids which have longer acyl chains, binary mixtures of zwitterionic and negatively charged lipids, and solutions of melittin either of high concentration or in high ionic strength buffer for which melittin is tetrameric [14]. With regard to the conformation of bound melittin, under the latter conditions the protein binds as an aggregate, whereas at its relatively low concentration of 0.118 mM used in the present study it binds as a monomer [15] *.

It should be noted that the model used in this study for the analysis of the data is a simplified one; it only represents a first attempt toward the description of the kinetics of the binding of melittin to vesicles. The macroscopic rate k which we measured here is actually the sum of the rates of insertion and deinsertion of the protein (which is already bound to the vesicle surface) into the bilayer. The present analysis considers the rate of deinsertion of the protein to be very small in comparison to that of insertion. The determination of both of these rates necessitates time-resolved measurements of the emission anisotropy on the millisecond scale as a function of the emission wavelength. (This will require an improvement in the sensitivity of our fluorometer by about one order of magnitude.) In this regard, the delineation of these two rates for below, at and above T_m is necessary in order to explain why the macroscopic rate k is observed to be a minimum at T_m for DMPC (Table I). Interestingly, it can easily be shown that, even if the rate of deinsertion of the protein into the bilayer is not negligible, the binding process is still cooperative in nature in the framework used in the present study.

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* It should be noted that studies in which modified melittin was used (either by attaching fluorescent probes [16–18] or [^{13}C]methyl groups [19]) reached conflicting conclusions concerning the state of aggregation of melittin bound to lipid vesicles. This was discussed in Ref. 15, in which study native melittin was used.