The Interaction of Melittin with E. Coli Membrane: the Role of Cardiolipin

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We studied the interaction of melittin peptide with E. Coli’s lipid membrane as a function of the molar peptide-to-lipid ratio (P/L). Two experimental methods were employed. The oriented circular dichroism (OCD) measured the peptide’s orientation in the membrane, whereas the lamellar x-ray diffraction (LXD) measured the membrane perturbed by peptides. The result shows that in the measuring range, P/L $\sim$ 1/150 to 1/20, all peptides binding to the membrane adopt a helical conformation, with about 40% of the m having a helical orientation normal to the surface (I-state) and the rest parallel to the membrane surface (S-state). In the same P/L range, it appears that the membrane is strongly perturbed by peptides in which the membrane is thinning, and such thinning increases with P/L. Furthermore, we mimiced the E. Coli membrane by two model lipid membranes, one containing cardiolipin while the other does not. We found that cardiolipin is the key lipid component in an E. Coli membrane which enhances the insertion of melittin in the low P/L region.

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I. INTRODUCTION

Antimicrobial peptides are gene-encoded evolutional weapons, which are widely used by animals and plants in their innate immune systems to fend off invading microbes [1–4]. Because of their amphiphilicity, the antimicrobial peptides are found to directly interact with a cell membrane, making the target cell die [5–8]. More interestingly, antimicrobial peptides are also found to exhibit high cell-type selectivity, that is, they can kill the invading cells but leave the host cells unharmed. At present, the factors which determine the selectivity are not all well understood.

The electrostatic effect is the first factor that has been recognized. This is because of the fact that almost all antimicrobial peptides are strongly cationic, so that they are attracted to the negatively charged lipids on the outer leaflets of bacterial membranes, whereas such an electrostatic effect is absent for mammalian membranes of which the outer leaflets are electrically neutral. Another factor that has been noticed is the lipid composition of the cell membrane. For example, antimicrobial peptides were found to have more difficulty in forming transmembrane pores in a membrane made of mixing phosphatidylethanolamine (PE) and phosphatidylcholine (PC) than in that of pure PC lipid [9]. Another example is that cholesterol contained in the membrane of lipid vesicles was found to suppress the leakage of vesicles induced by antimicrobial peptides [10, 11]. In this paper, we will study the effect of the lipid composition of E. Coli on an antimicrobial peptide’s activity.
E. Coli is a well-studied bacterium. The lipid composition of E. Coli’s membrane contains \( \sim 75\% \) phosphatidylethanolamine (PE), \( \sim 20\% \) phosphatidylglycerol (PG) and \( \sim 5\% \) cardiolipin (CL) [12]. The latter two lipids are negatively charged. They of course make E. Coli membrane very attractive to the positively charged antimicrobial peptides due to the electrostatic effect. Once peptides are attracted to bind on a membrane, however, the peptide’s further activity depends on the interaction between the peptide and the membrane, which in turn is determined by the lipid composition. CL is a unique phospholipid only found in the membranes of bacteria and mitochondria. It is a dimeric phospholipid in which two phosphatidyl moieties are linked by a central glycerol group [13, 14]. There is much evidence to support the conclusion that the full hydration lipid bilayers in oriented multilamellae [15] have the same properties as the lipid bilayers forming the vesicles in aqueous solution [16-19], having the same gel-to-fluid transition temperature and a similar undulation fluctuation. We also find that there are many reports showing that CL enhances peptide-inducing leakage of vesicles [20–23]. However how CL plays its role in peptide-membrane interactions is still not clear. In this study, we will pay attention to this issue using the oriented multilamellae membrane.

The antimicrobial peptide used in this study is the melittin from bee venom. Its interaction with bilayer membranes of rich PC lipids has been extensively studied [24–27]. It has been shown that melittin peptides binding to PC lipid membranes exhibit a helical conformation. In low peptide concentration (hereafter represented by the molar peptide-to-lipid ratio, \( P/L \)), all melittin peptides have their helical orientation parallel to the membrane surface (S-state). Upon exceeding a certain threshold \( P^*/L \), a fraction of melittin peptides turn their helical orientation normal to the membrane (I-state), forming transmembrane pores, as identified by neutron scattering experiments [28]. We will see in this study that the interaction of melittin with E. Coli membrane as a function of \( P/L \) is still compatible with this two-state model, but the interplay between the peptide’s activity and membrane’s response is not exactly the same as that found in the PC lipid membrane.

The E. Coli membrane used in this study was made of E. Coli polar extracted lipids. The compositions of E. Coli polar extracted lipids are PE/PG/CL (3:1:0.2 in molar ratio) [data sheet of Anvanti Polar Lipids (Alabaster, AL)]. To see the effect of CL on the peptide’s activity, we first mimic the E. Coli membrane by a model membrane consisting of DOPE/DOPG/CL with the same molar ratio (3:1:0.2) as the E. Coli membrane. Then we used another model membrane consisting of DOPE/DOPG (3:1) to represent a CL-deprived E. Coli membrane. These three lipid systems were measured as a function of \( P/L \) by the method of oriented circular dichroism (OCD) and the method of lamellar x-ray diffraction (LXD), both have been successfully employed in our previous studies [24–27]. OCD measured the peptide orientation in bilayers, whereas LXD measured the bilayer membrane thickness. We will show that CL essentially is the key lipid component for turning the helical orientation of membrane-bound melittin from the S-state to the I-state.
II. EXPERIMENT

II-1. Materials

E. Coli Polar Lipid Extract (E. Coli Polar), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1, 2-Dioleoyl-sn-Glycero-3-[Phospho -rac-(1-glycerol)] (Sodium Salt) (DOPG), and 1′, 2′, 2′-Tetraoleoyl Cardiolipin (CL) (Sodium Salt) were purchased from Avanti Polar Lipids (Alabaster, AL). Melittin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Two grades of melittin were purchased, the sequencing grade (product no. M-1407) and the grade of purity 93% HPLC (product no. M-2272). Both gave the same results in this study. Yang et al. also found no difference between Sigma melittin and pure synthetic melittin in this type of study, as long as there was no added Ca$^{2+}$ in the sample [33]. Polyethylene glycol (PEG400) was purchased from Merk Co. (Hohenfrunn, Germany). All materials were used as delivered.

II-2. Sample preparation

In this study, two experimental methods were used. One was oriented circular dichroism (OCD) for the measurement of the peptide orientation in lipid bilayers [29, 30]. Another was lamellar x-ray diffraction (LXD) for the measurement of the membrane thickness [31–36]. The samples used in both methods were in the form of oriented multilayers, a stack of parallel hydrated lipid bilayers on a solid substrate. The preparation of such oriented samples followed the method described in Ludtke et al. [37]. Briefly, lipid and peptide of chosen peptide-to-lipid molar ratio (P/L) were co-dissolved in a solvent of 1:1 (v/v) methanol and chloroform. The lipid concentration was about 1 mg per 20 µl solvent. An appropriate amount of the solution was spread onto a cleaned quartz surface–5 µl or less solution (depending on the P/L) onto a 12 mm diameter area for OCD, or 60 µl solution onto a 18 × 18 mm² area for LXD. When the solvent dried, the sample was vacuumed to remove the remaining solvent residue, and then slowly hydrated with water vapor until it appeared transparent. A good sample was visually smooth and showed at least five orders of Bragg diffraction by LXD. Three peptide/lipid systems were studied systematically, i.e., melittin in E. Coli polar, melittin in DOPE/DOPG/CL mixtures, and melittin in DOPE/DOPG mixtures.

II-3. OCD measurement

The sample chamber was a cylindrical construction, as described in Chen et al. [24]. The light beam of the CD spectropolarimeter was along the cylindrical axis, perpendicular to the two parallel quartz windows. One of the windows was the quartz plate; the sample was deposited on its inside surface. The space between the windows was sealed. The rim of this space was used to hold distilled water for a full hydration measurement, or a PEG solution for a less than full hydration measurement. The humidity corresponding to a polyethylene glycol (PEG400) solution was measured by a hygrometer in a calibration chamber provided by the hygrometer manufacturer (Rotronic Instrument Co., Huntington, NY). A typical concentration used in this study was 1 g of PEG400 in 4.0 g water, which
gave a 98.0% relative humidity (RH) at 30°C. The outer part of the sample chamber was a thermostat. The temperature was monitored by a Pt100 thermo-resistor and controlled by a computer via a feedback thermo-electric module. The temperature could be controlled from 10° to 40° C with a stability of ±0.1° C for several days. The cylindrical sample chamber was allowed to rotate around its axis for the purpose of rotational averaging.

The hydration equilibrium of the sample was ensured by an agreement of at least three OCD spectra measured over a period of 6 h. OCD was measured with a Jasco J-810 spectropolarimeter, with light incident normal to the sample surface [29]. The sample was allowed to rotate around the incident light at eight angles equally spaced in one complete rotation. The averaged spectrum of the measurements at eight rotational angles was used for analysis. The rotational average ensured the elimination of possible artifacts due to linear dichroism [29]. The background OCD spectra of pure lipid bilayers (i.e., without peptides) were measured separately and were removed from the spectra of the corresponding samples containing peptide.

The reason we chose 98% RH (rather than 100% RH) for this experiment was that for both OCD and LXD measurements the sample substrate was oriented vertically. At levels of humidity higher than 98% RH, the membranes on an open sample (i.e., on one substrate) would flow. This is not to say that it is impossible to make measurements at 100% RH. An oriented membrane sample could be covered with another substrate to prevent the sample flow, as was done previously for OCD [24, 29] and for LXD [7, 38, 39]. However, it would take a long equilibrating time to change the hydration of a covered (i.e., two-substrate) sample, and hydration changes are necessary in an x-ray experiment for the purpose of phase determination. Previous experiments have shown that the dependence of the threshold concentration on hydration is gradual. There is no qualitative difference between the states of peptides measured at 98% RH and at 100% RH [24, 25].

The OCD spectra for the membrane surface state (S state) and the insertion state (I state) of melittin were measured previously by Chen et al. [25] and Yang et al. [28] in DMPC bilayers in another laboratory. These spectra were re-measured and reproduced here using the instrument described here.

II-4. LXD measurement

The sample chamber for LXD was the same as that used in our previous studies [7, 31–39], except that the relative humidity was controlled by a series of PEG solutions enclosed inside the chamber. This was to ensure that the hydration levels of the sample were the same in the OCD and LXD measurements. The temperature was set at 30°C, the same temperature used for the OCD measurement. In addition to the measurement at 98% RH, a series of measurement were made at lower levels of humidity for the purpose of phase determination. A precise reading for these lower level humidities was not necessary, because the swelling method used for phase determination depended on the precise reading only of the lamellar repeat spacing.

LXD was measured with Cu Kα radiation generated by a sealed tube at 30kV/30mA by a θ-2θ scan from θ = 0.5 to 7.5° with a step size Δθ = 0.01° at one sec per step. The equilibrium of the sample at each humidity setting was ensured by an agreement of at
least three consecutive diffraction patterns whose average was subsequently analyzed. Only samples that produced at least 5 discernible diffraction peaks were accepted. Each peptide-lipid combination was measured with at least two separately prepared samples. Each sample was measured twice separately at least 10 hours apart to check the reproducibility.

The procedure for data reduction has been described in many previous papers [7, 38, 39]. Briefly, the procedure started with the background removal, and the absorption and diffraction volume corrections. Then the integrated peak intensities were corrected for the polarization and the Lorentz factors. The magnitude of the diffraction amplitude $F_h$ was the square root of the integrated intensity. The bilayer symmetry leads to either a positive or a negative phasing for $F_h$. The phases of the amplitudes were determined by the swelling method [40, 41]. Once the phases (positive or negative) were determined, the diffraction amplitudes were Fourier transformed to obtain the trans-bilayer electron density profiles, $\rho(z) = \Sigma h F_h \cos(Q_h z)$, where $Q_h$ was determined by the relation $(4\pi/\lambda)\sin\theta_h = Q_h = h2\pi/D$, here $\lambda$ is the x-ray wavelength and $D$ is the lamellar repeating distance. The profiles were not normalized to the absolute scale, but they gave the correct peak-to-peak distances, since the latter are independent of normalization [39].

III. RESULTS

III-1. Peptide orientation by OCD

The helical orientation of melittin was measured in three lipid compositions: E. Coli polar, DOPE/DOPG/CL (3:1:0.2), and DOPE/DOPG (3:1). In each lipid composition, the OCD was measured for a series of P/L, including one without peptide (P/L= 0) for the background removal. The raw data of the OCD measurements are shown in Fig. 1. The analysis of the OCD spectra follows Chen et al. [25]. Briefly we found that each spectrum can be fit very well to a linear combination of two mutually normalized bases, an I state spectrum and an S state spectrum, as $\alpha[I + (1 - \phi)S]$, where $\alpha$ is a normalization factor. The I and S spectra were obtained from the melittin in the DMPC bilayers, as described in Yang et al. [28]. According to the theory of OCD [29], the I spectrum represents a helix oriented perpendicular to the plane of bilayers and the S spectrum represents a helix oriented parallel to the plane. The linear decomposition of the spectra in Fig. 1 gives the fraction of melittin in the I state, $\phi$, as a function of P/L (Fig. 2).

III-2. Membrane thinning by LXD

Every OCD sample was measured by LXD under the same condition as the OCD was measured, and at a few lower humidity levels for the purpose of determining the phases of the diffraction amplitudes. Representative diffraction patterns, phasing diagrams, and the transmembrane electron density profiles are shown in Figs. 3, 4, and 5, respectively. It is very important to note that every diffraction pattern has only one Bragg series, indicating the homogeneity of the peptide-lipid mixtures. For every lipid mixture, we were concerned about the possibility of phase separations, but we did not find them. From each electron density profile, we measured the peak-to-peak distance (PtP) corresponding to the
FIG. 1: Oriented circular dichroism spectra of melittin in E. Coli polar membrane (a), in DOPE:DOPG:CL (3:1:0.2) (b) and in DOPE:DOPG (3:1) (c). All measurements were made for a series of P/L at 30°C and 98% RH. The lipid background has been removed from each spectrum. In each panel, the mutually normalized basis spectra for the S-state and the I-state were obtained from melittin in DMPC [27]. Each OCD spectrum can be fit with a linear combination of S-state and I-state bases to obtain the fraction of the peptide in the I-state.
phosphate-to-phosphate distance, and plotted the result as a function of P/L for each lipid composition (Fig. 6).

Membrane thinning linearly proportional to P/L has been noted since 1995 by Wu et al. [39] (see more references in [27]). This is because the inclusion of peptides at the interface stretches the membrane area. The stretching occurs under the condition that the peptides are not inserted transmembrane (the S state). The volume of the hydrocarbon region is conserved. Thus the fractional area increase $\Delta A/A$ is equal to the fractional thickness decrease of the hydrocarbon region $-\Delta h/h$. The thickness of the hydrocarbon region $h$ is $\text{PtP-10 Å}$ or PtP minus twice the length of the glycerol region (from the phosphate to the first methylene of the hydrocarbon chains). The latter is very close to $10 \text{ Å}$ [36, 42, 43].

We introduce the quantity $A_P$ as the area increase due to the addition of one peptide in the S state. Let $A_L$ be the cross sectional area per lipid which is calculated by

$$A_L = \frac{\text{chain volume}}{(h/2)}.$$  

Then we have

$$-\Delta h/h = \Delta A/A = (A_P/A_L)(P/L).$$

Thus $A_F$ is practically a directly measured quantity, i.e., from the measurement of PtP and the well known values for the lipid chain volumes [43].

### III-3. Thermodynamic analysis

Consider the free energy change when a peptide binds to a lipid bilayer from solution. Before the bound peptide-to-lipid ratio P/L exceeds the threshold P*/L, all peptides are bound in the headgroup region on the membrane surface, which has the effect of increasing the membrane area. Therefore the differential free energy change can be written as

$$\delta F = -\varepsilon_s \delta P + \sigma \delta A,$$

where $\varepsilon_s$ is the binding energy and $\sigma$ is the membrane tension. For a finite (rather than infinitesimal) P/L, the free energy change normalized to per lipid is

$$\Delta F/L = \Delta f = -\varepsilon_s(P/L) + 1/2K_A A_L (\Delta A/A)^2 = -\varepsilon_s(P/L) + 1/2K_A (A_P^2/A_L)(P/L)^2. \tag{1}$$
FIG. 3: Representative LXD diffraction patterns. An attenuator was used for the first order Bragg peak to prevent the photon count from exceeding $10^4$ per sec. The patterns were displaced for clarity. Note that each pattern consists of only one Bragg series, indicating the homogeneity of the sample.

FIG. 4: Representative phasing diagrams obtained by the swelling method. The phases were chosen so that a form factor, Fourier-constructed from the diffraction amplitudes measured at one humidity level, will go through all the data points [39].
FIG. 5: Representative electron density profiles constructed from the measured diffraction amplitudes, displayed for a series of P/L.

FIG. 6: Peak-to-peak distance (PtP) vs. P/L. The error bars represent the ranges of reproducibility from four measurements, two measurements for each of two independently prepared samples.
For P/L above the threshold, a fraction of the bound peptide molecules, $\phi P \equiv P_I$, participates in pore formation. We have to allow for a possible effect of the pore on the membrane thickness. Thus we modify $\Delta A = A_p P$ for P/L $< P^*/L$ to

$$\Delta A = A_p(P - P_I) + \beta A_p P_I = A_p P [1 - \phi (1 - \beta)]$$

(2)

for P/L $> P^*/L$. The parameter $\beta$ expresses the effect of a peptide in the pore relative to the effect of a peptide bound on the surface. We base our consideration on $-\Delta h/h = \Delta A/A$. If $\Delta A$ is negative, $\Delta h$ will be positive which indicates that the membrane will be thickening. On the other hand, $\Delta A$ positive indicates the membrane will be thinning. Then for P/L $> P^*/L$, the free energy change is given by [24, 25],

$$\Delta f = -\varepsilon_s (1 - \phi)(P/L) - \varepsilon_p \phi P/L + (1/2) K_A (A_p^2/A_L) [(1 - \phi) P/L + \beta \phi P/L]^2,$$

(3)

where $\varepsilon_s$ and $\varepsilon_p$ are the energy per peptide in the S state and the I state, respectively; $K_A$ is the area stretch modulus of the bilayer [44]. The meaning of the first two terms is obvious—they represent the two possible states for the peptide. The third term is the elastic energy for the thinning of the bilayer. The thinning is caused by the peptide adsorption in the bilayer. It is this term that describes the peptide-peptide interaction mediated by membrane deformation (thinning). This interaction is extended, involving all the peptides in the bilayer—a many-body effect.

The minimization of $\Delta f$ with respect to $\phi$ gives the relation

$$\phi = \frac{1}{1 - \beta} \left( 1 - \frac{P^*/L}{P/L} \right),$$

(4)

with the threshold concentration $P^*/L$ given by

$$P^*/L = \frac{\varepsilon_s - \varepsilon_p}{K_A (A_p^2/A_L)(1 - \beta)}.$$  

(5)

Eq. (4) explains why the fraction of the peptide in the I state $\phi$ is a linear function of $1/(P/L)$. Since the threshold concentration $P^*/L$ has been determined by OCD (Fig. 2), the parameter $\beta$ can be determined from the slope of $\phi$ vs. $1/(P/L)$. Thus all the parameters appearing in the free energy $\Delta f$ are independently determined, except for the energies of the S state and the I state relative to the peptide in solution. Only the relative energy $\delta \varepsilon = \varepsilon_s - \varepsilon_p$ is determined by Eq. (5). The physical meaning for each of the parameters has been discussed extensively in Lee et al. [27]. For Mel/PE:PG(3:1), when P/L $> P^*/L$ we have $\beta \sim -1.1$ and $\phi \sim 0.4$. Thus $\Delta A = A_p P [1 - \phi (1 - \beta)] = A_p P [1 - 0.88] > 0$. The results indicate that the membrane will be thinning, as shown in Fig. 6.

IV. DISCUSSION

In the measuring range of P/L, from 1/150 to 1/20, there appears a constant fraction, 40%, of melittin peptides in the I-state in E. Coli membrane (Fig. 2, open circles). The same
result happens to the model membrane DOPE/DOPG/CL (3:1:0.2) (Fig. 2, solid squares),
indicating that it is a representative of E. Coli, a mimic membrane. According to the two-
state model of the peptide’s activity, the threshold P*/L for these two lipid systems must
be lower than the lower bound of our measuring P/L range, that is, below 1/150. What is
the lipid component in E. Coli membrane that makes such a low P*/L? The answer is CL
lipid. The reason is as follows. Since DOPE/DOPG/CL (3:1:0.2) is a good representative
of the E. Coli-mimic membrane, the model membrane DOPE/DOPG (3:1) thus represents
an E. Coli-mimic membrane but deprived of CL. We see that the melittin’s activity on this
membrane exhibits a sigmoidal dependence on peptide concentration (Fig. 2, opened squares
with solid line). The threshold P*/L in this case can be well determined. It appears to be
around 1/100, which is larger than that which occurs in the E. Coli membrane. Therefore,
it is clear that CL is the key lipid in the E. Coli membrane that enhances the melittin
insertion in the low P/L region.

The membrane response to the peptide’s activity is revealed by the change of PtP as
a function of P/L. Fig. 6 shows that the PtP of the three lipid systems decreases linearly
with P/L. Such membrane thinning has been noticed since 1995 by Wu et al. [39] (see
more references in [27]). A simple explanation is the following. In the measured P/L
range, there is at least a 60% fraction of melittin peptides that are in the S-state (see
Fig. 2). These S-state peptides are located at the hydrophobic-hydrophilic interface of the
membrane, so that they will stretch the membrane’s surface, causing an increase in the
surface area. Since the volume of the membrane’s hydrocarbon region is approximately
conserved, an increase in surface area leads to a decrease in membrane thickness, that is,
membrane thinning. The more the peptides binding to the membrane, the larger the area
expanded. Thus the membrane thinning increases with peptide concentration, P/L, as
shown in Fig. 6. The interesting fact is that the degree of membrane thinning with P/L
is almost the same for the three lipid systems. This indicates that PE and PG are the
major lipids responsible for the thinning of the E. Coli membrane induced by melittin. In
other word, the CL contained in the E. Coli membrane does not have a contribution to
the membrane thinning. This can be understood, because, after all, the E. Coli membrane
contains only 0.2/(3+1+0.2) = 4.8% CL lipids. However, this lipid minority CL does have
a significant effect on peptide insertion, as we already saw from the discussion of the OCD
result (Fig. 2).

Finally, it is interesting to discuss the relationship between the peptide activity and
the membrane thinning. In our previous studies, we used PC lipids as the majority in
membranes and found that membrane thinning is correlated to the peptide state in the
following way [24–27]. The membrane thinning increases with P/L in the low P/L region,
where all peptides are in S-state, but it reaches a limit as P/L exceeds P*/L, where a
fraction of peptides turn to the I-state. We also proposed a thermodynamic free energy
model including thinning elastic energy to give an explanation. However, such a correlation
between peptide activity and membrane thinning does not exist in the present study (see
Fig. 2 and Fig. 6). The thinning of the E. Coli membrane induced by melittin does not
reach a limit when a fraction of peptides come out in the I-state. It is not unreasonable to
speculate that the majority lipid PE in E. Coli membrane is the key leading to a membrane
response differing from that which happens in PC lipid membrane.

V. SUMMARY

We have made OCD and LXD measurement to study the interaction of melittin peptide with E. coli membrane as a function of peptide concentration. The results can be summarized as follows: (1) Melittin peptides binding to E. Coli membrane adopt a helical conformation in which about 40% of them are in the I-state and the rest in the S-state. (2) The melittin peptides binding to E. Coli membrane induces membrane thinning as they do to the PC lipid membranes. But the correlation between membrane thinning and peptide state is different between the E. Coli and PC lipid membranes. (3) CL, the lipid minority contained in E. Coli membrane, plays a key role in enhancing the melittin insertion into the membrane, particularly in the low P/L region.

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