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# CALORIMETRIC INVESTIGATION OF POLYMYXIN BINDING TO PHOSPHATIDIC ACID BILAYERS

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**The cooperative binding process between the antibiotic peptide polymyxin-B and negatively-charged phosphatidic acid bilayers was investigated by differential thermal analysis and completed by fluorescence polarization measurements. The sigmoidal binding curves were analyzed in terms of the interaction energy within a domain formed by polymyxin and phosphatidic acid molecules. The formation of such a heterogeneous domain structure was favoured by high concentration of external monovalent ions. The cooperativity of the binding increased while a charge-induced decrease in the phase transition temperature of the pure lipid phase was observed with increasing ion concentration at a given pH. The reduced lateral coupling within the lipid bilayer in the presence of salt ions, as demonstrated by an increase in the lipid phase transition enthalpy, was considered to facilitate the cooperative domain formation. Moreover, an increase in the cooperativity of the polymyxin binding could be observed if phosphatidic acids of smaller chain length and thus of a lowered phase transition temperature were used. By the use of chemically-modified polymyxin we were able to demonstrate the effect of electrostatic and hydrophobic interaction. Acetylated polymyxin with a reduced positive charge was used to demonstrate the pure hydrophobic effect of polymyxin binding leading to a decrease in the phosphatidic acid phase transition temperature by about 20°C. The cooperativity of the binding was strongly reduced. Cleavage of the hydrophobic polymyxin tail yielded a colistinnonapeptide which caused an electrostatically-induced increase in the phosphatidic acid phase transition temperature. With unmodified polymyxin we observed the combined effects of electrostatic as well as hydrophobic interaction making this model system interesting for the understanding of lipid-protein interactions. Evidence is presented that the formation of the polymyxin-phosphatidic acid complex is a lateral phase separation phenomenon.**

## Introduction

Polymyxin as well as circulins are decapeptides with comparable structures and modes of action. These membrane active antibiotics are cyclic polycationic peptides containing a high percentage of positively-charged diaminobutyric acids [1]. The antibiotic affects the cell membrane [2] leading to an increased permeability for carbohydrates [3,4] or potassium ions [5]. The resulting breakdown of the osmotic equilibrium stops the RNA and DNA synthesis as well as the oxygen respiration [6,7]. Higher polymyxin concentrations lead to the formation of protrusions or blebs on the membrane surface [8,9] with a diameter of about 100–300 Å and finally to the lysis of the cell. The positive charges at the polymyxin are thought to be responsible for its specific binding to charged phospholipids [9,10].

Since the molecular mechanism of the antibiotic

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action is still unknown, we have investigated the polymyxin effect on model membranes containing negatively-charged lipids. Cooperative binding properties were found by fluorescence polarization [11] and this calorimetric investigation.

The cooperative binding process is strongly dependent on the ionic strength and pH of the electrolyte solution [12]. $Ca^{2+}$ is reported to inhibit polymyxin activity in model membranes [13] as well as biological membranes [8,14].

A comparable cooperative interaction between acid phospholipids and a basic polysoap was reported in a monolayer study [15]. Again the charge density of the interacting polyions were found to determine the cooperativity of the binding process. These studies shine some light on the charge-charge lipid-protein interaction as well as on the hydrophobic interaction between membrane proteins and phospholipid bilayers in biological system.

Our investigations on phosphatidic acid membranes result in a model proposal for the molecular mechanism of polymyxin action to charged lipid membranes. As demonstrated earlier polymyxin is able to bind up to 5 molecules of phosphatidic acid [11]. Due to elastic distortions of the lipid membrane, a domain of polymyxin-bound lipids may be formed in a cooperative way. The domain exhibits a heterogeneous structure with an inner core of strong polymyxin-posphatidic acid interaction due to electrostatic as well as hydrophobic interaction. This inner core is surrounded by an annular ring that is characterized by hydrophobic interaction only [12].

The aim of this paper was to elucidate our model. By the use of differential thermal analysis we could improve the lateral heterogeneous domain structure. Chemically-modified polymyxins have been incorporated into charged lipid bilayer membranes to discriminate between the electrostatic and hydrophobic effects on the phase transition of polymyxin-bound phosphatidic acid. The domain size could be estimated.

## Material and Methods

*Antibiotics and lipids*

Polymyxin and Colistin A from Sigma were checked for purity by TLC on cellulose plates in *n*-butanol/pyridine/glacial acetic acid/water (18 : 12 : 1 : 9, v/v) [16]. Phosphatidic acid-disodium salt was obtained from Fluka.

Acetylation of polymyxin was performed in a 200 ml aqueous solution containing 1 g (0.77 mM) of polymyxin B. After addition of 50 ml of a saturated sodium bicarbonate solution and 80 μl (0.85 mM) of acetic acid anhydride the reaction mixture was stirred for 1 h at 5°C. The volume was reduced by vacuum evaporation and the solution was desalted by gel chromatography on Bio-Gel P2 from BioRad. Separation of the obtained mono- and polyacetylated products was performed on a cation-exchange column (CM-Sephadex from Pharmacia) in a starting phosphate buffer at pH 7.5 and 0.067 M with increasing salt concentration. Monoacetylated polymyxin was eluted at 0.55 M and the diacetylated compound at 0.4 M salt concentration. The obtained fractions were desalted on BioGel P2, freeze-dried and checked for purity on TLC-cellulose plates as described above.

Colistin nonapeptide was prepared by enzymatic hydrolysis of Colistin A (polymyxin E). A solution of 1 g colistin sulfate in 100 ml 0.1 M phosphate buffer, pH 7, was mixed with 100 mg Ficin (Sigma) and incubated for 2 days at 37°C. Then the solution was heated to 80°C to precipitate the enzyme. After filtration the solution was acidified to pH 2.0 by 1 M HCl and extracted with *n*-butanol to remove the γ-acyl-diaminobutyric acid formed during the hydrolysis. Unhydrolyzed colistin was extracted from the aqueous solution with *n*-butanol at pH 8.0. The remaining aqueous solution was desalted on BioGel P2 and freeze-dried [17,18].

*Sample preparation and methods*

Calorimetric measurements were done with a microcalorimeter Triflux from Thermoanalyse (France). Sealable stainless-steel sample pans were used with lipid samples of about 3.3 mg in 300 μl buffer solution. The lipids were dissolved in chloroform and transferred into the pan. After evaporation of the solvent with a nitrogen stream the lipid films were dried under reduced pressure. Polymyxin was added from a stock solution (10 mg/ml). The sample was filled up with borate buffer (pH 9, adjusted with NaCl to the desired ionic strength) to a volume of 300 μl. The samples

were sonified above the lipid phase transition temperature with a Branson-Ultrasonifier at a power of 20 W. Complete incorporation of polymyxin into the lipid phase was concluded from the lack of phenylalanine absorption in the supernatant of the vesicle preparation after centrifugation. The reference pan contained the pure buffer. Calorimetric scans were taken with a heating rate of 0.5°C/min at increasing temperature. Repetitive scans showed no significant differences in $T_t$ or $\Delta H$ values. The molar enthalpies, $\Delta H_{cal}$, were calculated from the weighed peak areas and from the known amount of dispersed lipid. The phase transition temperatures were taken as the extrapolated onset temperatures, which were determined by the intersection of the tangent drawn at the point of the greatest slope on the leading edge of the peak with the extrapolated baseline [19]. The size of the cooperative unit involved in the phase transition $n = \Delta H_{van't\ Hoff}/\Delta H_{cal}$ was evaluated from the calorimetric $\Delta H_{cal}$ value and the $\Delta H_{van't\ Hoff}$ value taken from the integrated and normalized transition curves at $\theta = 0.5$ using the van't Hoff equation $(d\theta/dT)_{\theta=0.5} = \Delta H_{van't\ Hoff}/4R\ T_t^2$ [20], where $\theta$ is the degree of the transition, $T_t$ is the transition temperature and $R$ is the gas constant.

Fluorescence polarization measurements were performed by the use of diphenylhexatriene as optical probe. A Schoeffel Instrument RRS 1000 equipped with two sets of measuring devices to allow simultaneous observation of the fluorescence intensity parallel and perpendicular to the excitation light was used as described earlier [11–13]. Vesicles were prepared from dried lipid films by ultra sonication for 2 min at 20 W. Polymyxin B sulfate was added from a stock solution and the samples were resonicated for 1 min. The temperature during sonication was kept above the lipid phase transition. The final lipid concentration was 0.1 mg/ml of buffer solution brought to the given ionic strength by addition of NaCl.

## Results

*Calorimetric determination of sigmoidal binding curves of polymyxin to phosphatidic acid membranes and the effect of ionic strength*

The thermotropic behaviour of phosphatidic acid bilayers containing polymyxin B was investigated by differential thermal analysis. Typical results for dipalmitoyl phosphatidic acid at pH 9.0 and an ionic strength of $I = 0.1$ M are shown in

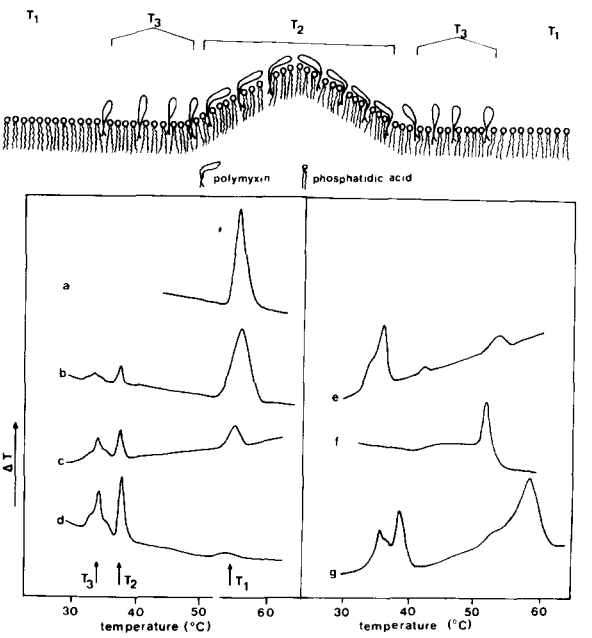


Fig. 1. Calorimetric scans (heating curves) of dipalmitoyl-phosphatidic acid vesicles containing different amounts of polymyxin or its derivatives. In accordance with our earlier results from fluorescence polarization measurements two phase transitions at $T_2$ and $T_3$ of the polymyxin-bound phosphatidic acid were observed in addition to the phase transition, $T_1$, of the unbound phosphatidic acid. All scans are taken at pH 9 and an ionic strength of $I = 0.1$ M. (a) Pure phosphatidic acid, phosphatidic acid vesicles containing: (b) 5 mol% polymyxin; (c) 15 mol% polymyxin; (d) 25 mol% polymyxin; (e) 36 mol% monoacetylated polymyxin; (f) 50 mol% diacetylated polymyxin; (g) 20 mol% polymxyin pH 9 and $I = 0.03$ M, but the antibiotic is added to the prepared vesicles so that it is only bound to the outer layer of the bilayer vesicle. The schematic drawing on top of the figure illustrates the proposed model. An inner core of strongly coupled 3:1 complexes between phosphatidic acid and polymyxin is characterized by electrostatic and hydrophobic interaction. The lipids are tilted to the membrane normal. The center part is surrounded by an annular ring that exhibits only hydrophobic interaction. No strong correlation between the single complexes occur. The peptide rings of polymyxin protrude out into the water phase and the lipid is accessible to external ions. The whole domain in turn is surrounded by a free phosphatidic acid layer. The temperatures $T_2$, $T_3$ are the ordered-disordered lipid phase transition temperatures of the lipid in the corresponding binding states. $T_1$ is the phase transition temperature of the unbound phosphatidic acid. The model is based on our earlier experiments [11,12] and the experiments that are reported in this paper.

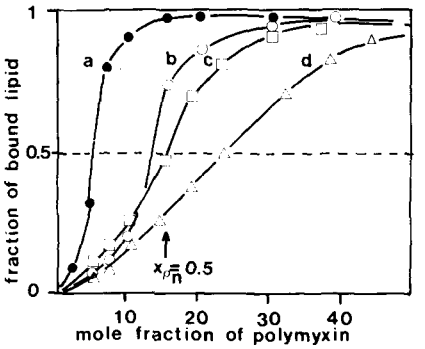


Fig. 2. Typical binding curves of polymyxin (a, b and c) and monoacetylated polymyxin (curve d) to dipalmitoylphosphatidic acid bilayers at pH 9.0 and different ionic strengths (a) $I = 0.2$ M; (b) $I = 0.1$ M; (c) $I = 0.03$ M and (d) $I = 0.1$ M. The fraction of bound lipid $\rho_n$ is normalized to the maximum fraction of lipid, $\rho$max, obtained from the experimental curves at high polymyxin concentration. The value of the mol fraction of polymyxin added to the vesicle preparation, $x_{\rho_n} = 0.5$, corresponding to the half-value $\rho_n = 0.5$ of bound lipid is marked in the binding curve at $I = 0.03$ M.

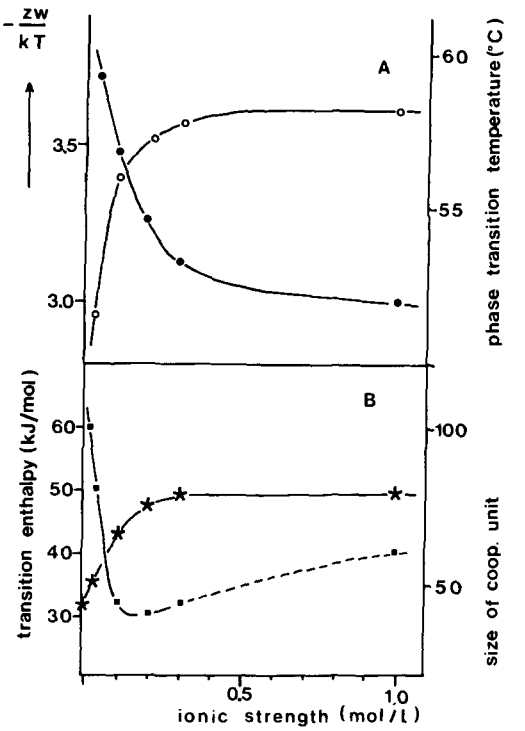


Fig. 3A. The interaction energy $-zw/kT$ within the polymyxin-bound lipid domain obtained by Eqn. 6 of the appendix is given as function of the ionic strength at pH 9.0 (○——○). The interaction energy increases while the phase transition temperature $T_t$ (●——●) of the pure phosphatidic acid obtained from calorimetric scans decreases. B. Transition enthalpy of dipalmitoylphosphatidic acid at pH 9.0 obtained from the weighed areas of the calorimetric peaks (×——×). The size of the cooperative unit (■——■) vs. ionic strength is given as the number of lipids that melt cooperatively.

Fig. 1. In the absence of polymyxin one sharp calorimetric peak was observed at $T_1 = 56$°C which characterizes the phase transition of the pure lipid. Addition of polymyxin up to 25 mol% causes the appearance of two additional calorimetric peaks at lower temperatures denoted $T_2$ and $T_3$, which have to be attributed to phosphatidic acid-polymyxin domains. Such a domain structure in the presence of polymyxin has already been postulated from our fluorescence polarization measurements [12].

From the peak area we were able to determine the amount of polymyxin-bound phosphatidic acid (peaks at $T_3$ and $T_2$) and the amount of free phosphatidic acid (peak at $T_1$). This procedure can be applied if the transition enthalpy of the free phosphatidic acid in a membrane preparation containing polymyxin is equal to the enthalpy change of pure phosphatidic acid in the absence of polymyxin, and if the average transition enthalpy of lipids bound within the domain is comparable to that one of free phosphatidic acid. A plot of the fraction of polymyxin-bound phosphatidic acid vs. the amount of polmyxin added to the lipid dispersion results in the binding curve. Fig. 2 shows examples at different ionic strengths for polymyxin (curves a, b and c) and for monoacetylated polymyxin (curve d). The curves are normalized to the maximum amount of bound lipid at the given ionic strength. The sigmoidal binding curves are characteristic for a cooperative binding process. Note that the slope of the inflectional tangent increases with increasing ionic strength which is equivalent to an increase in the cooperativity of the binding. According to the formalism given in the appendix a binding process is cooperative if an interaction energy in the polymyxin-phosphatidic acid complex exceeds the energy which is necessary to promote a free lipid molecule into the domain [11,12]. From binding curves as shown in Fig. 2 we calculated the average interaction energy $-zw/kT$ within the polymyxin-phosphatidic acid domain by the use of Eqn. 6 of the appendix. The increase in the magnitude of the interaction energy

with increasing ionic strength is shown in Fig. 3A together with the decrease in the phase transition temperature of the pure lipid as obtained by calorimetry. The corresponding $\Delta H$ values and the size of the cooperative unit of pure phosphatidic acid membranes are shown in Fig. 4B. The transition enthalpy change $\Delta H$ increases with ionic strength, whereas the size of the cooperative unit of the free phosphatidic acid involved in the phase transition decreases. Pronounced changes were observed in the ionic strength range up to 0.3 M.

*Electrostatic and hydrophobic interaction*

The effect of acetylated polymyxin B as well as colistin nonapeptide, the enzymatically cleaved polymyxin E derivative, on phosphatidic acid membranes was investigated. Differential thermal analysis curves of dipalmitoylphosphatidic acid vesicles after addition of monoacetylated and diacetylated polymyxin are also shown in Fig. 1. Beside the phase transition $T_1$ of the unaffected phosphatidic acid bilayer we observed two further transitions at $T_2$ and $T_3$ after addition of monoacetylated polymyxin. From our earlier ionic strength dependent results [12] we derived a domain structure composed of an inner core of a hydrophobically as well as electrostatically-bound polymyxin-phosphatidic acid complex characterized by an annular ring of lower melting, only hydrophobically-bound phosphatidic acid with the transition temperature $T_3$. The whole domain in turn is surrounded by an unchanged matrix of free phosphatidic acid melting at $T_1$.

Addition of monoacetylated polymyxin (Fig. 1e) resulted in a different calorimetric scan compared to unmodified polymyxin. The area under the peak $T_2$ diminished, whereas the peak area under $T_3$ remained unchanged. Both the phase transition at $T_3$ as well as at $T_2$ appeared at a somewhat higher temperature compared to unmodified polymyxin. $T_3$ was shifted by about 1°C, whereas $T_2$ was shifted by about 4°C. A much higher amount of acetylated polymyxin had to be added (36 mol%) if compared with unmodified polymyxin. Diacetylated polymyxin added at an equimolar concentration to phosphatidic acid into the water phase did not show calorimetric peaks that correspond to an antibiotic-lipid complex (Fig. 1f). Only a broad shoulder between 40 and 50°C

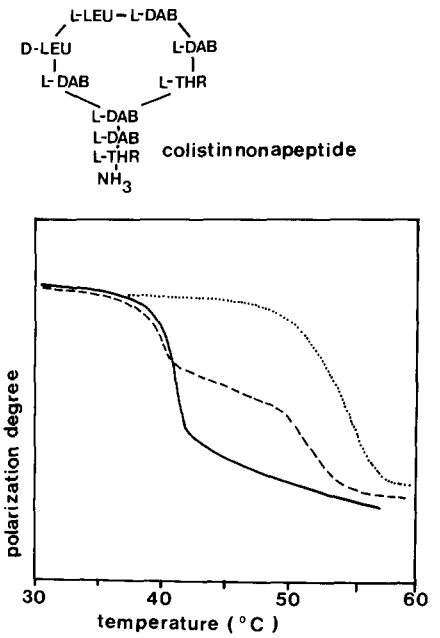


Fig. 4. Phase transition curves of pure dimyristoylphosphatidic acid membranes (full line) and after addition of a 1 : 1 molar ratio (broken line) or a 2 : 1 molar ratio (dotted line) of colistin nonapeptide at pH 9.0 and an ionic strength of $I = 0.03$ M. The fluorescence polarization degree of diphenylhexatriene incorporated into the membrane was measured continously as function of temperature. The chemical structure of the colistin nonapeptide is given: L-Leu = L-leucine, L-DAB = L-diaminobutyric acid, L-Thr = L-threonine.

was observed beside a downward shift of the $T_1$ transition. Obviously the binding potency of polymyxin to phosphatidic acid membranes decreases with decreasing charge of the peptide part.

The effect of decreasing positive charge on the binding properties is also clearly shown in Fig. 2d. The binding curve of monoacetylated polymyxin is compared to those of unmodified polymyxin. At each peptide concentration less amount of phosphatidic acid was bound to the monoacetylated polymyxin. Moreover the cooperativity of the binding was strongly reduced by neutralizing of one positive charge at the peptide. The maximum amount of bound lipid at the corresponding ionic strength was reached at about 30 mol% polymyxin, whereas about 50 mol% of acetylated polymyxin was needed to reach saturation.

The effect of the isolated charged peptide part of polymyxin on the phase transition temperature of dimyristoyl phosphatidic acid membranes at pH

9 and an ionic strength of $I = 0.03$ M is shown in Fig. 4. Colistin nonapeptide (Fig. 4) obtained by cleavage of the hydrophobic tail of polymyxin E was used to investigate the electrostatic effect. Diphenylhexatriene was the optical probe to measure the lipid phase transition curves by fluorescence polarization. Dimyristoyl phosphatidic acid vesicles were used due to the lower phase transition temperature. Addition of colistin nonapeptide in equimolar concentration with respect to the lipid led to an increased second step in the phase transition curve at about 52°C compared to 41°C of the pure lipid. Further addition of colistin nonapeptide to the water phase resulted in a single broad transition between 50 and 57°C. Obviously adsorption of the positively-charged peptide ring to phosphatidic acid membranes leads to a rigidification of the membrane. Due to the high water solubility of the colistin nonapeptide, a high concentration of the substrate had to be added to the aqueous vesicle dispersion.

*Lateral phase separation*

In the experiments presented so far polymyxin was added to the aqueous lipid dispersion before sonification to allow adsorption to both sides of a bilayer membrane. To exclude the possibility of a heterogeneous inside-outside distribution of polymyxin which could lead to different phase transitions $T_2$ and $T_3$ we also added polymyxin to the water phase of a phosphatidic acid vesicle preparation after sonification. Under these conditions polymyxin could only incorporate into the outer monolayer of a bilayer vesicle. The polymyxin concentration of 20 mol% was high enough to bind all lipids in the outer monolayer. A transfer of polymyxin into the inner half of the bilayer vesicle does not occur [21]. The calorimetric scan shown in Fig. 1g is in substantial agreement with the curves of vesicles sonified after polymyxin addition. Again a three-step phase transition was obtained. This demonstrates a lateral phase separation within one layer and excludes a different behaviour of polymyxin-bound phosphatidic acid in the inner or outer monolayer of a bilayer vesicle. However, the calorimetric peak at 57°C which corresponds to the free lipid in the inner layer was much broader as obtained from the pure phosphatidic acid or if polymyxin was added in low enough concentration, where domains and surrounding free lipids were observed. Obviously the complete bound outer layer influences the unaffected inner layer and thus lowers the cooperativity of the phase transition.

*Analysis of the calorimetric peaks at $T_2$ and $T_3$*

The low temperature part of the calorimetric scans of dipalmitoylphosphatidic acid membranes in the presence of 15 mol% of polymyxin at pH 9 are shown in Fig. 5 at different ionic strengths. The peak at $T_3$ is a superposition of a sharp signal with an underlying broad component. The contribution of the broad signal diminishes with increasing ion concentration. Simultaneously to the disappearance of the broad component, the average transition enthalpy, $\Delta H_a$, of the whole complex (area under $T_2$ and $T_3$) given in Table I decreases

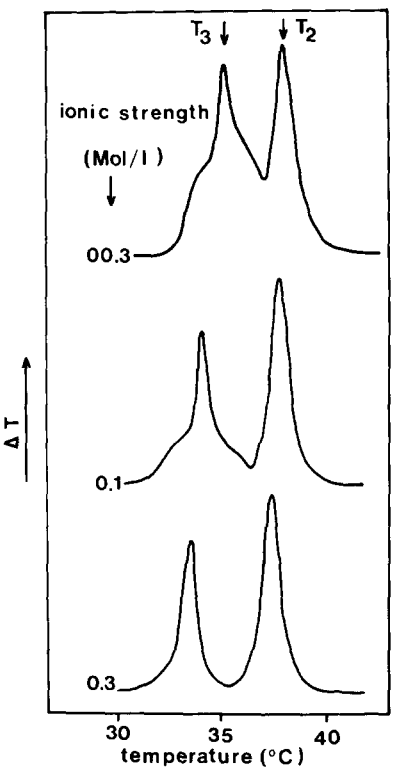


Fig. 5. Calorimetric scans of dipalmitoylphosphatidic acid containing 15 mol% of polymyxin at pH 9 and different ionic strength. The picture shows the transitions of the peptide-bound phosphatidic acid at $T_2$ and $T_3$. The $T_3$ peak is a superposition of a broad and a sharp component. The broad component diminishes with increasing ionic strength.

TABLE I

Average transition enthalpy ($\Delta H_a$) of the whole dipalmitoylphosphatidic acid-polymyxin domain melting at $T_2$ and $T_3$ given for pH 9.0 and different ionic strengths. The values are taken from the calorimetric scans of a membrane preparation containing 15 mol% of polymyxin as shown in Fig. 5. For comparison the transition enthalpy ($\Delta H_f$) of the free phosphatidic acid in the absence of polymyxin is given. The ratio $F_2/F_3$ is the area ratio under the calorimetric peaks at $T_2$ and $T_3$.

| Ionic strength (M) | $\Delta H_a$ (kJ/mol) | $\Delta H_f$ (kJ/mol) | $F_2/F_3$ |
|---|---|---|---|
| 0.03 | 38.9 | 32.7 | 0.5 |
| 0.1 | 33.5 | 41.0 | 0.9 |
| 0.2 | 29.3 | 42.3 | 1.0 |
| 0.3 | 18.0 | 44.0 | 1.1 |

with increasing ionic strength, whereas the transition enthalpy of the unbound phosphatidic acid $\Delta H_f$ increases in this concentration range. The transition enthalpy of the complex exceeds the value of the free phosphatidic acid only at low ionic strength ($I \leqslant 0.03$ M).

This result already indicates a relatively large transition enthalpy of the broad peak underlying the transition at $T_3$ which dominates at low ionic strength. This conclusion can also be derived from the peak area ratio $F_2/F_3$ under the calorimetric peaks at $T_2$ and $T_3$, which increases with increasing ionic strength.

In order to analyze the signal at $T_3$ we have separated the components by spectral deconvolution. Due to the symmetry of the calorimetric peaks we were able to fit the $T_3$ peak by the sum of two gaussian curves. The curve fitting is shown in the upper part of Fig. 6 for a phosphatidic acid membrane containing 25 mol% of polymyxin at pH 9 and at an ionic strength of 0.1 M. The calorimetric scan (solid line) is in good agreement with the calculated curve (broken line) which is a composition of a sharp and a broad signal (dotted lines). The lower part of Fig. 6 shows the signal components of the calorimetric scans at different polymyxin concentrations. At low polymyxin concentration the broad signal predomiates over the sharp signal which in turn increases with increasing polymyxin concentration. Superposition of the two gaussian curves yielded the approximated

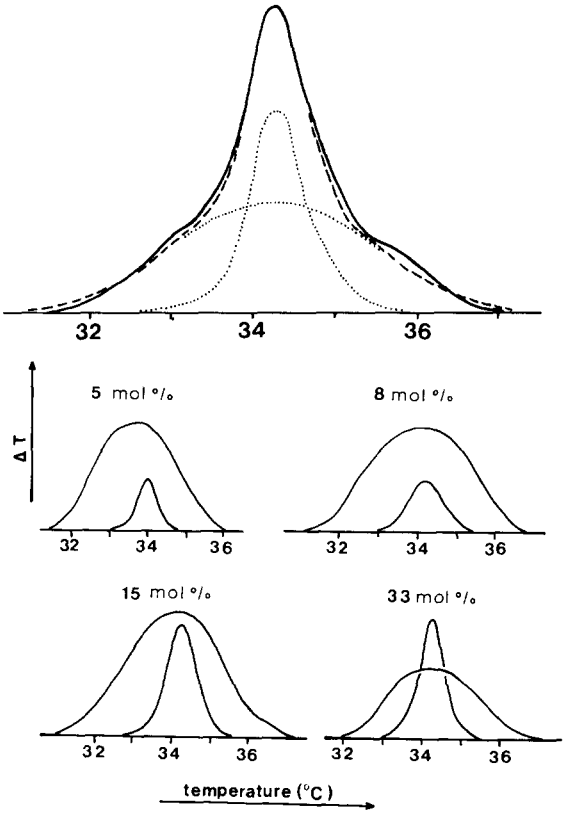


Fig. 6. A calorimetric scan of the $T_3$ peak of dipalmitoylphosphatidic acid at pH 9.0 and an ionic strength of $I = 0.1$ M in the presence of 25 mol% polymyxin is shown in the upper part. The experimental curve (full line) is fitted (broken line) by a superposition of two gaussian lines (dotted curves) to estimate the areas under the calorimetric peak. The signal is a superposition of a sharp and a broad peak. The relative components of the $T_3$ peak are shown in the lower part at different polymyxin concentrations. For low polymyxin concentration the broad component predominates.

eperimental curves at the given concentration. The primary incorporation of polymyxin-bound phosphatidic acid into the domain part with the broad melting is clearly demonstrated.

*Estimation of the domain size and the transition enthalpies of the different binding states*

The size of the cooperative unit of the inner domain which melts at $T_2$ can then be obtained from the ratio of the calculated van't Hoff enthalpy and the calorimetric enthalpy ($n = \Delta H_{\text{van't Hoff}}/\Delta H_{\text{cal}}$) where $n$ is the number of lipid molecules within the cooperatively melting unit.

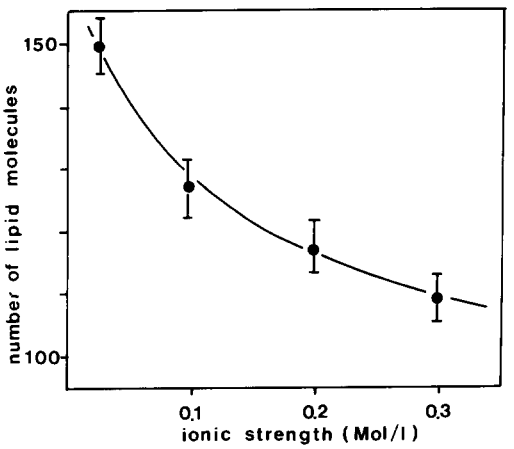


Fig. 7. Size of the cooperative unit of the inner core of the polymyxin-phosphatidic acid domain as function of ionic strength. The number, $n$, of cooperatively melting lipids corresponds to the minimal number of lipids within the domain.

An estimation of the domain size is only possible if the $H$ values of the different binding states within the domain are known.

To estimate $\Delta H_2$ we have to assume that the transition enthalpies of the broad and the sharp transition of the peak at $T_3$ are of comparable size. This is plausible because both binding states have the same transition temperature and differ only in the width of the phase transition. Moreover the average enthalpy change, $\Delta H_3$, of the domain that melts at $T_3$ is considered to be about 40 kJ/mol in conformity with the enthalpy change of the free phosphatidic acid. The phase transition temperatures $T_1$ and $T_3$ exhibit the same decrease with increasing ionic strength [12]. Obviously both states are accessible to external ions. The polymyxin peptide rings are thought to protrude out into the aqueous phase in the binding state with the transition temperature $T_3$. This value of $\Delta H_3$ also explains the high average transition enthalpy $\Delta H_a$ of the whole complex at low ionic strength, where the calorimetric peak area $F_3$ dominates $F_2$ (Table I).

Under these conditions $\Delta H_2$ can be estimated to about 20 kJ/mol. The relatively small value of the transition enthalpy of state 2 demonstrates a low lateral expansion of the lipids in the inner core of the domain during the thermotropic phase transition. Using the value $\Delta H_2 = 20$ kJ/mol and the width of the phase transition obtained from the integrated calorimetric scan we calculated the number of lipids, $n$, that participate in the lipid phase transition as cooperative unit (Fig. 7). The corresponding diameter of the inner core of the polymyxin-phosphatidic acid domain was estimated to 120 Å at pH 9.0 and an ionic strength of $I = 0.03$ M. The diameter decreased to 95 Å with increasing ionic strength.

*Binding of polymyxin to short chain phosphatidic acids*

The binding of polymyxin to dipalmitoyl-, dimyristoyl- and dilauroylphosphatidic acid bilayer membranes was investigated. Fluorescence polarization measurements yield the same type of binding curve as obtained by calorimetry. Diphenylhexatriene was used as probe molecule. Some typical phase transition curves in the absence and presence of polymyxin are shown in Fig. 8 at pH 6 and an ionic strength of $I = 0.03$ M. The dimyristoylphosphatidic acid membranes exhibit a phase transition at $T_t = 52°C$ and dilauroylphosphatidic acid at $T_t = 33°C$. The melting of dipalmitoylphosphatidic acid at this pH and ionic strength occurs at 66°C. Again addition of polymyxin leads to the appearance of a lower phase transition. The two transitions at $T_2$ and $T_3$ (e.g., calorimetric scans in Fig. 1) are not well resolved for dipalmitoylphosphatidic acid at the low ionic strength [12]. The applied electrolyte conditions, however, were necessary to allow the

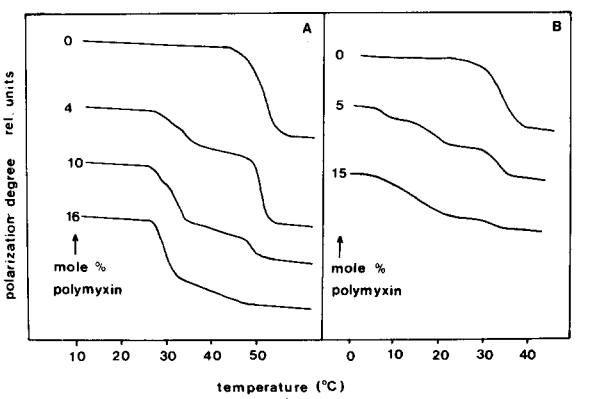


Fig. 8. Phase transition curves of (a) dimyristoylphosphatidic acid and (b) dilauroylphosphatidic acid in the presence of different amounts of polymyxin obtained by fluorescence polarization measurements using diphenylhexatriene as optical probe.

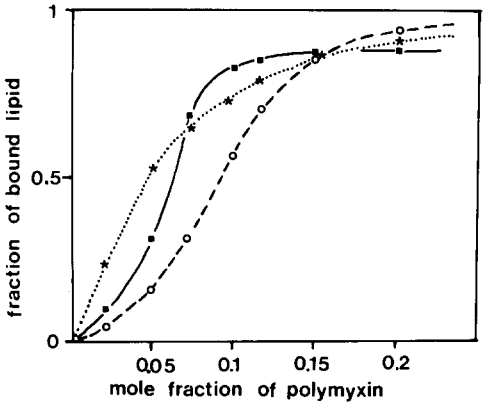


Fig. 9. Binding curves of polymyxin to dipalmitoyl- (× ——— ×), dimyristoyl- (○ ——— ○) and dilauroyl-phosphatidic acid (■ ——— ■) obtained from phase transition curves as shown in Fig. 8. The ionic environment is characterized by $I = 0.03$ M and pH 6.0.

determination of the low melting temperature of the polymyxin-dilauroylphosphatidic acid complex. Increasing pH and increasing ionic strength leads to a further decrease in the phase transition temperature. According to previous results [11,12] we can determine the amount of polymyxin-bound lipid with respect to the total lipid from the step height of the corresponding phase transition curve. The obtained binding curves are shown in Fig. 9. In our earlier paper [12] we reported the loss in cooperativity at low pH and low ionic strength for dipalmitoylphosphatidic acid. Such a non-cooperative binding curve is again shown in Fig. 9 (dotted line). However, dimyristoylphosphatidic acid, under the same environmental conditions, exhibits a cooperative binding curve (broken line in Fig. 9). The cooperativity is even more pronounced if dilauroylphosphatidic acid membranes were used (full line in Fig. 9). In agreement with the calorimetric data shown above as function of ionic strength we observed an increase in the cooperativity of the binding process if a lipid with a lowered phase transition temperature was used.

## Discussion

*Shift in transition temperature*

From differential calorimetry we obtained phase transition curves of phosphatidic acid membranes that exhibit two additional peaks after polymyxin addition. The results were in good agreement with our earlier data obtained from fluorescence polarization measurements [12]. From the calorimetric data it is now much clearer, that the phosphatidic acid-polymyxin domain consists of two distinct areas with different phase transition temperatures denoted $T_2$ and $T_3$. In addition the calorimetric data show, that the lowest peak at $T_3$ is a superposition of a broad and a sharp component which will be discussed later. The melting of the polymyxin-phosphatidic acid occurs at temperatures $T_2$ and $T_3$ which are lowered by about 20°C with respect to the unbound phosphatidic acid depending on pH and ionic strength. From the change in phase transition temperature with increasing ion concentration [12] we derived a model of a domain consisting of an inner core of strongly-bound phosphatidic acid with the melting temperature $T_2$ surrounded by an annular ring of less strongly-bound lipid with the melting temperature $T_3$. The whole domain is embedded within a matrix of free phosphatidic acid melting at $T_1$. The phase transition temperature $T_3$ decreased with increasing ionic strength in accordance with the phase transition temperature $T_1$ of the unbound phosphatidic acid while $T_2$ increased. We concluded, that polymyxin-bound phosphatidic acid in the binding state characterized by $T_3$ was accessible to external ions which implies the absence of electrostatic interaction between the positive charges at the polymyxin peptide ring and the negtively-charged phosphatidic acid. The binding may occur via hydrophobic interaction only. In the binding state corresponding to $T_2$, however, electrostatic as well as hydrophobic interaction was assumed.

This model could be approved by the present investigation. Addition of acetylated polymyxin carrying less positive charges to a phosphatidic acid membrane resulted in a calorimetric scan exhibiting only the lowered $T_3$ transition beside the $T_1$ transition of the unbound lipid. Addition of the peptide ring obtained by cleavage of the hydrophobic tail of polymyxin led to an increase in the phase transition temperature of phosphatidic acid by about 12°C. This gave evidence for the isolated electrostatic effect of positively-charged peptides bound to the surface of a negatively-charged lipid bilayer. This observed upward-shift

in the phosphatidic acid phase transition temperature is comparable to the shift induced by positively-charged polylysine [22].

The appearance of the lowest phase transition at $T_3$ after addition of polymyxin may be explained by a pure hydrophobic effect due to the incorporation of the hydrophobic polymyxin tail into the lipid bilayer. However, polymyxin needs to be charged to allow the interaction with the membrane. The diacetylated antibiotic did not affect the bilayer membrane. An upward shift with respect to $T_3$ leading to a phase transition at $T_2$ may be induced if additional electrostatic interaction between the diaminobutyric acid residues in the peptide ring and the phosphatidic acid membrane is considered. Both binding states were clearly separated from each other at pH 9.0 and ionic strength $I \geqq 0.1$ M. In Fig. 1g we demonstrated the existence of a lateral phase separation within one monolayer of the bilayer membrane excluding an asymmetric inside-outside distribution of polymyxin-bound phosphatidic acid. Since we have no reason to assume two different domains of different binding states we developed the model of one heterogeneous domain with an inner core of electrostatically and hydrophobically-bound lipid surrounded by an annular ring of only hydrophobically-bound lipid where the polymyxin peptide rings protrude out into the waterphase.

Now the question arises why incorporation of polymyxin lowers the phosphatidic acid phase transition temperature. We can use the same arguments that led to the explanation of the cooperative binding curves [11]. From the cooperative binding poperties we assumed a tilt of the lipid chains with respect to the membrane normal upon binding to polymyxin. Such a tilt may be responsible for the shift in a lipid phase transition temperature. Very recently Jähnig et al. [23] interpreted their data of a pH-induced shift in the phase transition temperature in terms of a tilt of the lipid chains. On tilting the lipid chain looses interaction with the neighbouring chain, which is equivalent to a reduced chain length resulting in a lowered phase transition temperature. If the highly asymmetric polymyxin incorporates into the membrane at least three lipids are bound and tilted with respect to the membrane normal. The elastic distortion leads to the cooperative domain formation of tilted polymyxin-bound lipids. If we neglect the electrostatic interaction such a tilt could result in a lowering of the phase transition from $T_1$ to $T_3$ in our system. Taking into account the electrostatic effect which is equivalent to a reduced charge density on the phosphatidic acid surface we will obtain an increased phase transition compared to $T_3$. This is fullfilled in the inner core of the domain that melts at $T_2$, which is about 5°C higher than $T_3$.

*Interaction energy within the domain*

We have analyzed the binding curves of polymyxin to dipalmitoylphosphatidic acid bilayers obtained from the differential thermal analysis data at pH 9.0 and at different ionic strengths (Fig. 2). The values for the attractive energy within the domain were calculated by the application of the theory of cooperative processes in membranes [24]. The theory is given in the appendix, the results are given in Fig. 3a. The magnitude of the interaction energy $-zw$ increases from $2.9 \cdot kT$ to $3.6 \cdot kT$ if the ionic strength is increased from $I = 0.03$ to 0.3 M. Simultaneously the phase transition temperature, $T_1$, of the pure phosphatidic acid obtained by differential thermal analysis decreases from 59 to 53°C in this concentration range. This can be easily explained by an increased dissociation of the second phosphatidic acid proton leading to an increased head group repulsion [25] which is accompanied by the observed increase of the enthalpy change of the transition upon increasing the ionic strength (Fig. 3b). Another indication of the weakened lipid interaction at high ionic strength (number of lipids that undergo the cooperative lipid phase transition) is the decrease in the cooperative unit from $n = 60$ lipids to $n = 30$ lipids for $I = 0.03$ M and $I = 0.2$ M, given in Fig. 3b.

Coming back to the observed cooperative binding of polymyxin to phosphatidic acid membranes. Obviously the cooperativity of this binding process is best pronounced if the phase transition temperature of the charged lipid is lowered, which can be reached either by the use of short chain lipids, by an increased ionic strength or high pH value [12]. We conclude that a reduced lateral interaction within the charged phospholipid matrix increases the cooperativity of the polymyxin binding.

The effect of lipid chain length is clearly demonstrated in Fig. 9. We have chosen a combination of pH and ionic strength where a non-cooperative binding in dipalmitoylphosphatidic acid membranes takes place. Under the same ionic conditions dimyristoylphosphatidic acid membranes exhibit a cooperative binding process. The cooperativity is even increased in dilauroylphosphatidic acid membranes. There seems to be a preferential binding to short chain phosphatidic acids.

The decrease in the phase transition temperature after polymyxin addition with respect to the free phosphatidic acid does not depend on the lipid chian length. The melting of the antibiotic-phosphatidic acid complex is always 20°C lower compared to the pure lipid in dipalmitoyl-, dimyristoyl- as well as in dilauroylphosphatidic acid.

*Influence of ion concentration on the domain structure and its formation*

The effect of increasing ionic strength on the formation and structure of the domain should be summarized: (1) the broad peak underlying the signal $T_3$ diminishes; (2) the ratio $F_2/F_3$ of the calorimetric peak areas increases; (3) the average transition enthalpy $\Delta H_a$ of the whole domain decreases, whereas $\Delta H_f$ of the free phosphatidic acid increases; (4) the cooperativity of the domain formation increases and (5) the diameter of the inner core of the domain increases.

Increasing concentration of monovalent ions leads to an expansion of the unbound charged lipid membrane. This may cause a mechanical force on the peptide domain which stabilizes the tilt of the bound lipids within the inner core of the domain. As a consequence the cooperativity of the binding process should increase and the amount of lipid in the binding state characterized by $T_2$ should increase with respect to the amount of lipid in the state characterized by $T_3$. However, the absolute diameter of the inner core of the domain decreases with ionic strength. This finding is consistent with our cooperative binding model. The cooperative binding process of polymyxin and phosphatidic acid was found to be initiated by the formation of condensation nuclei [21]. A high cooperativity of the binding could be explained by a relatively high number of nuclei, that grow with increasing polymyxin concentration. Therefore higher numbers of smaller domains have to be expected at high ionic strength.

Similar results have been found by application of hydrostatic pressure on polymyxin-containing phosphatidic acid membranes [26]. High pressure was reported to condense the membrane which is contrary to the effect of external monovalent ions. Accordingly a loss in cooperativity and a relative decrease of the amount of lipid in state 2 of the domain observed under 100 atm pressure is consistent with the present results.

Let us now consider the calorimetric peaks at $T_2$ and $T_3$ at low ionic strength. By the use of fluorescence polarization we observed one combined transition for $T_2$ and $T_3$ at low ionic strength [11,12]. With calorimetry we still observe two peaks at $T_2$ and $T_3$. However, the temperature difference between the peaks decreases and $T_3$ becomes very broad with decreasing ionic strength. Obviously the annular ring is more diffuse at low ionic strength with a less strong correlation between the single polymyxin-phosphatidic acid complexes. Increasing mechanical stress due to the ion-induced expansion of the free lipid matrix may lead to a defined belt of only hydrophobically-bound phosphatidic acid thus smoothing elastic distortions at the boundary between the tilted lipids within the inner core of the domain and nontilted lipids in the free phosphatidic acid matrix. If a stronger correlation between single polymyxin-phosphatidic acid complexes in the outer belt of the domain occurs at high ion concentration we have to expect a lowered transition enthalpy of the total domain as was observed in the experiments.

The low transition enthalpy of the inner core of the domain at $T_2$ fits into the model. The strong electrostatic interaction between polymyxin and phosphatidic acids allows only small lateral expansion of the lipid-antibiotic matrix at the phase transition. This is again in good agreement with results obtained by the application of hydrostatic pressure which showed a low compressibility of polymyxin-bound lipids within the domain [26].

## Appendix

The observed cooperative binding process is described by the Bragg-Williams-Theory [11,27]: The incorporation of polymyxin into the charged

lipid bilayer membrane is described by the energy, $\epsilon$, needed to transfer a lipid from the unbound to the domain-bound state. The process becomes cooperative if an attractive energy, $w$, between the lipid molecules bound to polymyxin is introduced. Moreover, the total attractive energy is proportional to the fraction, $\rho$, of already bound lipid and an environmental factor $z$, for the number of nearest binding sites. The molar fraction of bound lipid is given by Hartmann et al. [11].

$$\rho = \frac{\lambda \cdot \exp\{(\epsilon - z \cdot w \cdot \rho)/kT\}}{1 + \lambda \exp\{(\epsilon - z \cdot w \cdot \rho)/kT\}} \quad (1)$$

and describes the binding curve between polymyxin and the lipid. The absolute activity $\lambda$ of the binding process is given by $\lambda = \exp(\mu/kT)$ where $\mu = \mu^0 + kT \ln x$ is the chemical potential and $x$ is the mol fraction of polymyxin added to the lipid dispersion. The interaction energy $z \cdot w$ can now be calculated from the slope of the normalized binding curves according to $d\rho/dx$ at $\rho_n = \frac{1}{2}$, where $\rho_n$ is defined as $\rho/\rho\max$. The value $\rho_{max}$, the maximal amount of lipid that can be bound within the complex, can be derived from the experimental curve at high polymyxin concentration for a given pH and ion concentration.

With $\lambda = \exp(\mu/kT) = x \cdot \exp(\mu^0/kT) = \lambda_0 \cdot x$ we derive from Eqn. 1:

$$x = \frac{\rho_n}{1 - \rho_n} \cdot \frac{1}{\lambda_0} \cdot \exp\left[ - \frac{\epsilon - zw \cdot \rho_n}{kT} \right] \quad (2)$$

At the half value $\rho_n = 0.5$ we obtain

$$\frac{1}{\lambda_0} = x_{\rho_n = 0.5} \cdot \exp\left( \frac{\epsilon - \frac{zw}{2}}{kT} \right) \quad (3)$$

Differentiation of Eqn. 2 and substitution of Eqn. 3 into Eqn. 2 yields:

$$\frac{dx}{d\rho} = x_{\rho_n = 0.5} \cdot \exp\left[ \frac{zw \cdot (\rho_n - 0.5)}{kT} \right] \times \left[ \frac{1}{(1 - \rho_n)^2} + \frac{\rho_n}{(1 - \rho_n)} \cdot \frac{zw}{kT} \right] \quad (4)$$

Taking the position $\rho_n = 0.5$ Eqn. 4 simplifies to

$$\left( \frac{dx}{d\rho} \right)_{\rho_n = 0.5} = x_{\rho_n = 0.5} \cdot \left( 4 + \frac{zw}{kT} \right) \quad (5)$$

Eqn. 5 yields the value of the interaction energy within the phosphatidic acid-polymyxin domain

$$\frac{zw}{kT} = \frac{(dx/d\rho)_{\rho_n = 0.5}}{x_{\rho_n = 0.5}} - 4 \quad (6)$$

which can be derived from the slope of the binding curve at the half-value of the fraction of bound lipid and the value $x$ of the mol fraction of added polymyxin corresponding to $\rho_n = 0.5$.

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