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COOPERATIVE LIPID-PROTEIN INTERACTION

EFFECT OF pH AND IONIC STRENGTH ON POLYMYXIN BINDING TO PHOSPHATIDIC ACID MEMBRANES

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Summary

The binding of polymyxin-B to charged dipalmitoyl phosphatidic acid membranes has been studied as function of the external pH and of the ionic strength of the buffer solution. The phase transition curves were obtained by measuring the fluorescence depolarization of diphenyl hexatriene incorporated into the membrane with temperature. The molecular process of polymyxin binding was elucidated:

1. At an ionic strength of $I \geqslant 0.1$ mol/l a three step phase transition curve is found. A high-temperature step corresponds to the non-bound lipid. A lowered phase transition concerns to protein-bound lipid domains. This again is splitted into two steps. An inner core of the domain is characterized by a lipid-protein complex which is stabilized through hydrophobic and electrostatic interactions between polymyxin and the charged lipid. This core is surrounded by an outer belt of only hydrophobically bound molecules. This part shows a lower phase transition temperature than the inner core.

2. The binding curves of polymyxin to phosphatidic acid membranes depend strongly on the ionic strength of the water phase. The cooperativity of the binding process increases with increasing ionic strength and reaches a constant value at $I > 0.2$ mol/l. The maximum fraction of bound lipid decreases with increasing ionic strength.

3. The pH of the water phase strongly influences the cooperative binding process. At pH 6 a loss of cooperativity is observed at low ionic strength.

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Increasing the ion concentration to $I = 0.3$ mol/l recuperates the cooperativity of the binding process. At pH 3.0 no cooperative binding is obtained even at high ionic strength.

Introduction

Polymyxin-B, a decapeptide antibiotic is characterized by a heptapeptide ring containing four 2,4-diaminobutyric acids. An additional peptide chain covalently bond to the γ-amino group of this residue carries a fatty acid chain which is attached to the peptide through an amide bond. Totally the molecule carries five positive charged diaminobutyric acid residues at $pH < pK = 10.4$.

Polymyxin has strong effects on bacterial metabolism and growth [1]. One of the earliest detectable changes is an increase in the permeability of the cytoplasmic membrane with respect to polar molecules. The perturbation of the membrane structure may result from an association of the peptide with membrane lipids. Therefore this membrane-active antibiotic is a useful tool for studying the structure and function of biological membranes.

In addition such peptide antibiotics have strong affinities for membrane lipids and can be used as a model component to study the lipid-protein interaction in artificial membranes [2—4]. An increase in glucose permeability, for example, has been reported in phospholipid liposomes upon treatment with polymyxin-B [5,6].

Imai et al. [7] reported that phosphatidylcholine liposomes were sensitive to polymyxin if they contain negatively charged lipids. A preferential binding of polymyxin-B to negatively charged lipids has also been reported by Teuber and coworker [2,3]. Nevertheless, the molecular mechanism of polymyxin binding is still unknown.

Very recently we reported a cooperative binding model [8] for the interaction of polymyxin with charged lipid bilayers. Insertion of the fatty acid tail into the lipid matrix weakens the hydrophobic interaction between the phospholipid molecules and thus lowers the lipid phase transition temperature by about 20°C. A lateral phase separation leads to a mosaic-like organisation of the membrane. Such a domain formation induced by the interaction of lipids with external [9] or integral [10] model peptides has already been observed by different physical techniques. This lateral phase separation may be triggered by external charges or by the pH value of the solution [11,12]. The outstanding property of polymyxin is its cooperative lipid-protein interaction.

The present paper investigates the influence of pH and of the ionic strength on the cooperative binding of polymyxin-B to charged phosphatidic acid membranes. An extended insight in this lipid-protein interaction is obtained. One result is the determination of the protein-bound domain structure. An inner core characterized by hydrophobic and electrostatic interactions is surrounded by a belt of lipids which do not interact electrostatically with polymyxin. This domain structure is strongly influenced by the free part of the lipid membrane. The cooperativity of the binding process may be influenced by both the pH and the ionic strength of the solution.

Materials and Methods

Lipids. Dipalmitoyl phosphatidic acid was purchased from FLUKA (Neu-Ulm, F.R.G.) and recrystallized from *n*-hexane/methanol (9 : 1, v/v). Polymyxin-B sulfate was delivered from Sigma (München, F.R.G.). Diphenyl hexatriene was a product of Aldrich.

Vesicle preparation. Lipid vesicles were prepared from a chloroform solution of the lipid and 2 mol% diphenyl hexatriene. After evaporation of the solvent by a nitrogen stream the buffer solution containing the corresponding salt concentration was added and the probe was sonicated for 5 min with a Branson sonifier at $P = 30$ W and at $T = 70°C$. The lipid concentration was 0.03 mg/ml in buffer solution. Polymyxin-B sulfate was added from a buffer solution and the probe was resonicated for 2 min. Within this time of sonification an equilibrium distribution between the inner and the outer monolayer of the bilayer vesicle is reached. The penetration time without sonification is about 2 h. This result will be published in a further contribution.

The temperature profiles are fully reversible showing a small hysteresis effect. The vesicle preparations were controlled by electron microscopy. No change in vesicle size distribution was observed. The electron microscopy study of giant bilayer vesicles is still in progress.

The following buffer solutions were used: a citrate buffer at pH 3.0, a phosphate buffer at pH 6.0, and a borate buffer at pH 9.0. The ionic strength was adjusted by addition of sodium chloride.

Measurement of the fluorescence polarisation. The fluorescence polarisation was recorded with a Schoeffel instrument RRS 1000 equipped with two sets of a monochromator/photomultiplier system at the emission side in an arrangement perpendicular to the excitation light beam. This enabled us to measure the fluorescence intensity parallel and perpendicular to the light vector of the excitation light continuously with temperature. The relative polarization degree $(I_\parallel - I_\perp)/(I_\parallel + I_\perp)$ was calculated by an analogue computer. The temperature was controlled to an accuracy of 0.2 degree.

Binding curves were obtained from the phase transition curves. The height of the transition steps, labelled as H_1, H_2 and H_3 in Fig. 1, are a measure of the lipid fractions in the corresponding phase (for a detailed information see Ref. 8).

Results

Influence of polymyxin on the thermotropic phase transition of phosphatidic acid membranes

Typical phase transition curves of dipalmitoyl phosphatidic acid after treatment with polymyxin-B are shown in Fig. 1. Diphenyl hexatriene was incorporated as probe molecule to determine the thermotropic phase transition from polarisation measurements. The results are given for pH 3 at an ionic strength of $I = 0.1$. Under these conditions the lipid phase transition of the pure dipalmitoyl phosphatidic acid membrane occurs at $T_1 = 68°C$ (curve 1 in Fig. 1). Treatment of this membrane preparation with polymyxin causes two additional phase transition steps at $T_2 = 56°C$ and $T_3 = 45°C$ (curves II and III).

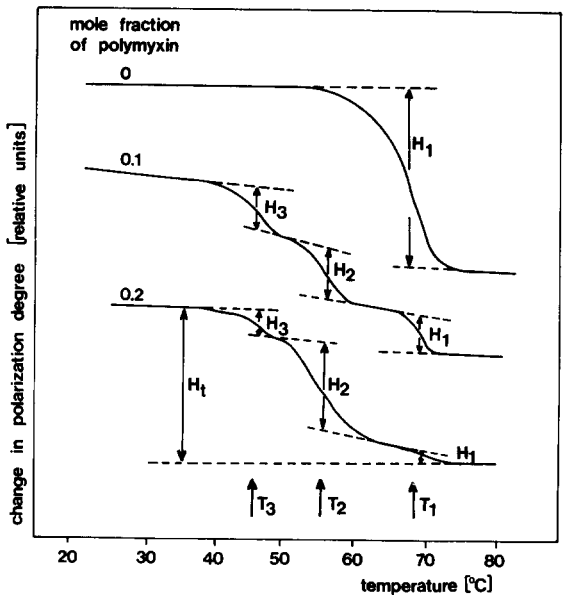

Fig. 1. Thermotropic phase transition curves of dipalmitoyl phosphatidic acid membranes in the absence and in the presence of polymyxin-B at pH 3.0 and an ionic strength of I = 0.1 M. Diphenyl hexatriene was used as probe molecule to determine the polarisation degree.

Simultaneously the step height H_1 at T_1 decreases relative to the total height of the phase transition curve. Moreover the ratio of H_3/H_2 decreases with increasing polymyxin content. Analogue curves were obtained at various pH and ionic strength values.

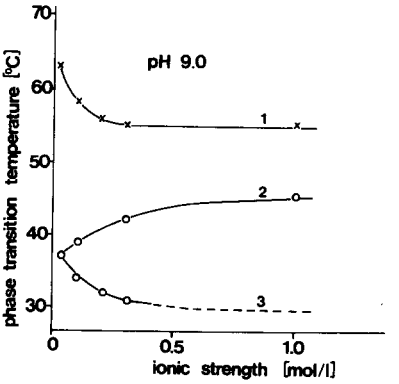

Fig. 2. The phase transition temperatures T_1, T_2 and T_3 given in Fig. 1 are shown as function of the ionic strength at pH 9.0.

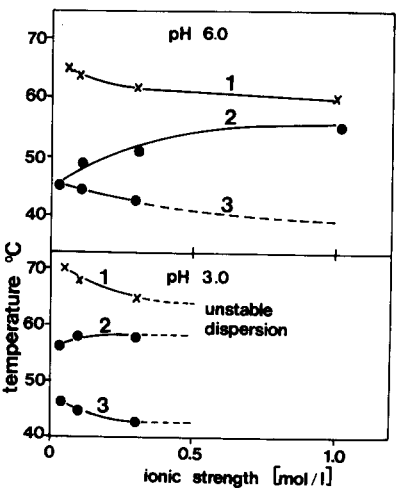

Fig. 3. Influence of the ionic strength on the phase transition temperatures T_1, T_2 and T_3 at pH 6.0 and at pH 3.0.

The effect of pH and ionic strength on the phase transition temperatures

The phase transition temperatures denoted T_1, T_2 and T_3 in Fig. 1 are investigated as function of the ionic strength at pH 3, pH 6 and pH 9. The results are given in Figs. 2 and 3. Let us first consider the results at pH 9.0. The high-temperature step ($T_1 = 63°C$) at $I = 0.03$ mol/l decreases to about $T_1 = 55°C$ with increasing ionic strength (curve 1 in Fig. 2). This effect is due to a decrease in the surface potential of a charged membrane with increasing ion concentration and leads to a decrease of the phase transition temperature [13]. The same behaviour is observed for the transition step denoted T_3 in Fig. 1. A decrease from $T_3 = 37°C$ to $T_3 = 30°C$ is found with increasing ionic strength (curve 3 in Fig. 2).

A totally different behaviour is given for T_2 (curve 2 in Fig. 2). At low ionic strength T_2 and T_3 coincide whereas with increasing ionic strength T_3 increases from $T_2 = 37°C$ to $T_2 = 45°C$. A corresponding behaviour is observed at pH 6 and at pH 3. Again the phase transition at low temperature splits into two steps with increasing ion concentration. At pH 6 the transition temperature T_3 was not measurable at $I > 0.3$ mol/l. At pH 3.0 the dispersion became unstable at $I > 0.3$ mol/l leading to a coagulation and a precipitation of the vesicles.

T_1 agrees exactly with the phase transition temperature of the phospholipid membrane in the absence of polymyxin-B at the given pH and the given ionic strength. The transitions at T_2 and T_3 are attached to the polymyxin-bound lipid domains including the domain boundary.

Binding curves of polymyxin-B to dipalmitoyl phosphatidic acid vesicles

The step height of the phase transition demonstrated in Fig. 1 is taken as a measure of the number of lipid molecules being present in the corresponding lipid phase. H_2 and H_3 both belong to the protein bound domains whereas H_1 represents the free lipid phase. Now the ratio $\rho = H_2 + H_3/H_t$ gives the fraction of polymyxin-bound lipid, where $H_t = H_1 + H_2 + H_3$ is the difference in the polarization degree of the total transition.

The fraction of bound lipid, ρ, is given in Fig. 4 as function of polymyxin concentration with variable ionic strength and at pH 9.0. Sigmoidal curves are obtained in all cases symbolizing the cooperativity of the binding process.

An increase of the ionic strength leads to an increase in the cooperativity up to $I = 0.2$ mol/l. This could be derived from the slope of the tangent touching the middle part of the curves. At $I > 0.2$ mol/l the cooperativity remains nearly constant. High ionic strength inhibits complete lipid binding to polymyxin-B. The maximal fraction of bound lipid first increases from $I = 0.03$ to $I = 0.1$ by about 5% $\rho = 0.95$ to $\rho = 1.0$. Further increase of the ion concentration leads to a dramatic decrease of the maximal fraction of bound lipid from $\rho = 1.0$ to $\rho = 0.35$ at an ionic strength of $I = 1.0$ mol/l (e.g. Table I). Moreover the amount of polymyxin necessary to yield the same relative fraction of bound lipid changes with ion concentration. This is also demonstrated in Table I. At $I < 0.1$ mol/l a mol fraction of about $c_{1/2} = 0.15$ of polymyxin with respect to the lipid is necessary to reach the half-height $\rho_{1/2}$ of the binding curve. This value drops dramatically to $c_{1/2} = 0.06$ at $I = 0.2$ mol/l but increases again to $c_{1/2} = 0.15$ with increasing ion concentration. The cooperativity of the binding process, however, remains present at all ion concentrations. This situation is

TABLE I

The maximal fraction δ_{max} of polymyxin-bound lipid (e.g. Fig. 2) depends on the ionic strength of the solution (I). $c_{1/2}$ is the polymyxin concentration, where the fraction of bound lipids δ reaches the value $\delta_{1/2} = \frac{1}{2}\delta_{max}$.

	I				
	0.03	0.1	0.2	0.3	1.0
δ_{max}	0.95	1.0	0.73	0.43	0.34
$c_{1/2}$ at $\delta_{1/2}$	0.16	0.15	0.06	0.075	0.15

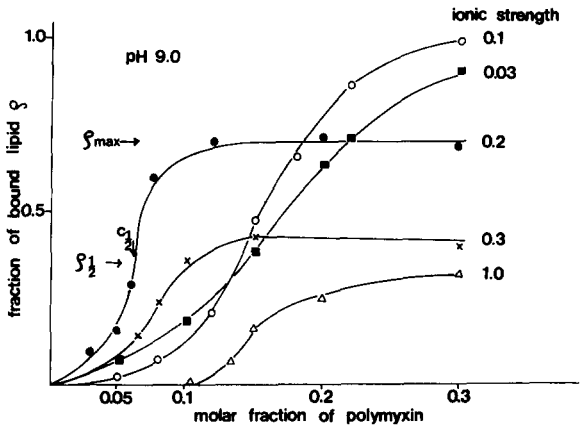

Fig. 4. Binding curves of polymyxin-B to phosphatidic acid membranes at pH 9.0 with variable ionic strengh. ρ_{max} is the maximal fraction of bound lipid obtained in our experiments. ρ_{max} differs with ionic strength. It is indicated for $I = 0.2$ M. $\rho_{1/2}$ is half the value of ρ_{max}. The corresponding fraction of polymyxin is indicated as $c_{1/2}$. Again only the values for $I = 0.2$ M are given by the arrows.

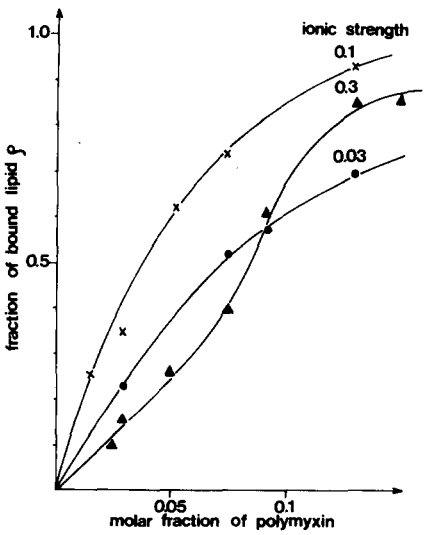

Fig. 5. Binding curves of polymyxin to phosphatidic acid membranes at pH 6. Note that the cooperativity of the binding process is lost at $I < 0.3$ M.

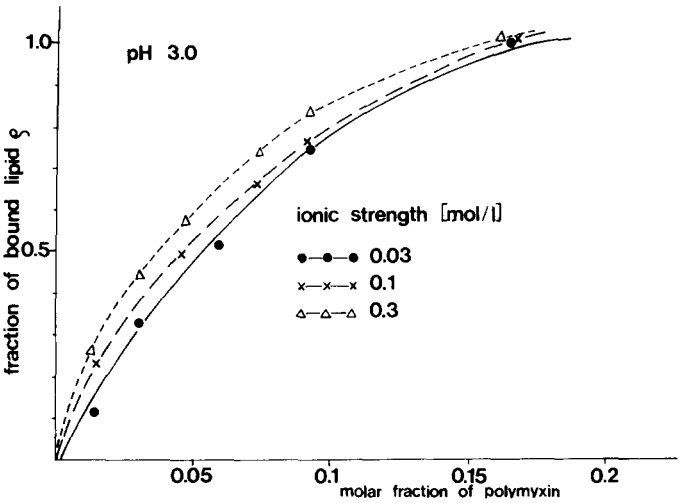

Fig. 6. Binding curves of polymyxin to phosphatidic acid membranes at pH 3.0. No cooperativity could be observed.

changed at pH 6. No cooperativity is found for the binding process at low ionic strength $I = 0.03$ and $I = 0.1$ mol/l. Again the maximal fraction of bound lipid increases in this concentration range. Further increase in the ionic strength up to $I = 0.3$ mol/l brings back a sigmoidal binding curve exhibiting the cooperative process. At pH 3 a non-cooperative binding mechanism is observed at ionic strengths up to $I = 0.3$ mol/l.

Discussion

Change in transition temperature

In our earlier paper [8] we reported a two step phase transition curve for phosphatidic acid membranes after treatment with polymyxin-B. This is correct at the low ionic strength were these measurements have been performed. A model has been proposed where the paraffin tail of the polymyxin sticks into the hydrophobic part of the membrane while the charged ring peptide will lay on the membrane surface. The result of the chain-chain interaction is an expansion of the lipid lattice which is observable by the lowering of the transition temperature. The asymmetry of the peptide molecule will cause an asymmetric distortion of the lipid matrix leading to a tilt of the lipid chains with respect to the membrane surface. The consideration of the repulsive and attractive elastic forces led to a cooperative binding model. A domain of a polymyxin/phosphatidic acid mixture is formed, which exhibits a curvature in order to minimize the elastic distortion energy at its boundary.

The first result of this paper now is the evidence of an inhomogeneous structure within the lipid-protein domain. An increase of the ionic strength of the dispersion at a given pH splits the transition of the peptide complex into two transition steps denoted in Fig. 1 as T_3 and T_2, where $T_3 < T_2$. The behaviour of the phase transition temperature T_3 with increasing ionic strength is equivalent to the phase transition temperature of the undisturbed lipid matrix at T_1.

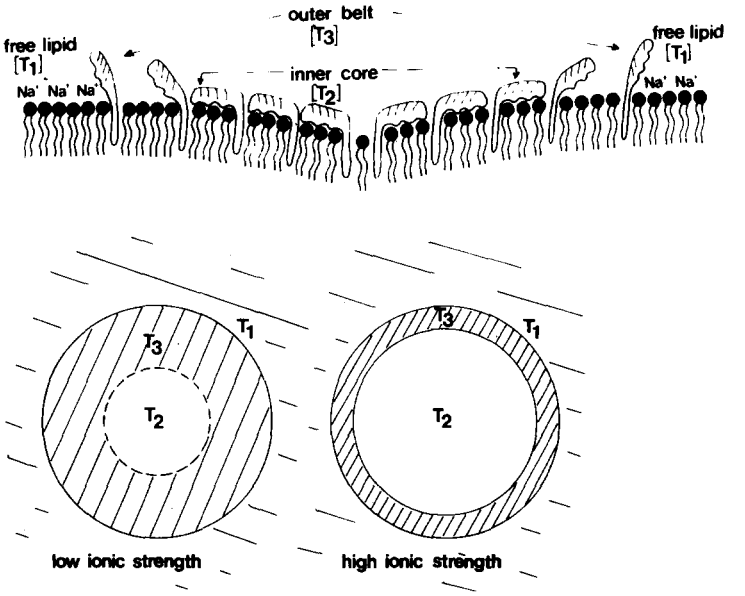

Fig. 7. Model proposal for the domain structure of the polymyxin/phosphatidic acid complex. A separation into three areas of different binding properties has been concluded from the experimental results.

T_1 and T_3 decrease with increasing ionic strength. Therefore we have to conclude that the lower phase transition at T_1 characterizes a polymyxin-bound lipid area where the chain-chain interaction between the lipid chains and the paraffin tail of the polymyxin dominates. An increase in ionic strength decreases the surface potential and reduces the phase transition temperature of the total membrane. This is also valid for the expanded peptide-bound domain.

Increasing the ionic strength leads to the appearance of a further transition at T_2 and we find $T_1 < T_2 < T_3$. But in contrast to T_1 there is an increase in T_2 with increasing ionic strength. Now we have to take into account the electrostatic interaction of the peptide. Pure electrostatic interaction, for example the interaction of polylysine with phosphatidic acid membranes, leads to an increase of the lipid phase transition temperature by about 10°C [9]. This is also valid for a pentalysine, but not for the lysine dimer. Applied to the polymyxin molecule we have to consider both the decrease in transition temperature due to chain-chain interaction and a countercurrent effect due to the electrostatic interaction. At high ionic strength there must exist an area of weak electrostatic interaction beside an area of strongly bound lipid due to the interaction of the positively charged diaminobutyric acid residues with the negatively charged phosphatidic acid.

A model is given in Fig. 7. The inner core with the characteristic phase transition temperature T_2 is assigned to a strongly bound lipid domain including both electrostatic as well as hydrophobic interaction. The effect of the lattice expansion exceeds the electrostatic effect. In total a reduction in the transition temperature compared to the free lipid is observed. This area is surrounded by a belt were mainly hydrophobic interaction takes place. Here the transition temperature is reduced compared to the inner core. The tilt of the lipid chains

in the inner core (see Fig. 7) leads to strong elastic distortion energy at the boundary. The splaying of the lipid is minimized by the curvature of the domain and by the belt surrounding the inner core. If this part is characterized by only weak electrostatic interaction then we obtain a smooth transition to the unmodified lipid matrix.

Now let us consider the effect of ion concentration. Increasing concentration of monovalent ions leads to an expansion of charged lipid membranes caused by a reduction of the surface potential. This effect in the non-bound lipid area creates a mechanical force on the protein domain which stabilizes the tilt in the domain. Therefore the size of the inner core increases relative to the outer belt. This is also observed in the experiments. The step height H_3 decreases relative to H_2 with increasing ion concentration showing the growth of the inner core relative to the outer belt.

Another explanation may be the solubility of the protein within the membrane. This is determined by the hydrophobic and hydrophilic interaction. But there is also a good solubility of the polar group of the peptide within the waterphase. Therefore it might be reasonable that there exists a belt as demonstrated in Fig. 7 of membrane-bound peptides but with their headgroup protruding out into the waterphase. It is well-known from peptide chemistry that proteins may be precipitated with increasing external concentration of monovalent ions. This could also be considered for membrane-bound particles. Thus the solubility of the peptide headgroup within the waterphase is reduced crowding the peptide of the belt into the inner core.

At low ionic strength both the transition T_2 and T_3 coincide due to the lack of additional elastic forces established by the outer matrix. The transition from the inner core to the outer belt may be smooth so that only one broad transition, as reported in Ref. 8, may be observable. At pH 3 the pure lipid matrix is rather rigid. Thus again creates mechanical stress against the tilt of the peptide-bound lipids. A defined belt protects the inner core. Both transitions are observable even at low ionic strength.

Cooperativity of the binding process

At pH 9 sigmoidal binding curves suggest a cooperative binding process between polymyxin-B and phosphatidic acid membranes. Such a cooperative binding process can be described by the Bragg-Williams theory [14,15]. The binding of phosphatidic acid to polymyxin may be taken as a transition from state A (free lipid) to state B (bound lipid). The average fraction of bound lipid is given by [8,14,15]:

$$\rho = \frac{\lambda \cdot \exp\left(-\dfrac{\epsilon - z \cdot w \cdot \rho}{kT}\right)}{1 + \lambda \cdot \exp\left(-\dfrac{\epsilon - z \cdot w \cdot \rho}{kT}\right)}$$

λ the activity of the transition A $\rightleftharpoons$ B is given by $\lambda = \exp(\mu/kT)$ where μ is the chemical potential $\mu = \mu_0 + RT \ln c$, if c is the concentration of the ligand. ϵ, the reaction energy necessary to promote a lipid from A $\rightarrow$ B is of the order of the melting heat ($\epsilon \approx 1\ kT$). The term $W = z \cdot w \cdot \rho$ denotes the total interac-

tion energy in the cluster. It is proportional to the fraction of lipid, ρ, which is already bound to the peptide. z, the number of nearest neighbours is assumed by $z = 6$ for the hexagonal lipid lattice. The equation can be solved numerically by plotting ρ as function of λ which again is a function of peptide concentration c. The s-shaped experimental binding curves given in Fig. 4 have been fitted thus exhibiting the interaction energy within the cluster as a function of the ionic strength. This now is a measure of the cooperativity of the binding process. At low ionic strength the attraction interaction energy is found to be $z \cdot w \approx 2.4\,kT$. It increases to about 3.5 kT at an ionic strength of $I = 0.3$ mol/l and remains constant up to $I = 1$ mol/l. This shows the increase of the cooperativity of the binding process with increasing ion concentration. The maximal fraction, ρ_{max}, of bound lipids, however, decreases with increasing ionic strength (Fig. 2 and Table I). Another parameter is the peptide concentration $c_{1/2}$ where the bound lipid fraction ρ reaches the half-value $\rho_{1/2}$ of the maximal fraction ρ_{max} (see Table I). Up to $I = 0.2$ mol/l the number of peptide molecules necessary to occupy half of the available binding places decreases. Further increase in ionic strength increases $c_{1/2}$.

Again two effects are countercurrent. One is the charge neutralization by the external monovalent ions inhibiting the polymyxin binding. As a consequence the number of maximal bound peptide molecules decreases. This has mainly to be taken into account at high ionic strength $I > 0.2$ mol/l. The second important point is the fluidity of the membrane. It increases with increasing ion concentration mainly in the range up to $I = 0.2$ mol/l (e.g. Fig. 2). This is the same range where the cooperativity of the binding process increases. That is why we conclude a strong influence of the membrane fluidity on the cooperative binding process. Increasing fluidity seems to support the cooperative process. The number of molecules necessary to reach the half-value of ρ_{max} decreases with increasing fluidity up to $I = 0.2$ mol/l. Further increase of the ionic strength only protects the binding places but this does not alter the properties of the domain formation, because the fluidity of the membrane remains constant at this ion concentration range. Thus the membrane fluidity seems to control the process of domain formation whereas the concentration of counterions at the membrane surface determines the number of binding places available for polymyxin interaction. As a consequence the half-concentration $c_{1/2}$ to reach $\rho_{1/2} = \frac{1}{2}\,\rho_{max}$ increases at $I > 0.2$ mol/l. An interesting case are the measurements at pH 6. Increasing pH leads to a decrease in membrane fluidity (compare Figs. 2 and 3) which can be concluded from the increase in phase transition temperature. At low ion strength no cooperativity is observed in the binding curve (Fig. 5). Increasing the ionic strength to $I = 0.3$ mol/l creates a fluidity which is comparable to that of pH 9 at $I = 0.05$ mol/l. The binding curve at pH 6 and an ionic strength of $I = 0.3$ mol/l again exhibits cooperative binding properties. The curve agrees well with the curve obtained at pH 9 and $I = 0.05$ mol/l. So again fluidity seems to trigger the binding properties which can be switched from a cooperative to a non-cooperative binding process by ion-induced fluidity changes. At pH 3.0 the membrane is characterized by a high rigidity ($T_1 = 70°C$ at $I = 0.03$ mol/l). No cooperative binding curve could be obtained even at high ionic strength ($I = 0.3$ mol/l). A further increase in I could not be performed due to precipitation of the membrane dispersion.

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