

# Lipid dependence of peptide-membrane interactions

## Bilayer affinity and aggregation of the peptide alamethicin

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Membrane incorporation and aggregation of the peptide alamethicin have been investigated as a function of lipid type. Head group and acyl chain regions both contribute to modulate alamethicin incorporation. Specifically, the peptide prefers thin membranes and saturated chains; incorporation is reduced by the presence of cholesterol. Aggregation of the peptide in the bilayer is virtually insensitive to changes in lipid composition. These findings show some analogies to results obtained with intrinsic membrane proteins and cast doubt on the use of global membrane parameters for interpreting lipid-peptide interactions.

Alamethicin; Lipid-protein interaction; Lipid specificity

### 1. INTRODUCTION

The details of how the diversity of lipids coexisting in biological membranes affects membrane protein function are still poorly understood. Many questions remain, e.g. about the relevance of global membrane parameters like viscosity or acyl chain 'order' [1,2]. In this context we have studied the influence of lipid type on membrane interactions of a small 20 amino acid peptide, alamethicin, best known for its ability to form voltage-dependent pores [3–5]. Its membrane-water partitioning can be probed by changes in the circular dichroism [6,7]. The corresponding isotherms are characterized by a sharp bend upwards near a 'critical concentration',  $c^*$  (see fig. 1),

where massive aggregation of the peptide in the membrane appears to set in [6,7]. Changes in  $c^*$  induced by varying the aqueous salt concentration or the cholesterol content of the bilayer have been shown to correlate quantitatively with the pore ac-

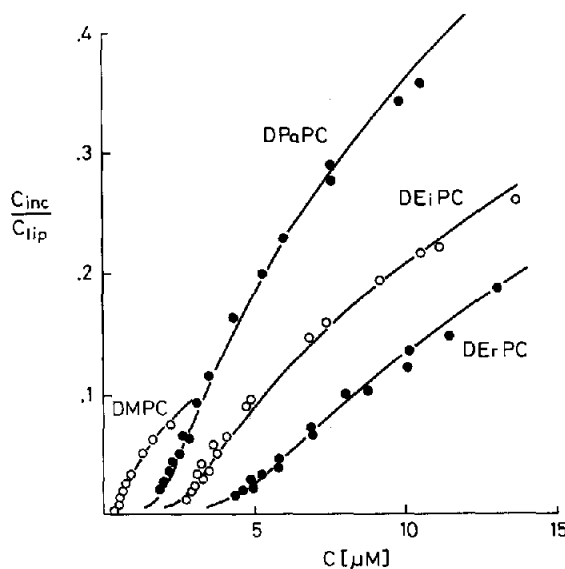


Fig.1. Incorporation isotherms (incorporated peptide per lipid versus aqueous peptide concentration,  $c$ ) for various lipids.

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**Abbreviations:** PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DEiPC, dieicosenoyl-PC (DiC20:1); DErPC, dierucoyl-PC (DiC22:1); DLiPC, dilinoleoyl-PC (DiC18:2); DMPC, dimyristoyl-PC (DiC14:0); DOPC, dioleoyl-PC (DiC18:1); DPaPC, dipalmitoleoyl-PC (DiC16:1); POPC, 1-palmitoyl-2-oleoyl-PC (C16:0/C18:1); Chol, cholesterol

tivity of alamethicin [8]. This is consistent with the long held notion of the alamethicin pore being formed by combining a variable number of peptide monomers [3,9]. The easily accessible quantity  $c^*$  therefore relates simple physical parameters (partition coefficient, aggregation constant) to function (pore activity on planar bilayers).

## 2. MATERIALS AND METHODS

Lipids were purchased from Avanti (Birmingham, AL). The alamethicin was prepared as described [7] in its neutral form carrying a glutamine residue in position 18. Incorporation isotherms were obtained by titrating sonicated small unilamellar lipid vesicles to a fixed concentration of alamethicin in 10 mM Tris-HCl, pH 7.5 (with 0.1 M NaCl added where indicated) at 20°C, observing the change in ellipticity at 224 nm on a Cary 61 instrument. Titrations were performed for at least 3 different alamethicin concentrations. The experimental and evaluation procedures were as described previously [7]. The critical concentration,  $c^*$ , related to the point where the isotherms start to bend upwards, has been shown to combine a partition coefficient of peptide monomers,  $\Gamma$ , and an isodesmic aggregation constant,  $K$  (i.e., the equilibrium constant for the addition of an incorporated monomer to an aggregate of arbitrary size):

$$c^* = (\Gamma \cdot K)^{-1} \quad (1)$$

Using the incorporated peptide to lipid ratio,  $r$ , as the concentration variable in the bilayer phase and distinguishing the different aggregate species by  $r_1$ ,  $r_2$  etc. (for the monomer, dimer etc.) these parameters are defined by the following equations (valid at small aqueous concentration  $c$ ):  $r_1 = \Gamma \cdot c$  and  $r_{i+1} = K \cdot r_i \cdot r_1$ . Activity corrections become important at higher  $c$ : they are not explicitly considered here. For a comprehensive description of the underlying theoretical approach see [7]. The non-ideality parameter  $z$  as defined in [7] varied between about 2.5 and 3.5, and reached about 4 to 4.5 for the higher cholesterol contents (for the special case of DMPC see [7]).

Independent ultracentrifugation experiments to determine  $\Gamma$  have been carried out as follows: lipids were dispersed in buffer, alamethicin was incorporated using 5 freeze-thaw cycles, and the solutions centrifuged for 3 h at 60000 rpm in a TST60 swinging bucket rotor. Peptide concentrations in the supernatant were determined from the ellipticity at 224 nm. Residual lipid in the supernatant was determined by phosphorus assay [10]; the signal was then corrected for lipid-associated peptide in the supernatant in an iterative procedure (using the known  $\theta_\infty$ -values of table 1). These corrections were usually small, of the order of a few percent. In each of these experiments, a series of different lipids were handled and spun simultaneously, together with some blanks containing only alamethicin. The resulting ratios of partition coefficients were fairly reproducible as given by the standard deviations in table 1.

## 3. RESULTS

The association of alamethicin with vesicles made out of a series of different lipids was in-

vestigated by circular dichroism titrations. The ellipticity of membrane-incorporated peptide,  $\theta_\infty$ , and the critical concentration,  $c^*$ , were determined and are compiled in table 1.  $c^*$  is related to water-membrane partitioning and aggregate formation as defined in eqn 1. The following conclusions can be drawn from a comparison of the different lipids.

- (i) The ellipticity value of the incorporated peptide increases with increasing chain length of otherwise similar lipids (mono-unsaturated phosphatidylcholines), reflecting the adaptation of the peptide to the increasing hydrophobic thickness of the bilayers, presumably by extending its  $\alpha$ -helical parts. (The deviation of the C20 chain lipid, DEiPC, is within the error range of  $\pm 300^\circ \text{ cm}^2 \text{ dmol}^{-1}$ .) Concomitantly, interaction of the peptide with thicker membranes is rendered less favorable as shown by the increase of  $c^*$ .
- (ii) Alamethicin appears to favor the more saturated with respect to the unsaturated chains (cf. table 1c). The DMPC results at 31°C might be influenced by the phase transition of the lipid, the temperature interval of which is considerably broadened upon incorporating alamethicin. It may be more significant to compare the unsaturated lipid with DMPC at higher temperatures, e.g. 40°C, or with a mixed chain lipid such as POPC: a decrease in  $c^*$  then remains, though small.  $c^*$  clearly increases for the doubly unsaturated chains.
- (iii) Including negatively charged lipid headgroups should not give rise to direct electrostatic interactions with the uncharged alamethicin species used here. If the vesicles contain 20% phosphatidylglycerol,  $c^*$  decreases, indicating increased affinity of the peptide as compared to the fully neutral lipids. This effect is accompanied by an increase in the peptide's helicity as measured by  $\theta_\infty$ , suggesting that the head group charge might lead to a reorganization of lipid packing, e.g. via changes in the hydration. In this context, it is interesting to note a similar increase in  $\theta_\infty$  upon adding salt to the aqueous medium (cf. table 1 and [7]). The experiments with negatively charged lipids were done with 0.1 M NaCl added to the buffer and compared to results obtained with DOPC bilayers under the same conditions.

Table 1  
Lipid dependence of alamethicin parameters<sup>a</sup>

Lipid	$\theta_{\infty}$ (deg cm <sup>2</sup> dmol <sup>-1</sup> )	$c^*$ ( $\mu$ M)	$c^*/c^*_{\text{DOPC}}$	$\Gamma_{\text{DOPC}}/\Gamma$
DPaPC	-12350	2.2	0.8	$0.95 \pm 0.15^b$
DOPC	-12950	2.7	1	1
DEiPC	-12750	3.2	1.2	$1.0 \pm 0.3$
DErPC	-13550	5.4	2.0	$1.7 \pm 0.2$
DMPC, 31°C	-13450	0.8	0.3	<sup>c</sup>
DMPC, 40°C	-12450	1.5	0.56	
POPC	-13750	2.5	0.93	$0.75 \pm 0.15$
DOPC	-12950	2.7	1	1
DLiPC	-12750	3.8	1.4	$1.55 \pm 0.15$
DOPC (NaCl)	-13550	2.1	1	1 <sup>d</sup>
DOPC/DOPG, 20%	-14250	1.5	0.7	$0.9 \pm 0.25$
POPC	-13750	2.5	1	1 <sup>e</sup>
POPC/POPE, 25%	-13050	3.5	1.4	$1.5 \pm 0.2$
DOPC (NaCl)	-13550	2.1	1	1 <sup>f</sup>
DOPC/Chol, 10%	-13150	3.0	1.43	
DOPC/Chol, 25%	-14750	4.4	2.1	$2.65 \pm 0.15$
DOPC/Chol, 40%	-11250	8	3.8	

<sup>a</sup>  $\theta_{\infty}$ , ellipticity per mol residue of lipid-associated peptide;  $c^*$ , critical aqueous concentration;  $\Gamma$ , partition coefficient of peptide monomers from ultracentrifugation of lipid dispersions

<sup>b</sup> Lipid chain length dependence

<sup>c</sup> Lipid unsaturation dependence

<sup>d</sup> Head group charge dependence, measured in the presence of 0.1 M NaCl

<sup>e</sup> Head group type dependence; ratios in the last two columns are with respect to POPC

<sup>f</sup> Cholesterol dependence, in the presence of 0.1 M NaCl

(iv) Alamethicin appears to prefer phosphatidylcholine headgroups over phosphatidylethanolamines. This is in accord with the higher peptide concentrations needed to observe a given pore activity in PE as compared to PC bilayers [3-5].

(v) Adding cholesterol to the phospholipids leads to a striking increase in  $c^*$ . This is not what one would expect from concepts established by studying pure lipid bilayers. There cholesterol is found to increase chain ordering in a way similar to increasing chain saturation. Special packing constraints are known to exist at cholesterol concentrations around 20 to 25 mol%, and these may indeed be reflected by the conspicuously large  $\theta_{\infty}$  value of the peptide at 25% cholesterol. But otherwise acyl chain order does not seem to be a relevant parameter, since cholesterol strongly dimin-

ishes the affinity of alamethicin, whereas saturated chains tend to have the opposite effect.

Since the critical concentration,  $c^*$ , combines contributions from the water-membrane partitioning of the peptide and from its aggregation in the membrane, an attempt was made to separate the two effects by measuring alamethicin incorporation at aqueous concentrations well below  $c^*$  (around 1  $\mu$ M) where aggregation should be of minor importance. In order to avoid optical contributions from large amounts of vesicle material, we preferred to evaluate  $\Gamma$  from ultracentrifuged coarse dispersions. The experimental system is not exactly the same as with the small vesicles, so the absolute values of  $\Gamma$  cannot a priori be assumed to be identical, but we find a similar order of magnitude, around  $10^3 \text{ M}^{-1}$  for DOPC, as estimated from vesicle titrations [6]. In any case,

there should be no problem to compare different lipids by considering the ratios of partition coefficients found in that way; these are given in table 1.

Direct comparison with the ratios of critical concentrations reveals that the main effect of varying lipid type comes from the change in the partition coefficient. The aggregation constant,  $K$ , must therefore remain remarkably invariable.

#### 4. DISCUSSION

Interaction of the amphipathic peptide alamethicin with lipid bilayers of varying composition is found to be influenced by both the head group and acyl chain properties of the lipids. The latter dependence (on parameters such as lipid chain length or degree of unsaturation) makes it highly unlikely that alamethicin is only adsorbed to the interface, but gives further evidence for a deep insertion of the peptide into the hydrophobic core of the bilayer [11–13]. Incorporation into the bilayer is found to be favored by thin membranes. This agrees with the current view of alamethicin inserting into the bilayer in a predominantly  $\alpha$ -helical conformation [14]. This helix would have a length of about 25 Å if the C-terminal glutamines are excluded, not much longer than the hydrophobic thickness of DPaPC bilayers [15]. However, alamethicin appears to adapt its conformation to membrane thickness and lipid packing as shown by the corresponding variations in the ellipticity,  $\theta_{\infty}$  (table 1).

Considering changes in lipid chain unsaturation, alamethicin appears to prefer the more saturated chains showing a relatively high conformational and packing order. On the other hand cholesterol, known to increase the order of fluid lipid chains, strongly inhibits alamethicin incorporation (table 1). It is therefore not possible to describe alamethicin-bilayer interactions satisfactorily using global parameters such as lipid 'order' or viscosity. Similar conclusions have been reached previously upon reconstituting membrane proteins such as the Ca/Mg-ATPase of sarcoplasmic reticulum in bilayers of varying composition [1]. In fact, there are striking parallels even on a more quantitative level, if we compare ATPase activity (estimated from the drawing in [1]) to changes of the critical concentration of alamethicin. Perhaps there are common principles, related to the steric

and energetic requirements for accommodating a peptide of a given secondary structure in a lipid environment. The strong inhibitory effect of cholesterol might be related to an incompatibility of its rigidity with the bend of the peptide helix around proline 14 [16]. Preferential accumulation of alamethicin in cholesterol-depleted domains has previously been postulated [17] from considering the conductance of single pores in mixed DOPC/cholesterol bilayers.

From an energetic point of view, bilayer incorporation of the peptide and its association to aggregates involve comparable free energies. Using conventional concentration variables on a mol per volume basis, the standard free energy of monomer incorporation,  $\Delta G_{inc}^0$ , is about  $-18$  kJ/mol for DOPC ( $= -RT \ln \Gamma / \bar{V}_L$  with  $\Gamma$  about  $10^3$  M $^{-1}$  and a partial molar volume  $\bar{V}_L$  of  $0.8$  cm $^3$ /mol). From  $c^*$ , the corresponding aggregation constant  $K$  is then found to lie in the range of 400–800 (eqn 1), and the standard free energy of association,  $\Delta G_{agg}^0 = -RT \ln K$  around  $-16$  kJ/mol. The changes in the partition coefficient induced by variation of the lipid composition of the membrane, as given in table 1, correspond to only small alterations of the free energy of incorporation, of the order of 2 kJ/mol. Such a small figure is in line with theoretical predictions [18,19].

Remarkably and unsuspectedly, the aggregation constant  $K$  was found to be insensitive to changes in membrane composition. This result clearly recalls the situation found with reconstituted bacteriorhodopsin, which was shown not to change its aggregation state upon large alterations of bilayer thickness [20]. The invariance of alamethicin aggregation parameters was nevertheless unexpected in view of the demonstration that the average size of alamethicin pores increases upon incorporating the peptide in bilayers formed from monoolein of increasing chain length [5]. However, similar results have never been reported for double chain lipids; in contrast, single channel analyses of Boheim [4,9] would indicate that the pore sizes are very similar in DOPC and DErPC membranes, respectively. In any event, the aggregate size distribution may not match the pore size distribution. Not all aggregates need be pores; also large aggregates could possibly form several small pores.

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