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PRESSURE-INDUCED CHANGES IN THE MOLECULAR ORGANIZATION OF A LIPID-PEPTIDE COMPLEX

POLYMYXIN BINDING TO PHOSPHATIDIC ACID MEMBRANES

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Summary

The effect of 100 atm pressure on the organization of the lipid-peptide complex formed between polymyxin and dipalmitoyl phosphatidic acid has been investigated. Phase transition curves were obtained by electron paramagnetic resonance by measuring the partition coefficient of the spin label, 2, 2, 5, 5-tetramethylpiperidine-*N*-oxyl. The three-step phase transition curve previously obtained with fluorescence polarization measurements was confirmed, demonstrating three distinct phosphatidic acid domains in the bilayer. Pressure increases binding of polymyxin to phosphatidic acid bilayers and alters the proportions of the two domains that differ in the mode of binding between phosphatidic acid and polymyxin. The binding curves of polymyxin to phosphatidic acid bilayers were determined and it was shown that application of pressure reduces the cooperativity of the binding curve.

Introduction

Polymyxin, a cyclic polycationic peptide with a fatty acid side chain, is a useful tool for studying the structure and function of biological membranes [1]. It is well established that polymyxin alters bacterial membrane structure [2,3] but the mechanism of its antimicrobial activity is still unknown. Disorganization of the membrane structure was proposed to be the primary effect

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of polymyxin on bacteria [4]. Binding of polymyxin to bacteria may occur by an initial electrostatic interaction with negatively charged phospholipids [5,6].

The lipid requirement for polymyxin sensitivity was investigated in liposomes by measuring changes in glucose permeability [6,7]. These studies indicate that polymyxin-induced permeability changes are dependent on the presence of negatively charged lipids. However, the fatty acid side chain that exhibits hydrophobic interaction with the lipid matrix also needs to be taken into account. Insertion of the fatty acid chain of the antibiotic into the lipid matrix perturbs the normal packing density of the membrane. The resulting expansion in surface area of phosphatidic acid bilayers lowers the lipid phase transition temperature by approx. 20°C [8]. The binding of polymyxin to these negatively charged membranes is a cooperative process. Moreover, a recent study demonstrated that both the electrostatic and the hydrophobic interactions need to be considered for polymyxin binding [9]. A model for the binding of polymyxin to a phosphatidic acid bilayer is shown in Fig. 1. The central domain (T_2) of each cluster contains polymyxin bound to phosphatidic acid by hydrophobic and electrostatic interactions. A second domain (T_3), consisting of an annular ring of polymyxin bound to the phosphatidic acid bilayer by only hydrophobic interaction, has been postulated based on evidence from an earlier fluorescence polarization study [9]. The cluster containing these two domains is surrounded by a phosphatidic acid bilayer (T_1) that exhibits a slightly lower phase transition temperature and cooperativity than pure phosphatidic acid.

Due to the presence of both hydrophilic and hydrophobic parts, polymyxin is useful as a model substance for the study of lipid-protein interactions in membranes as well as for antibiotic action. The fluid-mosaic model of biological membranes [10] distinguishes between intrinsic and extrinsic proteins; changes in physical properties of the phospholipid bilayer can be used as indicators of each of these protein classes within a lipid matrix [12]. Polymyxin has the advantage of exhibiting properties of both extrinsic and intrinsic proteins, due to its structure and mode of binding. Therefore, it is an excellent model protein for studying phospholipid-protein interactions in membranes.

High pressure has been reported to antagonize the effects of inhalation anesthetics on lipid bilayers [13,14] and to alter phase separation phenomena in bilayers of binary mixtures of phospholipids [15—17]. Moreover, hydrostatic pressure is reported to influence the enzyme activity of the ($Na^+ + K^+$)-ATPase as a consequence of the modified lipid-protein interaction [18]. The phospholipid-peptide system investigated here has been shown to exhibit dis-

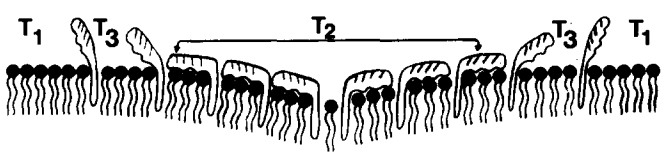

Fig. 1. Model proposed in Ref. 9 for the phosphatidic acid-polymyxin cluster. An inner domain of polymyxin bound to a phosphatidic acid layer by both electrostatic and hydrophotic interactions (T_2) is surrounded by an annular ring (T_3) that exhibits only hydrophobic interaction which is in turn surrounded by a free phosphatidic acid domain (T_1).

ordering and expansion of the lipid matrix by inhalation anesthetics as well as alteration of the structural lipid-peptide complex by these agents [14]. The effect of high pressure on the structure of the complex formed between polymyxin and phosphatidic acid as well as the cooperativity of this binding process are investigated in this paper.

Materials and Methods

Dipalmitoyl phosphatidic acid (disodium salt, 99% pure, and a Ca^{2+} content of less than 2 ppm, obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.) was checked by thin-layer chromatography and used without further purification. Polymyxin-B sulfate was also purchased from Sigma Chemical Co. All measurements were performed in a pH 9.0 sodium borate buffer containing 91.6 ml of 0.05 M sodium borate and 18.4 ml of 0.2 M boric acid and 0.25 M NaCl.

Lipid dispersions were prepared by sonication of 20 mg of phosphatidic acid in 4 ml of the above buffer for 5 min at 55°C. Polymyxin was then added from a stock solution of 5 mg/ml to the dispersion which was then resonicated for 2 min at 55°C, and centrifuged at $1000 \times g$. The pellet (approx. 0.4 ml) was resuspended after addition of 40 μl of a $3 \cdot 10^{-3}$ M solution of TEMPO (2, 2, 6, 6-tetramethylpiperidine-*N*-oxyl) in the above buffer. 10 μl of the lipid dispersion were transferred to a 1 mm inner diameter quartz sample cell pressurizable to 100 atm of helium. The probes were placed in an electron paramagnetic resonance (EPR) cavity thermostatically controlled to ±0.1°C.

EPR spectra were measured on a Varian E-104A Spectrometer with on-line digitized recording. Curve fitting for the evaluation of the fluidity parameter, $f = H/(H + P)$, as a function of temperature to derive phase transition curves was performed using a DEC PDP 11/03 computer. H is the intensity of the EPR signal of spin probe dissolved in the hydrophobic lipid phase and P is the signal height of spin probe in the polar (water) phase. The standard deviation for the determination of f was less than 1%.

Results

The effect of pressure on the phase transition curves of phosphatidic acid membranes containing polymyxin

The phase transition curves of pure dipalmitoyl phosphatidic acid at pH 9.0 and 0.3 M Na^+ are shown in Fig. 2 at 1 and 100 atm. Application of 100 atm helium pressure leads to an increase in the lipid phase transition temperature by 1.5°C. A typical phase transition curve for dipalmitoyl phosphatidic acid membranes containing 4 mol% polymyxin is shown in Fig. 3. A three-step phase transition is observed. This phase transition curve, determined in this study with EPR spectroscopy, is in good agreement with earlier experiments using fluorescence polarization [9]. The transitions at T_3 (30°C) and T_2 (40°C) are assigned to the two lipid-peptide domains. The highest temperature step of the phase transition curve at T_1 (50°C) comes from the phosphatidic acid bilayer with which the lipid-peptide complex is surrounded. This phase transition is lower and broader in pure phosphatidic acid bilayers (Fig. 2). Each step in the

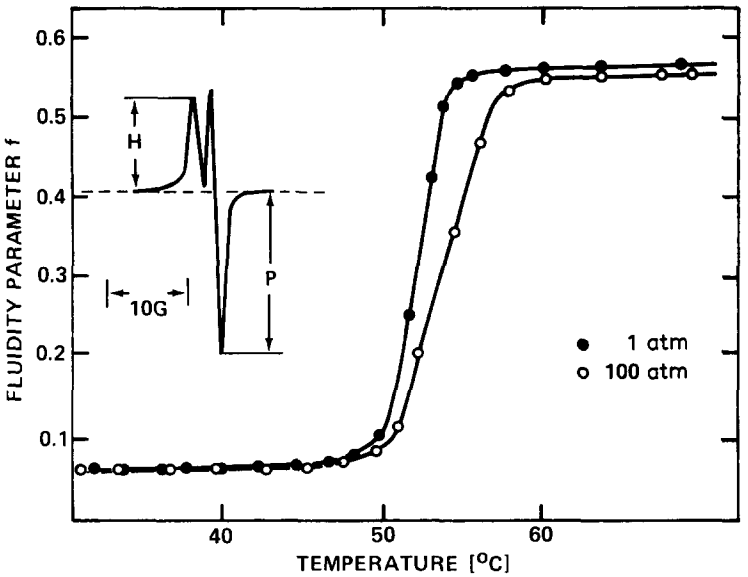

Fig. 2. Phase transition curve of dipalmitoyl phosphatidic acid membranes at 1 and 100 atm of helium pressure by EPR. The insert gives the TEMPO spectrum. The fluidity parameter is defined as $f = H/(H + P)$.

phase transition curve shown in Fig. 3 corresponds to melting of phospholipids in different lipid-peptide domains. The height of the change in the fluidity parameter (H) is a measure of the mole fraction of spin probe which is dissolved in the lipid matrix. Based on quantitative assignments of mole fractions determined by fluorescence polarization, the height of each phase transition step, H_1, H_2 and H_3, indicated on the curve at atmospheric pressure in Fig. 3 is considered to represent the relative amount of lipid in the corresponding

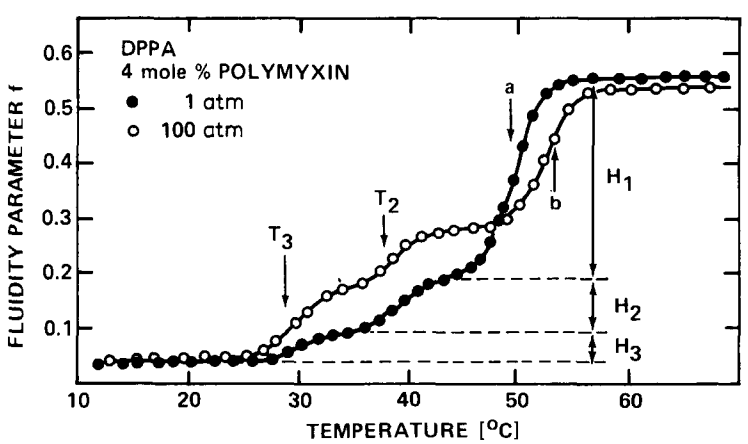

Fig. 3. Phase transition curves of dipalmitoyl phosphatidic acid (DPPA) membranes containing 4 mol% polymyxin at 1 and 100 atm of helium pressure. T_1, T_2 and T_3 identify the temperatures of each phase transition step; H_1, H_2 and H_3 are the amplitudes of the fluidity change corresponding to each domain. The difference ΔT_1, between arrows a and b, that is the shift of the phase transition temperature (T_1) of the phosphatidic acid domain, with pressure, is about 3°C.

domains. We use the ratio, $(H_2 + H_3)/(H_1 + H_2 + H_3)$, as a measure of the mole fraction, ρ, of phosphatidic acid in the cluster.

Application of 100 atm of helium pressure causes three effects; (a) the fraction of polymyxin-bound lipid ($\rho = (H_2 + H_3)/(H_1 + H_2 + H_3)$) increases with increasing pressure; (b) the ratio, H_2/H_3, reflecting the proportion of the two domains within the phosphatidic acid-polymyxin cluster decreases with increasing pressure; and (c) the phase transition temperature of the surrounding phosphatidic acid matrix (T_1) is increased compared to pure dipalmitoyl phosphatidic acid after application of 100 atm of pressure whereas the phase transition temperatures of the peptide-bound domains (T_2, T_3) are nearly unaffected.

Pressure effect on the binding curve

The relative step height of $(H_2 + H_3)/(H_1 + H_2 + H_3)$ is used as a measure of the fraction of polymyxin-bound lipids, ρ. This ratio is plotted in Fig. 4 as a function of the mole fraction of polymyxin added to the dipalmitoyl phosphatidic acid dispersion, thus yielding a binding curve. A sigmoidal curve is obtained at atmospheric pressure in agreement with our earlier results [8,9], indicating a cooperative binding process. At 100 atm the cooperativity of the binding is drastically reduced. Moreover, as already described in the case of one polymyxin concentration (Fig. 3), the amount of lipid in the cluster increases at high hydrostatic pressure.

The slight shift of the mid-point of the curve shown in Fig. 4 at atmospheric pressure compared to results obtained with fluorescence polarization may be due to the different method of membrane preparation; in the present experiments we used centrifuged multilamellar bilayers whereas in our earlier experi-

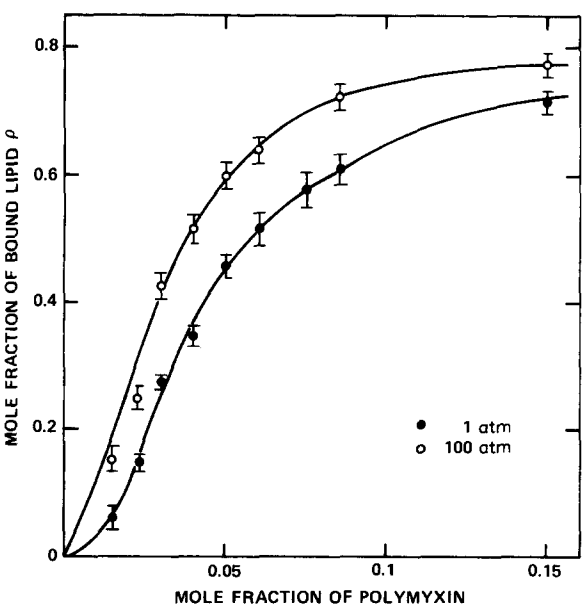

Fig. 4. Binding curve of polymyxin to dipalmitoyl phosphatidic acid membranes at pH 9.0. The data are obtained from measurements equivalent to the one shown in Fig. 2. The curves are taken at 1 and 100 atm of helium pressure.

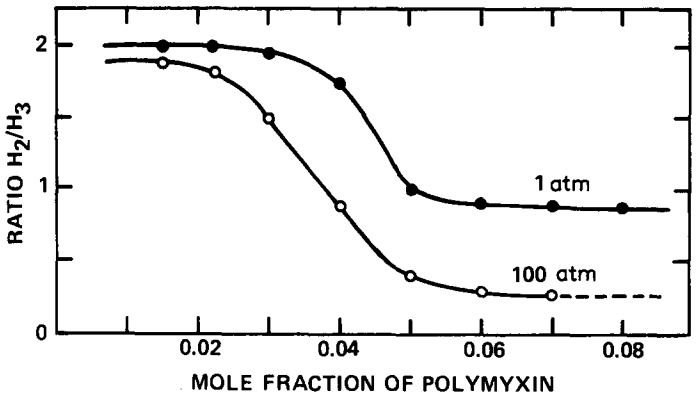

Fig. 5. The ratio, H_2/H_3, is given as a function of polymyxin concentration for 1 and 100 atm of helium pressure. Note that the ratio, H_2/H_3, corresponds to the proportions of the two lipid-peptide domains.

ments we used bilayer vesicles. The change in bilayer preparation was necessary to perform the TEMPO-partitioning measurements, which allowed the application of high pressure to the sample.

Concentration and pressure-dependent organization of the lipid-peptide complex

The step height ratio, H_2/H_3, gives a measure of the distribution of the two domains within the lipid-polymyxin cluster. The concentration dependence of the ratio, H_2/H_3, at atmospheric pressure is given in Fig. 5 and is compared to the corresponding graph at 100 atm. In the curve at atmospheric pressure, the ratio H_2/H_3 is constant up to 2 mol% polymyxin content, then decreases with increasing polymyxin concentration. The curve at 100 atm exhibits a similar shape but the relative amount of lipid in the T_3 domain is increased. The redistribution is most pronounced at higher polymyxin concentrations ($c > 3$ mol%).

Discussion

Cooperative lipid-peptide interaction

In previous work [8,9] we have shown that the binding of polymyxin to negatively charged phosphatidic acid bilayers is a cooperative process. The cooperativity of the binding is dependent on the pH and ion concentration of the buffer solution. Decreasing fluidity (low pH and low ion concentration) led to loss of the cooperative binding properties. Moreover, a domain structure of the polymyxin-phosphatidic acid cluster was postulated in which there are two types of phospholipid-peptide complex: a central domain of lipid attached to polymyxin by hydrophobic and electrostatic interactions is surrounded by an annular ring of polymyxin bound to lipids by only hydrophobic interactions which, in turn, is surrounded by a bilayer membrane of essentially pure phosphatidic acid.

Here we report the effect of high pressure on the biophysical properties of this model system. EPR data yield the same type of cooperative binding curve

as that previously determined by fluorescence polarization [9]. The results of this study clearly show that polymyxin binding to phosphatidic acid bilayers causes phase separation into three distinct domains that differ greatly in their fluidity, phase transition temperature and compressibility. There may be several possible models that accommodate the incorporation of three different phospholipid domains in a bilayer membrane. However, in order to provide continuity with previous publications [8,9] and clarity in presentation, in the following discussion we shall interpret the results in terms of the annular ring model described in Introduction and Fig. 1.

Organization of the lipid-bound complex

It is necessary to consider the change in the amount of phosphatidic acid in the whole lipid-peptide cluster as well as the change in the relative amount of lipid in the central domain (T_2) and the annular ring (T_3). Application of 100 atm of helium pressure leads to an increase in the total amount of lipid (ρ) within the cluster (Fig. 4). This is shown more clearly in Fig. 6a as a function of polymyxin concentration where $\Delta\rho$ is the difference between the amount of bound lipid measured at 100 and at 1 atm pressure. An explanation consistent

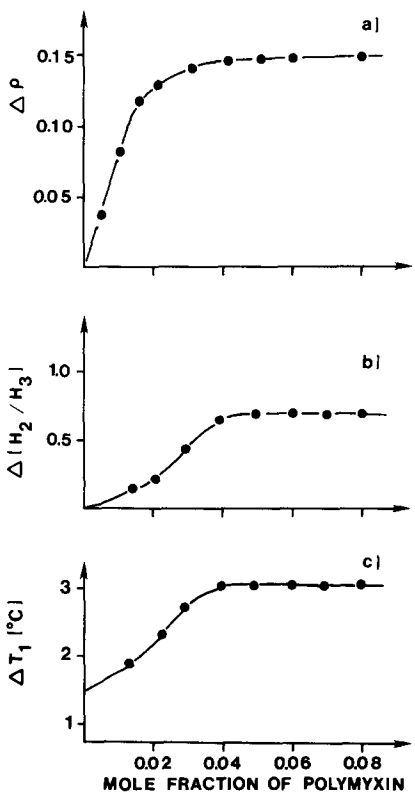

Fig. 6. Pressure-induced changes in domain organization and phase transition temperature as function of the mole fraction of total polymyxin: (a) difference ($\Delta\rho$) of the mole fraction of phosphatidic acid in the two phosphatidic acid-polymyxin domains ($H_2 + H_3$)/($H_1 + H_2 + H_3$) measured at 100 and 1 atm pressure; (b) $\Delta(H_2/H_3)$ is the difference between the ratios of H_2/H_3 obtained at 100 and 1 atm pressure; (c) Changes in the phase transition temperature (T_1) after application of 100 atm pressure.

with the model in Fig. 1 is that a small fraction of the polymyxin is dissolved in the free phosphatidic acid matrix (T_1) and that, as high pressure causes a more ordered phosphatidic acid phase, it leads to expulsion of the dissolved polymyxin. A similar exclusion of a lipid-solvated drug from a phosphatidic acid bilayer by pressure was recently observed [14]. The high packing density of dipalmitoyl phosphatidic acid bilayers under pressure may be the cause of this effect.

Further support for the existence of polymyxin dissolved in the free phosphatidic acid domain is provided by the phase transition curves (Figs. 2 and 3) where it is seen that the pressure elevation of the phase transition temperature (ΔT_1) of domains of nearly pure phosphatidic acid increases with increasing total polymyxin concentration. Moreover, the phase transition temperature (T_1) at atmospheric pressure of this domain is lower than pure phosphatidic acid. The difference, ΔT_1, of the phase transition temperature of the free phosphatidic acid phase obtained at 100 and 1 atm pressure as a function of polymyxin concentration is shown in Fig. 6c. An increasing amount of polymyxin will be extruded from the phosphatidic acid domain (T_1) as a function of pressure and will be included into the lipid-peptide cluster. Consequently, as is seen in Fig. 6a, the amount of polymyxin-bound lipid in the cluster increases.

Now the question arises as to how polymyxin distributes into the two domains, T_2 and T_3, of the cluster. Fig. 5 shows that the amount of lipid in the annular ring (H_3) increases with pressure because the ratio H_2/H_3 is smaller at 100 atm compared to 1 atm pressure over the observed concentration range. The difference in this ratio $\Delta(H_2/H_3)$ between 100 and 1 atm is given in Fig. 6b as a function of polymyxin concentration. The increase in $\Delta(H_2/H_3)$ corresponds to the changes shown in Fig. 6a and c. Pressure extrudes the dissolved polymyxin from the free phosphatidic acid phase into the lipid-peptide cluster. The phase transition temperature (T_1) is therefore increased by ΔT (Fig. 6c). More phosphatidic acid is then bound within the complex. The greater proportion of the phosphatidic acid ($\Delta\rho$) which becomes incorporated within the lipid-peptide cluster adds preferentially to the annular ring (T_3), leading to a decrease in the ratio H_2/H_3. The difference in this ratio, $\Delta(H_2/H_3)$, exhibits a comparable dependence on polymyxin concentration as was already discussed for $\Delta\rho$ and ΔT_1 in Fig. 6. In summary, pressure causes polymyxin dissolved in the free phosphatidic acid matrix to add to already existing clusters of phosphatidic acid and polymyxin. Consequently, more phosphatidic acid is bound to the domain, T_3, depicted as an annular ring in the model of Fig. 1.

Another important result of the high-pressure study is the non-compressibility of the lipid-peptide cluster. Both phase transition temperatures of the inner domain (T_2) and its annular ring (T_3) are constant up to 100 atm of pressure. This means that the volume and the molecular packing of phospholipids in the two domains are not altered by pressure. The molecular structure of the phosphatidic acid-polymyxin cluster that best fits our results includes an expanded bilayer surface, of which the area is dominated by the projected area of extrinsic polymyxin bound to negatively charged head-groups. The close packing of the polymyxin may result in an incompressible complex that exhibits a cooperative but low-temperature phase transition. These unusual

properties may cause the membrane instability responsible for the bacteriocidal action of polymyxin.

This study shows that 100 atm of pressure cause significant changes in the proportion of lipid-peptide clusters in phosphatidic acid bilayer membranes. The resulting reorganization of the membrane could lead to changes in the function of membrane bound proteins that control ion permeability and neurochemical transmission. This information may be of value in understanding the effects of high pressure on living organisms such as the high-pressure nervous syndrome [20] and adaptation to pressure.

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