Sigmoidal Concentration Dependence of Antimicrobial Peptide Activities: A Case Study on Alamethicin

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ABSTRACT The transition of the state of alamethicin from its inactive state to its active state of pore formation was measured as a function of the peptide concentration in three different membrane conditions. In each case the fraction of the alamethicin molecules occupying the active state, \( \phi \), showed a sigmoidal concentration dependence that is typical of the activities of antimicrobial peptides. Such a concentration dependence is often interpreted as due to peptide aggregation. However, we will show that a simple effect of aggregation cannot explain the data. We will introduce a model based on the elasticity of membrane, taking into consideration the membrane-thinning effect due to protein inclusion. The elastic energy of membrane provides an additional driving force for aggregation. The model produces a relation that not only predicts the correct concentration dependence but also explains qualitatively how the dependence changes with membrane conditions. The result shows that the membrane-mediated interactions between monomers and aggregates are essential for the strong cooperativity shown in pore formation.

INTRODUCTION

It is well known that the activities of membrane-active antimicrobial peptides, whether on bactericide, hemolysis, or liposome lysis, often exhibit a sigmoidal (sometimes described as all-or-none) dependence on the peptide concentration (Steiner et al., 1988; Boman et al., 1994; Shai, 1999). These phenomena are often interpreted as an aggregation effect of the peptides, although the data were rarely analyzed quantitatively. In this paper, we will use the example of alamethicin to measure and analyze the concentration dependence of the peptide activity. We conclude that the sigmoidal dependence cannot be explained as a simple aggregation effect of peptide, rather the phenomenon requires an additional driving force that is provided by a membrane-thinning effect induced by the peptide inclusion.

Gene-encoded membrane-active antimicrobial peptides, such as alamethicin, magainin, and protegrin (also bee venom toxin melittin), have been shown to exhibit two distinct oriented circular dichroism spectra (Olah and Huang, 1988; Wu et al., 1990), clearly indicating that there are two distinct states of binding to lipid bilayers (Huang, 2000). In one state, the I state, the peptide molecules induce formation of transmembrane pores as shown by neutron diffraction (He et al., 1995, 1996a), presumably that is how the antimicrobial peptides kill the target cells. The other state, the S state, is an inactive state because no transmembrane pores were detected (Yang et al., 2001). Thus, the factors that determine the state of a peptide in a cell membrane will determine the susceptibility of the cell to the peptide. At present, these factors are not well understood.

One important factor appears to be the concentration of the peptide molecules bound to the membrane. In all the cases we have studied (Huang and Wu, 1991; Ludtke et al., 1994; Heller et al., 1998; Yang et al., 2001), we found that a peptide at low concentrations favors the S state, whereas at high concentrations favors the I state. This is consistent with the above-mentioned sigmoidal dependence of the peptide activity on the peptide concentration. Our purpose here is to study what causes such a cooperative phenomenon, that is, a superlinear increase of peptide activity with concentration.

We measured the transition of alamethicin from the S state to the I state as a function of the peptide concentration through a coexistence region by using the method of oriented circular dichroism (OCD). The measurement was done in three different conditions: in fully hydrated diphytanoyl phosphatidylycholine (DPhPC) bilayers, in slightly dehydrated DPhPC bilayers, and in fully hydrated 5:1 DPhPC and diphytanoyl phosphatidylethanolamine (DPhPE) mixture bilayers. In each case the fraction of alamethicin molecules occupying the I state shows a sigmoidal dependence on the peptide concentration. We compared the data with a Debye’s model for micelles that has satisfactorily described the micellization of soap solutions (Debye, 1949; Blankschtein et al., 1986). We found that a simple effect of aggregation is insufficient to explain the strong cooperativity exhibited by the pore formation of alamethicin. We will introduce an elasticity model based on the fact that a bilayer membrane in its fluid state is an elastic body. The inclusion of a protein in the membrane induces a strain field around the protein. This strain field mediates protein-protein interactions at a distance (Harroun et al., 1999) and contributes to a peptide concentration-dependent elastic energy that influ-
ences the relative energy level between the S state and the I state. We will show that the experimental data can be described by a phenomenological relation based on the elasticity theory of membrane (Huang, 1986, 1995).

MATERIALS AND METHODS

1,2-Diphtanoyl-sn-glycerol-3-phosphatidylcholine (DPhPC) and 1,2-diphytanoyl-sn-glycerol-3-phosphatidylethanolamine (DPhPE) were purchased from Avanti Polar Lipids (Alabaster, AL). Alamethicin was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). It is a mixture of components, principally alamethicin I (85% by high-performance liquid chromatography) and alamethicin II (12%), which differ by one amino acid of components, as described by a phenomenological relation based on the elasticity theory of membrane (Huang, 1986, 1995). In a previous publication (Huang and Wu, 1991; Ludtke et al., 1994; Heller et al., 1998; Yang et al., 2001), they are defined as two extreme spectra in the sense that all other spectra fall in between and can be expressed as linear superpositions of the two. The extreme spectra were searched separately and were removed from the corresponding spectra of the samples containing alamethicin. An example of low peptide concentration is shown in Fig. 2.

The method of obtaining the OCD spectra for the I state and the S state has been demonstrated for at least four different peptides in previous publications (Huang and Wu, 1991; Ludtke et al., 1994; Heller et al., 1998; Yang et al., 2001). They are defined as two extreme spectra in the sense that all other spectra fall in between and can be expressed as linear superpositions of the two. The extreme spectra were searched by measuring the OCD of a peptide in many different lipid bilayers and as a function of PIL, temperature, and hydration. Here one extreme OCD was found in the sample of PIL = 1/15 in DPhPC. This spectrum represents a helix oriented parallel to the light or perpendicular to the plane of bilayers, according to the theory of OCD (Olah and Huang, 1988; Wu et al., 1990). When a sample exhibited this spectrum, it also produced a neutron diffraction pattern of transmembrane pores (He et al., 1995, 1996a).

The cylindrical sample chamber was allowed to rotate around its axis for the purpose of rotational averaging.

Circular dichroism was measured with light incident normal to the substrate surface (Olah and Huang, 1988; Wu et al., 1990). The resulting spectrum was the OCD. Data were collected on a Jasco J-810 spectropolarimeter. Because the state of alamethicin is sensitive to the hydration level, the equilibrium of the sample was ensured by an agreement of at least three OCD spectra measured over a period of 6 h after each humidity setting. Each OCD spectrum presented in Figs. 2, 3, and 4 was an average of eight measurements at eight rotational angles equally spaced in one complete rotation. Such rotational averaging diminishes possible spectral artifacts due to the linear dichroism that could be caused by imperfections in the sample, strain in the quartz plates, or an imperfect alignment of the windows (Wu et al., 1990). We did not detect any significant change of spectrum with temperature from 10° to 40°C. This seems to indicate that the entropic contribution to the change of state is negligible. All data presented here were measured at 25°C. The background OCD spectra of lipid bilayers were measured separately and were removed from the corresponding spectra of the samples containing alamethicin. An example of low peptide concentration is shown in Fig. 2.

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1996a). We called this the S state of alamethicin. For some peptides, the two extreme spectra could be obtained from one sample at two different hydrations or temperatures (e.g., melittin see Yang et al., 2001). In that case, the two spectra are relatively normalized. However, when the two extreme spectra were obtained from two separate samples as in this case, there was a problem of normalization, i.e., the two spectra were not normalized to each other. This problem was solved as follows. Suppose that there is a cross point between the I and the S spectra, then this isodichroic point must be common to all spectra provided that they are all normalized correctly. We could easily find such a point by varying the hydration level of the sample (Fig. 3A). The relative normalization was achieved by adjusting the amplitudes of all other spectra to cross this isodichroic point (Fig. 3C). Each normalized spectrum was then fitted by a linear superposition of the I and S spectra (Fig. 3C) from which the fraction of alamethicin in the I state, \( \phi \), was obtained. The example in Fig. 3C shows that the fit is excellent.

RESULT AND ANALYSIS

Fig. 4 shows the normalized OCD spectra of alamethicin in DPhPC for a series of \( P/L \)s, all in full hydration. The fraction of alamethicin occupying the I state as a function of \( P/L \) is shown in Fig. 5, along with the data for alamethicin in DPhPC equilibrated at 98% relative humidity, and alamethicin in 5:1 DPhPC/DPhPE mixtures in full hydration. The error bars, about \( \pm 0.05 \), represent the standard deviations of the numerical fits.

We make three remarks before we proceed with analyses.

First, the system of alamethicin in DPhPC bilayers has been studied by one of us (H.W.H.) for over 10 years. When we first discovered the concentration dependence of the state (or orientation) of alamethicin, the threshold concentration \( (P/L)^* \) (at full hydration) was measured at \( \sim 1/120 \) (Huang and Wu, 1991). But in our 1995 study (Wu et al., 1995) we found \( (P/L)^* \) shifted to \( 1/40 \). This latter value was again measured in the 1996 (He et al., 1996b) and in the 1997 (Heller et al., 1997) studies. In our current study, we found \( (P/L)^* \) shifted back to \( 1/120 \) (Fig. 5), the same value originally measured in 1991. We have obtained alamethicin and DPhPC from the same companies. As far as we know, the source of alamethicin has been the same. However, according to Avanti (S. Burgess, personal communication), DPhPC has been made with phytol from different sources over the years. Whether the different results were due to any differences in the materials is not clear, although we suspected so in our previous investigation (Wu et al., 1995). What is clear is that the state of a peptide is sensitive to many physical and chemical variables. Therefore, to study the state of a peptide, one has to take care in

FIGURE 3  (A) OCD spectra of one alamethicin sample in DPhPC bilayers at \( P/L = 1/15 \), equilibrated at various relative humidities. The spectra obtained near full hydration (relative humidity > 98%) are identical and were identified as the I state spectrum. The spectra obtained at lower hydration levels are quite different, but all share a common point. (B) OCD spectra of one alamethicin sample in DPhPC bilayers at \( P/L = 1/150 \) (Fig. 2) are essentially independent of the degree of hydration. These spectra were identified as the S state spectrum. (C) All spectra were (relatively) normalized to have the same isodichroic point. An intermediate spectrum was fitted by a linear combination of the normalized I and S spectra. An appropriate lipid background has been removed from each spectrum.
keeping all conditions except the variables of interest constant.

Second, in our samples, all of the alamethicin molecules appeared to be bound to lipid bilayers. Negligible amounts of alamethicin, if any, were in the water layers between lipid bilayers. This is because the thickness of the water layer between two lipid bilayers is generally less than the width of the alamethicin helix (Wu et al., 1995; Hung et al., 2000). Furthermore, the samples showed a pure I state in which all peptide molecules were perpendicularly oriented to the bilayers, indicating that all were participating in the S to I transition.

Third, each of the three types of sample produced a single series of Bragg diffraction peaks (data not shown), indicating that the peptide and lipids were mixed into homogeneous bilayers.

![Image](https://example.com/image1.png)

**FIGURE 4** OCD spectra of alamethicin in DPhPC bilayers at various P/Ls from 1/200 to 1/15, all in full hydration. The spectra were normalized to have the same isodichroic point.

![Image](https://example.com/image2.png)

**FIGURE 5** Fraction of alamethicin molecules occupying the I state, $\phi$, is expressed as a function of the peptide concentration $P/L$. Three sets of data are shown: (■) alamethicin in DPhPC in full hydration; (●) alamethicin in 5:1 DPhPC/DPhPE mixtures in full hydration; (□) alamethicin in DPhPC equilibrated at 98% relative humidity.

**Model analysis**

**Micellar model**

The fraction of alamethicin molecules occupying the active state of pore formation, $\phi$, shows a sigmoidal concentration dependence in each of the three conditions we have measured (Fig. 5). The most commonly invoked interpretation for a sigmoidal concentration dependence is that there is molecular aggregation. In the case under consideration, one would assume that the peptide aggregates to form pores in the I state. Indeed, transmembrane pores were detected by neutron diffraction when alamethicin was in the I state (He et al., 1995, 1996a). No pores were detected in the S state. The diffraction data were consistent with alamethicin forming pores in the barrel-stave fashion. Furthermore, the pore size distribution was surprisingly narrow, limited to $n$ and $n \pm 1$ monomers, with $n \sim 11$ for alamethicin in DPhPC (He et al., 1996a).

Let us examine the aggregation effect by using a Debye’s model that provides a good description for the formation of micelles in soap solutions (Debye, 1949; Blankschtein et al., 1986). Consider the equilibrium kinetics between peptide monomers $A$ and peptide aggregates $A_n$, in which $n$ is the number of peptide monomers composing a transmembrane pore.

$$nA \leftrightarrow A_n.$$  

Let $C_n$ be the concentration of the pores, $C_i$ the concentration of the monomers, and $C$ the total concentration of the peptide molecules. Then we have

$$C = C_i + nC_n.$$  

In equilibrium, the following relation holds.

$$C_i^n = KC_n.$$  

in which $K$ is the equilibrium constant and also, for simplicity, the activity coefficients have been assumed to be unity. If we now define $C_c$ by $K \equiv C_c$, the combination of Eqs. 2 and 3 gives

$$\frac{C}{C_c} = \frac{C_i}{C_c} + n \frac{C_n}{C_c} = \frac{C_i}{C_c} + n \left(\frac{C_i}{C_c}\right)^n.$$  

As long as $n$ is sufficiently large, say $>10$, Eq. 4 has a simple implication. For $C < C_c$, we have $(C_i/C_c)^n \ll (C_i/C_c)$. In this case, the density of pores is negligible and so we have $C_i \approx C$. But for $C > C_c$, the $C_n$ terms dominates Eq. 4. $C_i$ needs to exceed $C_c$ by only a very small amount in order that almost all of $C - C_c$ be accommodated entirely by the pore density. $C_c$ is called the critical micellization concentration, equivalent to $(P/L)^*$ here. Thus, the essential implication of a simple aggregation effect is $C_i \approx C$, $C_n \approx 0$ for $C < C_c$ and $C_i \approx C_c$, $nC_n \approx C - C_c$ for $C > C_c$.
Therefore, the prediction of the micellar model in the S-to-I transition region is

\[ \phi = 1 - \frac{(P/L)^*}{P/L}. \]  

(5)

**Elasticity model**

We know that what makes alamethicin bind to a lipid bilayer is the hydrophobic interaction, the attraction between the hydrophobic surface of the alamethicin helix (Fox and Richards, 1982) and the hydrophilic-hydrophobic interface of the lipid bilayer. Let the energy of this interaction be \(-\varepsilon_e\). This is, however, not the total free energy of binding. For a peptide to adsorb on the interface, it needs to be embedded in the headgroup region of the lipid bilayer. This has a consequence of expanding the area of the bilayer and causing a local thinning in the hydrocarbon region (Wu et al., 1995; Ludtke et al., 1995). The elastic energy of such a deformation in the lipid bilayer, estimated to be \(-2k_B T\) (the Boltzmann constant times the absolute temperature), is a significant part of the total binding energy (Huang, 1995). According to the elasticity theory (Huang, 1986), the deformation extends over a range \(\sim 2\) nm in plane (the value depends on the elastic constants of the membrane, which can vary significantly from one bilayer to another). When two bound peptide molecules approach each other, the elasticity energy gives rise to a repulsive force between them over a range \(\sim 3.5\) nm (Huang, 1995). Thus, it was predicted that the peptide molecules do not aggregate in the S state. Indeed, a number of experiments that were designed to observe peptide aggregations in membranes all reported negative results (Gazit et al., 1994, 1995; Hirsch et al., 1996; Schümann et al., 1996). In particular, the fluorescence energy transfer experiment by Schümann et al. (1996) concluded that the peptide molecules were randomly distributed except that no two molecules were within \(\sim 2\) nm of each other even at high concentrations (\(P/L \sim 1/20\)).

When the peptide concentration in the S state is sufficiently high such that the average distance between two neighboring molecules is within the repulsion range, the resulted membrane thinning caused by the peptide adsorptions tends to be approximately uniform (Harroun et al., 1999). This thinning can be measured directly by x-ray diffraction, and we have found that the degree of thinning is directly proportional to \(P/L\) (Wu et al., 1995; Ludtke et al., 1995; Heller et al., 2000). Under the circumstances, the elastic energy of thinning is proportional to the square of \(P/L\), so the total free energy of alamethicin binding to the lipid bilayer can be written as (Huang, 1995),

\[ F = -\varepsilon_s (P/L) + \alpha (P/L)^2. \]  

(6)

Here the energy is normalized to per lipid and \(\alpha (P/L)^2\) is the elastic energy of membrane thinning mentioned above with \(\alpha\) as the proportionality constant. Then the chemical potential of the S state contains a positive term of elastic energy that increases linearly with \(P/L\). We have proposed that this is the reason the chemical potential of the S state crosses over that of the I state as \(P/L\) increases and that explains the S to I transition at high peptide concentrations (Huang, 1995).

We now extend Eq. 6 to the transition region where a fraction of alamethicin molecules, \(\phi(P/L)\), form pores, and the rest, \(1 - \phi(P/L)\), remain in the S state. We propose the following phenomenological energy in the fashion of the Landau theory (Landau and Lifshitz, 1969)

\[ F = -\varepsilon_s (1 - \phi)(P/L) - \varepsilon_1 \phi(P/L) + \alpha[(1 - \phi)(P/L) + \beta\phi(P/L)]^2, \]  

(7)

in which \(-\varepsilon_1\) is the interaction energy for a peptide in a pore, and we assume that pores also cause membrane thinning. But the thinning effect of a pore (normalized to per peptide) is different from that of a peptide adsorbed in the headgroup region. We express this difference by the factor \(\beta\). Minimization of the free energy with respect to \(\phi\), \(\partial F/\partial \phi = 0\), gives the relation between \(\phi\) and \(P/L\),

\[ (1 - \beta)\phi = 1 - \frac{\varepsilon_s - \varepsilon_1}{2\alpha(1 - \beta)} \frac{P/L}{P/L^*}. \]  

(8)

Because \(\phi = 0\) when \(P/L = (P/L)^*\), we have

\[ (P/L)^* = \frac{\varepsilon_s - \varepsilon_1}{2\alpha(1 - \beta)}, \]  

(9)

and consequently

\[ \phi = \frac{1}{1 - \beta} \left( 1 - \frac{(P/L)^*}{P/L} \right). \]  

(10)

We note that \(\varepsilon_s > \varepsilon_1\), because at very low concentrations, where the quadratic term is absent (Huang, 1995), the S state has a lower energy level than the I state. Then Eq. 9 implies that \(\beta < 1\). This makes sense. The contribution of a pore to membrane thinning (normalized to per peptide) should be less than the contribution by a peptide in the S state, otherwise the insertion transition would not occur by increasing the concentration. As seen from Eq. 10, \(\beta\) is the ratio of the lower boundary to the higher boundary of the transition (or coexistence) region, \(\beta = (P/L)^*/(P/L)^{**}\). \((P/L)^{**}\) is the threshold concentration for all of the alamethicin molecules to form pores.

The first thing to notice in Eq. 10 is that if we did not include the contribution of pores in the membrane thinning effect, i.e., if \(\beta = 0\), the two models would predict the same \(\phi\) vs. \(P/L\) relation. The equivalence of these two models is reminiscent of the equivalence of the Bragg-Williams approximation and the Landau theory for the Ising model (Huang, 1987). Debye’s micellar model, like the Bragg-
CONCLUSION

We have presented a measurement of the sigmoidal concentration dependence for the change of the state of alamethicin, from its inactive state of adsorbing in the headgroup region of a lipid bilayer to the active state of forming transmembrane pores. Our quantitative analysis shows that a simple aggregation effect does give rise to a sigmoidal concentration dependence. However, the experiment shows that the cooperativity of pore formation is stronger (steeper increase in $\phi$) than that predicted by a simple aggregation model. Thus, a micellar model as described by Eq. 3 that includes no interactions between monomers and aggregates does not agree with the experiment. Many investigators have proposed that the energetics of peptide-membrane interaction must include a term arisen from the elastic deformation in membrane caused by the peptide inclusion (for review, see Aranda-Espinoza et al., 1996). Here we show that this elastic energy produces a membrane-mediated interaction between monomers and pores, and this is essential for the action of pore formation by alamethicin.

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